PURIFICATION AND REGULATION OF
CTP:PHOSPHOCHOLINE CYTIDYLTRANSFERASE

By

JASBINDER SINGH SANGHERA

B.Sc., The University of London, 1982

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in
THE FACULTY OF GRADUATE STUDIES

(Department of Biochemistry)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1989

© Jasbinder Singh Sanghera, 1989
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of BIOCHEMISTRY

The University of British Columbia
Vancouver, Canada

Date 27th April 1989
ABSTRACT

CTP:phosphocholine cytidylyltransferase was purified to homogeneity using a procedure involving aggregation of enzyme with exogenous lipid and selective dissociation with detergents and chromatography on conventional and FPLC columns. SDS-PAGE of purified enzyme showed a single protein of molecular weight ~42 Kd. Native-PAGE showed the protein to migrate at a molecular weight of ~90 Kd, indicating that the native enzyme may be a dimer. IEF-PAGE revealed the enzyme to have a pI of ~5.8. 2-D-PAGE showed the enzyme to have at least two isoforms.

Attempts to generate polyclonal antibody to the purified enzyme in rabbits were unsuccessful. Even after two booster injections, the antibody titre was still weak indicating that the enzyme may not be a good antigen. However, Dr Harris Jamil was able to generate antibodies in the chicken. This antibody detected cytidylyltransferase by the Western-blotting technique and inhibited enzyme activity in a concentration-dependent manner, but was unable to immunoprecipitate the cytidylyltransferase in solution. Using this antibody, the cytidylyltransferase was found to occur in a variety of isoforms.

Kinetic studies using the purified enzyme showed the $K_m$ for CTP and phosphocholine to be 0.31 mM and 0.15 mM respectively. Activation of cytidylyltransferase by commercial and microsomal lipids showed the enzyme to be activated by anionic phospholipids. The presence of oleate did not greatly enhance the activation of the enzyme by these lipids.
The purified cytidylyltransferase was shown to be a substrate for cAMP-dependent protein kinase. Phosphorylation of the enzyme led to inactivation and increased recovery of the enzyme in the cytosol, while dephosphorylation by alkaline phosphatase led to activation and increased recovery of the enzyme in the microsomes. A serine residue(s) was phosphorylated on cytidylyltransferase. Twice as much phosphate was incorporated if cytidylyltransferase were dephosphorylated prior to phosphorylation. Incubation of hepatocytes with $^{32}$Pi and then detection of cytidylyltransferase with antibody after 2-D-PAGE and Western-blotting, showed the cytidylyltransferase may be phosphorylated \textit{in vivo}.

Incubation of hepatocytes with Ca$^{2+}$, Ca$^{2+}$-mobilizing agents and phospholipase A$_2$ resulted in translocation of cytidylyltransferase from the cytosol to the microsomes. Incubation of hepatocytes with Ca$^{2+}$ resulted in an increase in PC and LPC formation. Incubation of hepatocytes with phospholipase A$_2$ resulted in an increase in PC formation.

Regulation of cytidylyltransferase activity by reversible phosphorylation and by Ca$^{2+}$ may be important during short term regulation of enzyme activity by hormones.
# Table of Contents

## Phosphatidylcholine-Structure and Function

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1. 1. 1. 1.) Structure of Phosphatidylcholine</td>
<td>1</td>
</tr>
<tr>
<td>(1. 1. 2. 1.) Component of biological membranes</td>
<td>1</td>
</tr>
<tr>
<td>(1. 1. 2. 2.) Component of lung surfactant</td>
<td>3</td>
</tr>
<tr>
<td>(1. 1. 2. 3.) Component of lipoproteins</td>
<td>3</td>
</tr>
<tr>
<td>(1. 1. 2. 4.) Component of bile</td>
<td>4</td>
</tr>
<tr>
<td>(1. 1. 2. 5.) Acyl donor to cholesterol esters</td>
<td>4</td>
</tr>
<tr>
<td>(1. 1. 2. 6.) Precursor for sphingomyelin synthesis</td>
<td>5</td>
</tr>
<tr>
<td>(1. 1. 2. 7.) Precursor for prostaglandin synthesis</td>
<td>5</td>
</tr>
</tbody>
</table>

## The Enzymes and Pathways of Phosphatidylcholine Biosynthesis

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1. 2. 1. 1.) The CDP-choline (Kennedy) pathway</td>
<td>7</td>
</tr>
<tr>
<td>(1. 2. 1. 2.) Choline kinase</td>
<td>7</td>
</tr>
<tr>
<td>(1. 2. 1. 3.) CTP:phosphocholine cytidylyltransferase</td>
<td>11</td>
</tr>
<tr>
<td>(1. 2. 1. 4.) CDP-choline:1,2-diacylglycerol cholinephosphotransferase</td>
<td>15</td>
</tr>
<tr>
<td>(1. 2. 2. 1.) Phosphatidylethanolamine N-methylation pathway</td>
<td>17</td>
</tr>
<tr>
<td>(1. 2. 2. 2.) Phosphatidylethanolamine N-methyltransferase</td>
<td>18</td>
</tr>
<tr>
<td>(1. 2. 3. 1.) Base exchange pathway</td>
<td>22</td>
</tr>
</tbody>
</table>

## Regulation of Key Enzymes Involved in Lipid Synthesis by Phosphorylation/Dephosphorylation

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1. 3. 1. 1.) Hydroxymethylglutaryl-CoA reductase</td>
<td>26</td>
</tr>
<tr>
<td>(1. 3. 1. 2.) Acetyl-CoA carboxylase</td>
<td>28</td>
</tr>
<tr>
<td>(1. 3. 1. 3.) Glycerol phosphate acyltransferase</td>
<td>32</td>
</tr>
</tbody>
</table>
(1. 3. 2. 1.) Regulation of enzymes involved in PC biosynthesis by reversible phosphorylation ..................................34
(1. 3. 2. 2.) Choline transport and choline kinase ...........................................34
(1. 3. 2. 3.) CTP:phosphocholine cytidylyltransferase ..................................35
(1. 3. 2. 4.) CDP-choline:1,2-diacylglycerol cholinephosphotransferase 38
(1. 3. 2. 5.) Phosphatidylethanolamine N-methyltransferase ..................38

IN VolVEMENT OF CALCIUM IN PC BIOSYNTHESIS
(1. 4. 1. 1.) Ca\(^{2+}\)-an important cellular second messenger .........................41
(1. 4. 1. 2.) Mode of action of vasopressin and other \(\alpha_1\)-adrenergic agonists ..............................................................42
(1. 4. 1. 3.) Regulation of PC synthesis by vasopressin and other \(\alpha_1\)-adrenergic agonists ..............................................................44
(1. 4. 2. 1.) Agents which are activated by Ca\(^{2+}\) ........................................47
(1. 4. 2. 2.) Protein kinase C .........................................................................47
(1. 4. 2. 3.) Involvement of protein kinase C in PC synthesis .......................49
(1. 4. 2. 4.) Ca\(^{2+}\)-calmodulin-dependent protein kinase ..........................51
(1. 4. 2. 5.) Involvement of Ca\(^{2+}\)-calmodulin-dependent protein kinases in PC synthesis ..........................................................55
(1. 4. 2. 6.) Phospholipase A\(_2\) ......................................................................56
(1. 4. 2. 7.) Involvement of phospholipase A\(_2\) in PC synthesis .....................58

(1. 5. 1. 1.) Aim of thesis ................................................................................61

EXPERIMENTAL PROCEDURES

MATERIALS
(2. 1. 1. 1.) Animals .....................................................................................63
(2. 1. 1. 2.) Chemicals .................................................................63

GENERAL PREPARATIVE AND ANALYTICAL PROCEDURES

(2. 2. 1. 1.) Preparation of rat hepatocytes .........................................63
(2. 2. 1. 2.) Subcellular fractionation of whole rat liver or isolated hepatocytes .................................................................65

PURIFICATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

(2. 2. 2. 1.) Purification by aggregation/deaggregation of cytosolic form ...............................................................................67
(2. 2. 2. 2.) Purification by conventional and high performance techniques ...........................................................................68
(2. 2. 2. 3.) Purification by lipid-protein aggregation and resolution on FPLC ........................................................................69

PREPARATION OF SUBSTRATE AND LIPID ACTIVATORS OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

(2. 2. 3. 1.) Synthesis of $[^3]$H phosphocholine ........................................71
(2. 2. 3. 2.) Lipid extraction ..................................................................72
(2. 2. 3. 3.) Preparation of PC:oleic acid vesicles .....................................73
(2. 2. 3. 4.) Separation of lipids ..............................................................73
(2. 2. 4. 1.) Assay of CTP:phosphocholine cytidylyltransferase ....................74
(2. 2. 4. 2.) Analysis of choline-containing metabolites by TLC ..................74
(2. 2. 4. 3.) Liquid scintillation counting ..................................................75
(2. 2. 5. 1.) Estimation of protein .............................................................75

ANALYSIS OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE BY POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

(2. 2. 6. 1.) Preparation of sample for SDS-PAGE .....................................76
(2. 2. 6. 2.) SDS-PAGE .................................................................77
(2. 2. 6. 3.) 2D-PAGE .................................................................77
(2. 2. 6. 4.) IEF- and native-PAGE .............................................78

IMMUNOLOGICAL STUDIES
(2. 2. 7. 1.) Preparation of polyclonal antibody in rabbits ................78
(2. 2. 7. 2.) Separation of serum from blood .....................................79
(2. 2. 7. 3.) Detection of CTP:phosphocholine cytidylyltransferase
               with antibody using the Western-blot technique ..................79

EXPERIMENTS WITH HEPATOCYTES
(2. 2. 8. 1.) Digitonin-mediated release of CTP:phosphocholine
               cytidylyltransferase from hepatocytes ..................................81
(2. 2. 8. 2.) Radiolabelling studies with hepatocytes ........................81

PHOSPHORYLATION STUDIES
(2. 2. 9. 1.) Phosphorylation of CTP:phosphocholine cytidylyltransferase in vitro ....................................................82
(2. 2. 9. 2.) Identification of the amino acid phosphorylated on the
               cytidylyltransferase ..................................................................84
(2. 2. 9. 3.) Phosphorylation of CTP:phosphocholine cytidylyltransferase in vivo .................................................................85

AFFINITY COLUMN
(2. 2. 10. 1.) Preparation of aminophenylphosphorylcholine affinity
               column ..................................................................................85
(2. 2. 10. 2.) Preparation of diazo compound ....................................86
RESULTS

PURIFICATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

(3.1.1.1.) Purification by aggregation/deaggregation of cytosolic form ................................................................. 89

(3.1.2.1.) Purification by conventional and FPLC techniques .......... 94

(3.1.2.2.) Stabilization of cytidylyltransferase after Mono-Q column ............................................................................... 106

(3.1.2.3.) Chromatography of cytidylyltransferase after Mono-Q on CTP-agarose .............................................................. 109

(3.1.2.4.) Chromatography of cytidylyltransferase after Mono-Q on aminophenylphosphorylcholine affinity column ........... 111

(3.1.2.5.) Chromatography of cytidylyltransferase after Mono-Q on Superose 12 HR 10/30 column ............................................. 114

(3.1.2.6.) Chromatography of cytidylyltransferase after Mono-Q on hydroxylapatite column .................................................. 114

(3.1.2.7.) Chromatography of cytidylyltransferase after Mono-Q on hexyl-agarose column ...................................................... 116

(3.1.2.8.) Chromatography of cytidylyltransferase after Mono-Q on Green A column .............................................................. 119

(3.1.2.9.) Aggregation and isolation of cytidylyltransferase by density gradient centrifugation ...................................................... 119

(3.1.2.10.) Other purification steps tried ................................................. 121

(3.1.3.1.) Purification by lipid-protein aggregation and resolution on FPLC ................................................................. 124

CHARACTERIZATION OF PURIFIED CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

(4.1.1.1.) Native-PAGE ................................................................. 134
(4. 1. 1. 2.) IEF-PAGE  ........................................................................134
(4. 1. 1. 3.) 2D-PAGE  ........................................................................134

ANTIBODY GENERATION
(4. 1. 2. 1.) Generation of polyclonal antibodies in rabbits ...............134
(4. 1. 2. 2.) 2D-PAGE and detection by antibody  ....................................140

KINETICS
(4. 1. 3. 1.) Determination of $K_m$ for CTP and phosphocholine ..........140
(4. 1. 3. 2.) Dependence of rate of reaction on the amount of protein 140
(4. 1. 3. 3.) Dependence of rate of reaction on time  ..............................142
(4. 1. 3. 4.) Dependence of rate of reaction on temperature .................142
(4. 1. 4. 1.) Activation of CTP:phosphocholine cytidylyltransferase with commercial lipids .................................................................142
(4. 1. 4. 2.) Activation with microsomal lipids .........................................145
(4. 1. 5. 1.) Association of cytidylyltransferase with various membrane fractions in vitro .................................................................149
(4. 1. 6. 1.) Gel filtration of purified cytidylyltransferase and molecular weight estimation ...............................................................149

REGULATION OF CTP-PHOSPHOCHOLINE CYTIDYLTRANSFERASE BY REVERSIBLE PHOSPHORYLATION
(5. 1. 1. 1.) Effect of treatment of post mitochondrial supernatant with oleate ......................................................................................153
(5. 1. 1. 2.) Effect of treatment of post mitochondrial supernatant with BSA .......................................................................................153
(5. 1. 1. 3.) Effect of treatment of post mitochondrial supernatant with oleate ± BSA ..............................................................................153
5.1.1.4) Treatment of post mitochondrial supernatant with oleate under reversible phosphorylation conditions

5.1.2.1) Treatment of post mitochondrial supernatant with Mg$^{2+}$ and ATP

5.1.2.2) Treatment of post mitochondrial supernatant with protein kinase inhibitor

5.1.2.3) Treatment of post mitochondrial supernatant with cAMP-dependent protein kinase

5.1.2.4) Treatment of cytosol with cAMP-dependent protein kinase and chromatography on Sepharose 6B

5.1.3.1) Treatment of post mitochondrial supernatant with alkaline phosphatase

5.1.3.2) Treatment of post mitochondrial supernatant with NaF

5.1.4.1) Fractionation of post mitochondrial supernatant after reversible phosphorylation

5.1.4.2) Fractionation of post mitochondrial supernatant after treatment with BSA

5.2.1.1) Incubation of pure cytidylyltransferase with oleate in the presence of washed microsomes

5.2.2.1) Incubation of pure cytidylyltransferase under phosphorylating conditions in the presence of washed microsomes

5.2.2.2) Incorporation of $^{32}$P into cytidylyltransferase in vitro

5.2.3.1) Incubation of pure cytidylyltransferase under dephosphorylating conditions in the presence of washed microsomes

5.3.1.1) Incubation of pure cytidylyltransferase with phosphatidylcholine vesicles under phosphorylating conditions
Incubation of pure cytidylyltransferase with phosphatidylcholine vesicles under dephosphorylating conditions ................................................................. 179

Time course of incorporation of $^{32}P$ into the cytidylyltransferase ................................................................. 181

Analysis of the amino acid phosphorylated in the cytidylyltransferase ................................................................. 185

Incorporation of $^{32}P$ into pure cytidylyltransferase \textit{in vitro} and analysis by 2D-PAGE ................................................................. 185

Incorporation of $^{32}P$ into pure cytidylyltransferase \textit{in vivo} and analysis by 2D-PAGE ................................................................. 185

REGULATION OF CYTIDYLTRANSFERASE BY CALCIUM

Effect of Ca$^{2+}$ and EGTA on post mitochondrial supernatant ................................................................. 189

Fractionation of post mitochondrial supernatant after treatment with Ca$^{2+}$ and EGTA ................................................................. 191

Treatment of post mitochondrial supernatant with various cations ................................................................. 193

Treatment of post mitochondrial supernatant with Ca$^{2+}$ in the presence and absence of calmodulin ................................................................. 193

Digitonin-mediated release of cytidylyltransferase from hepatocytes by Ca$^{2+}$ ................................................................. 195

Digitonin-mediated release of cytidylyltransferase from hepatocytes by Ca$^{2+}$ in the presence of verapamil ................................................................. 197

Digitonin-mediated release of cytidylyltransferase from hepatocytes by ionophore A23187 ................................................................. 197

Digitonin-mediated release of cytidylyltransferase from hepatocytes by vasopressin ................................................................. 199
(6.1.3.1.) Subcellular fractionation after treatment with Ca\textsuperscript{2+} and verapamil .............................................202

(6.1.3.2.) Subcellular fractionation after treatment with vasopressin and ionophore A23187 ...........................................205

(6.1.4.1.) Effect of Ca\textsuperscript{2+} on PC synthesis .................................................205

(6.1.4.2.) Effect of Ca\textsuperscript{2+} on PC degradation .................................................209

TREATMENT WITH PHOSPHOLIPASE

(6.2.1.1.) Digitonin-mediated release of cytidylyltransferase by phospholipase C and A\textsubscript{2} .............................................211

(6.2.2.1.) Subcellular fractionation after treatment with phospholipase A\textsubscript{2} .................................................214

(6.2.3.1.) Effect of phospholipase A\textsubscript{2} on PC synthesis .............................................215

DISCUSSION

PURIFICATION AND CHARACTERIZATION OF CYTIDYLTRANSFERASE

(7.1.1.1.) Purification of CTP:phosphocholine cytidylyltransferase .............................................221

(7.2.1.1.) Analysis by PAGE .................................................222

(7.2.1.2.) Lipid activation of purified cytidylyltransferase .............................................223

(7.2.1.3.) Generation of polyclonal antibodies .................................................224

REGULATION OF CYTIDYLTRANSFERASE BY REVERSIBLE PHOSPHORYLATION

(7.3.1.1.) Regulation of cytidylyltransferase by reversible phosphorylation in vitro in post mitochondrial supernatant .............................................225
(7.3.1.2.) Using purified cytidylyltransferase and washed microsomes .......................................................... 226

(7.3.1.3.) Incorporation of $^{32}$P into cytidylyltransferase ...................... 227

(7.3.1.4.) Using purified cytidylyltransferase with phosphatidylcholine vesicles .................................................... 228

(7.3.1.5.) Phosphorylation of cytidylyltransferase in vivo .................... 231

REGULATION OF CYTIDYLTRANSFERASE BY CALCIUM

(7.4.1.1.) In post mitochondrial supernatant ........................................ 231

(7.4.2.1.) Digitonin-mediated release of cytidylyltransferase from hepatocytes ............................................................. 232

(7.4.2.1.) Ca$^{2+}$ stimulates PC synthesis ............................................. 233

(7.4.2.2.) Ca$^{2+}$ stimulates PC degradation ........................................... 235

EFFECT OF PHOSPHOLIPASE A$_2$ ON CYTIDYLTRANSFERASE

(7.5.1.1.) Phospholipase A$_2$ decreases digitonin-mediated release of cytidylyltransferase from hepatocytes .......... 237

(7.5.2.1.) Phospholipase A$_2$ stimulates PC synthesis ........................... 237

(7.6.1.1.) Future directions .................................................................. 239
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Commercial source of research materials</td>
<td>87</td>
</tr>
<tr>
<td>2. Purification of cytidylyltransferase by aggregation/deaggregation</td>
<td>96</td>
</tr>
<tr>
<td>3. Purification of cytidylyltransferase by conventional and FPLC techniques</td>
<td>104</td>
</tr>
<tr>
<td>4. Summary of purification of cytidylyltransferase after Mono-Q by various techniques</td>
<td>123</td>
</tr>
<tr>
<td>5. Summary of cytidylyltransferase purification by lipid-protein aggregation and resolution on FPLC</td>
<td>132</td>
</tr>
<tr>
<td>6. Effect of phosphorylating and dephosphorylating conditions on pure cytidylyltransferase in the presence of washed microsomes</td>
<td>176</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Structure of phosphatidylcholine</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Phosphatidylcholine (PC) biosynthesis via the CDP-choline (de novo) pathway</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Phosphatidylcholine (PC) synthesis by successive N-methylation from phosphatidylethanolamine (PE)</td>
<td>19</td>
</tr>
<tr>
<td>4.</td>
<td>Pathway of conversion of citrate into lipids in mammals</td>
<td>25</td>
</tr>
<tr>
<td>5.</td>
<td>Model for regulation of HMG-CoA reductase activity</td>
<td>29</td>
</tr>
<tr>
<td>6.</td>
<td>Comparison of purification schemes for cytidylyltransferase</td>
<td>90</td>
</tr>
<tr>
<td>7.</td>
<td>Ammonium sulfate precipitation of aggregated and non-aggregated cytidylyltransferase</td>
<td>92</td>
</tr>
<tr>
<td>8.</td>
<td>Chromatography of aggregated cytidylyltransferase on 1st Sepharose 6B column</td>
<td>93</td>
</tr>
<tr>
<td>9.</td>
<td>Chromatography of dissociated cytidylyltransferase on 2nd Sepharose 6B column</td>
<td>95</td>
</tr>
<tr>
<td>10.</td>
<td>SDS-PAGE of sample after purification by aggregation/deaggregation</td>
<td>97</td>
</tr>
<tr>
<td>11.</td>
<td>Chromatography of cytidylyltransferase on DEAE-cellulose</td>
<td>99</td>
</tr>
<tr>
<td>12.</td>
<td>Chromatography of cytidylyltransferase eluted from DEAE-cellulose, on phospho-cellulose column</td>
<td>100</td>
</tr>
<tr>
<td>13.</td>
<td>Chromatography of cytidylyltransferase eluted from phospho-cellulose, on Mono-Q column</td>
<td>102</td>
</tr>
<tr>
<td>14.</td>
<td>Chromatography of cytidylyltransferase eluted from Mono-Q, on Mono-S column</td>
<td>103</td>
</tr>
</tbody>
</table>
15. SDS-PAGE of sample after purification by conventional and FPLC techniques ................................................................. 105

16. Stabilization of partially purified cytidylyltransferase activity .... 108

17A. Chromatography of cytidylyltransferase eluted from Mono-Q, on CTP-agarose column ..................................................... 110

17B. SDS-PAGE of sample after purification on CTP-agarose ........... 112

18. Chromatography of cytidylyltransferase eluted from Mono-Q, on aminophenylphosphorylcholine affinity column ..... 113

19. Chromatography of cytidylyltransferase eluted from Mono-Q, on Superose 12 HR 10/30 column ............................................. 115

20. Chromatography of cytidylyltransferase eluted from Mono-Q, on hydroxylapatite column ................................................... 117

21. Chromatography of cytidylyltransferase eluted from Mono-Q, on hyxyl-agarose column ....................................................... 118

22. Chromatography of cytidylyltransferase eluted from Mono-Q, on Green-A column ............................................................... 120

23. Isolation by density gradient centrifugation of aggregated cytidylyltransferase .................................................................... 122

24. Chromatography of cytidylyltransferase, after aggregation with lipid and acid precipitation, on DEAE-Sepharose CL-6B column ......................................................................................... 126

25A. Chromatography of cytidylyltransferase, after DEAE-Sepharose CL-6B, on hydroxylapatite column ........................................ 127

25B. SDS-PAGE of sample after purification on DEAE-Sepharose CL-6B and hydroxylapatite .......................................................... 129

26A. Chromatography of cytidylyltransferase, after hydroxylapatite, on Mono-Q column ............................................................... 130

26B. SDS-PAGE of sample after purification on Mono-Q column .......... 131
27. Native-PAGE of purified cytidylyltransferase .................................................135
28. IEF-PAGE of purified cytidylyltransferase .......................................................136
29. 2D-PAGE of purified cytidylyltransferase .........................................................137
30. Detection of antibody against cytidylyltransferase, in the serum from a rabbit, using the Western-blot technique .............................139
31. 2D-PAGE of cytidylyltransferase and detection by antibody from chicken ..........................................................141
32. Determination of Km of the purified cytidylyltransferase for CTP and phosphocholine ..................................................143
33. Linearity of the cytidylyltransferase catalyzed reaction with varying amounts of cytidylyltransferase ................................144
34. Linearity of the cytidylyltransferase catalyzed reaction at various time intervals .....................................................................144
35. Rate of cytidylyltransferase catalyzed reaction at various temperatures ........................................................................146
36. Activation of cytidylyltransferase with commercial lipids ..........147
37. Activation of cytidylyltransferase with microsomal lipids ..........148
38. Association of cytidylyltransferase with various membranes in the presence or absence of oleate ........150
39. Chromatography of cytidylyltransferase on Sephacryl S-300 and molecular weight estimation ........................................152
40. Treatment of post mitochondrial supernatant with oleate ..........154
41. Treatment of post mitochondrial supernatant with BSA ..........154
42. Treatment of post mitochondrial supernatant with oleate in the presence or absence of BSA ........................................156
43. Treatment of post mitochondrial supernatant with oleate under reversible phosphorylating conditions ................................156
<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>44.</td>
<td>Treatment of post mitochondrial supernatant with Mg\textsuperscript{2+} and ATP</td>
</tr>
<tr>
<td>45.</td>
<td>Treatment of post mitochondrial supernatant with protein kinase inhibitor</td>
</tr>
<tr>
<td>46.</td>
<td>Effect of cAMP-dependent protein kinase on cytidylyltransferase activity in post mitochondrial supernatant</td>
</tr>
<tr>
<td>47.</td>
<td>Treatment of cytosol with cAMP-dependent protein kinase and chromatography on Sepharose 6B</td>
</tr>
<tr>
<td>48.</td>
<td>Treatment of post mitochondrial supernatant with alkaline phosphatase</td>
</tr>
<tr>
<td>49.</td>
<td>Treatment of post mitochondrial supernatant with NaF</td>
</tr>
<tr>
<td>50.</td>
<td>Fractionation of post mitochondrial supernatant after phosphorylating or dephosphorylating conditions</td>
</tr>
<tr>
<td>51.</td>
<td>Fractionation of post mitochondrial supernatant after treatment with BSA</td>
</tr>
<tr>
<td>52.</td>
<td>Incubation of pure cytidylyltransferase with oleate in the presence of washed microsomes</td>
</tr>
<tr>
<td>53.</td>
<td>Incubation of pure cytidylyltransferase with cAMP-dependent protein kinase in the presence of washed microsomes</td>
</tr>
<tr>
<td>54.</td>
<td>Phosphorylation of cytidylyltransferase with [\gamma^{32}\text{P}]ATP</td>
</tr>
<tr>
<td>55.</td>
<td>Incorporation of 32P into cytidylyltransferase under phosphorylating conditions in the presence of washed microsomes</td>
</tr>
<tr>
<td>56.</td>
<td>Effect of alkaline phosphatase on pure cytidylyltransferase in the presence of washed microsomes</td>
</tr>
<tr>
<td>57.</td>
<td>Incubation of 32P-labeled cytidylyltransferase under dephosphorylating conditions in the presence of washed microsomes</td>
</tr>
</tbody>
</table>
58. Effect of cAMP-dependent protein kinase on alkaline phosphatase-treated cytidylyltransferase in the presence of phosphatidylcholine vesicles ..........................................................178

59. Incorporation of $^{32}$P into cytidylyltransferase under phosphorylating conditions in the presence of phosphatidylcholine vesicles ..........................................................180

60. Effect of alkaline phosphatase on pure cytidylyltransferase activity in the presence of phosphatidylcholine vesicles ............182

61. Incubation of $^{32}$P-labeled cytidylyltransferase under phosphorylating conditions in the presence of phosphatidylcholine vesicles ..........................................................183

62. Time course of amount of $^{32}$P incorporated into normal and alkaline phosphatase-treated cytidylyltransferase ..................184

63. Analysis of amino acid phosphorylated on cytidylyltransferase ...186

64. Incorporation of $^{32}$P into cytidylyltransferase in vitro and analysis by 2D-PAGE ..............................................................................187

65. Treatment of post mitochondrial supernatant with Ca$^{2+}$ .............190

66. Treatment of post mitochondrial supernatant with EGTA .............190

67. Time course of treatment of post mitochondrial supernatant with Ca$^{2+}$ and EGTA ..............................................................192

68. Fractionation of post mitochondrial supernatant after treatment with Ca$^{2+}$ and EGTA ..............................................................192

69. Fractionation of post mitochondrial supernatant after treatment with various concentrations of Ca$^{2+}$ ..........................................194

70. Treatment of post mitochondrial supernatant with various cations ..............................................................194

71. Treatment of post mitochondrial supernatant with Ca$^{2+}$ in the presence or absence of calmodulin ..........................................196
72. Effect of Ca\(^{2+}\) on digitonin mediated release of cytidylyltransferase ................................................................. 198

73. Effect of verapamil on digitonin mediated release of cytidylyltransferase ................................................................. 200

74. Effect of ionophore A23187 on digitonin mediated release of cytidylyltransferase ................................................................. 201

75. Effect of vasopressin on digitonin mediated release of cytidylyltransferase ................................................................. 203

76. Effect of Ca\(^{2+}\) on cytidylyltransferase translocation ................................................................. 204

77. Effect of vasopressin on cytidylyltransferase translocation ................................................................. 206

78. Effect of ionophore A23187 on cytidylyltransferase translocation ................................................................. 206

79. Effect of Ca\(^{2+}\) during pulse on PC synthesis ................................................................. 208

80. Time course of Ca\(^{2+}\) effect during pulse on PC synthesis ................................................................. 208

81. Effect of Ca\(^{2+}\) during pulse-chase on PC synthesis ................................................................. 210

82. Effect of Ca\(^{2+}\) on LPC formation ................................................................. 212

83. Effect of Ca\(^{2+}\) on GPC formation ................................................................. 213

84. Effect of phospholipase C on digitonin mediated release of cytidylyltransferase ................................................................. 216

85. Effect of phospholipase A\(_2\) on digitonin mediated release of cytidylyltransferase ................................................................. 217

86. Effect of phospholipase A\(_2\) on cytidylyltransferase translocation ................................................................. 218

87. Effect of phospholipase A\(_2\) on PC synthesis ................................................................. 220
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>aqueous counting scintillant</td>
</tr>
<tr>
<td>Ado-Met</td>
<td>S-adenosyl-methionine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3':5'-monophosphate</td>
</tr>
<tr>
<td>CK</td>
<td>choline kinase</td>
</tr>
<tr>
<td>CM</td>
<td>carboxy-methyl</td>
</tr>
<tr>
<td>cpm</td>
<td>count per minute</td>
</tr>
<tr>
<td>CPT</td>
<td>cholinephosphotransferase</td>
</tr>
<tr>
<td>CT</td>
<td>CTP:phosphocholine cytidylyltransferase</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DG</td>
<td>diglyceride</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegration per minute</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminotetraacetate</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis (aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EK</td>
<td>ethanolamine kinase</td>
</tr>
<tr>
<td>E.R.</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>GPC</td>
<td>glycerophosphorylcholine</td>
</tr>
</tbody>
</table>
h

hour

Km

Michaelis-Menten constant

l

liter

LCAT

lecithin cholesterol acyltransferase

LPC

lysophosphatidylcholine

LPE

lysophosphatidylethanolamine

M

molar

MEM

modified Eagle's medium

min

minute

PAGE

polyacrylamide gel electrophoresis

PBS

phosphate buffered saline

PC

phosphatidylcholine

PDME

phosphatidyldimethylethanolamine

PE

phosphatidylethanolamine

PEMT

phosphatidylethanolamine methyltransferase

PG

phosphatidylglycerol

PI

phosphatidylinositol

PMME

phosphatidylmonomethylethanolamine

PS

phosphatidylserine

ref.

reference

S.D.

standard deviation

SDS

sodium dodecyl sulfate

TG

triglyceride

TLC

thin layer chromatography

TPA

12-o-tetradecanoyl-phorbol-13-acetate

Tris

tris( hydroxymethyl)aminomethane

TRLP

total rat liver phospholipid
$V_{\text{max}}$ maximum velocity
ACKNOWLEDGEMENT

I am most grateful to my supervisor, Dr. Dennis E. Vance, who encouraged and guided me through difficult periods of the project. I am also grateful to Dr. Steven Pelech, who aided me in the early stages of the study and during the writing of the thesis. I thank Harris, Brenton, Zemin Yao, Neale, Jean, Amandeep, Sandi, Rocky, Sharlene, Suzanne, Marilyn, Penny, Kerry Ko, Harry, Fateem, Maggie, Howard, Rosemary, Zing-Mei, Z. C, and Kozo for their ideas and technical aid. Finally, I wish to thank my father, Karnail Singh; mother, Gurdev Kaur; wife, Kuljit; sister, Parminder; brother, Sukhdev; son, Bhavendep; and maternal-uncle, Surinder Atwal for their encouragement and support.
INTRODUCTION

PHOSPHATIDYLCHOLINE-STRUCTURE AND FUNCTION

Structure of phosphatidylcholine (1. 1. 1. 1.).

Phosphatidylcholine (PC) is ubiquitous and quantitatively the most important phospholipid in eucaryotes with little occurrence in procaryotes. It comprises between 20-40% of the total lipid in human cells (1). PC is comprised of a glycerol backbone to which fatty acyl chains and a choline head group are esterified. It is the choline head group which distinguishes PC from other phospholipids. Although a variety of fatty acids can be esterified to the glycerol backbone, usually saturated fatty acids are esterified in the C1 position, while unsaturated fatty acids are esterified in the C2 position. A space-filling model and structure of PC showing location of the charged groups is represented in Figure 1.

FUNCTIONS OF PHOSPHATIDYLCHOLINE
Component of biological membranes (1. 1. 2. 1.).

PC has a diverse range of functions within the body. The numerous functions of PC arise from its amphipathic nature - the choline headgroup is hydrophilic, while the fatty acyl chains are hydrophobic. This feature allows PC (and other phospholipids) to readily form membrane bilayer structures when hydrated, whereby the hydrophilic groups are exposed to the outside and the hydrophobic groups are in the interior of the bilayer. Thus PC, together with other phospholipids, serve as structural matrix for the
Figure 1. Structure of Phosphatidylcholine (PC)
protein components of all biological membranes as detailed in the fluid mosaic model of Singer and Nicholson (2). In addition, the membrane bilayer serves as anchorage for glycoproteins, and is a non-aqueous medium for reactions involving lipid soluble metabolites. In eucaryotes, PC accounts for over 50% of the membrane phospholipid.

Component of lung surfactant (1. 1. 2. 2.).

PC is a major component of lung surfactant (which is almost 80% phospholipid by weight, of which PC accounts for 80-90% (3)). Surfactant is the surface active material which lines the alveoli of the lungs to lower the surface tension at the air-alveolar interface and thus prevent alveolar collapse during expiration. Surfactant is synthesized and secreted by the type II alveolar epithelial cells (4-6). Dipalmitoyl PC is the major PC species present in surfactant (7). Whether dipalmitoyl PC is made via the CDP-choline pathway or arises mainly via deacylation/reacylation of PC remains undecided. Deficiency in surfactant production is thought to be responsible for Infant Respiratory Distress Syndrome (8), which is the major cause of death of the newborn.

Component of lipoproteins (1. 1. 2. 3.)

The plasma lipoproteins facilitate the transport of cholesterol, cholesterol-esters, and triglycerides between the intestines, liver, and adipose. The outside coat of the mature lipoprotein particles contains phospholipid, of which PC accounts for 60% of the phospholipid present in human lipoproteins and 75% in rat
lipoproteins (9). The importance of PC in lipoprotein assembly is clearly demonstrated in choline deficiency. Choline deficiency, which blocked the CDP-choline pathway for PC synthesis, led to a decrease in VLDL secretion with no effect on HDL secretion (10). It was further established that the inhibitory effect of choline deficiency on VLDL secretion can be compensated by the methylation of PE to form PC.

Component of bile (1. 1. 2. 4.).

Bile salts, which are secreted by the liver, are amphiphilic compounds and they dissolve at the oil-water interface with their hydrophobic faces pointing into the oil and the hydrophilic surfaces pointing into the aqueous phase of the luminal content. Bile salts are essential for the efficient solubilization, digestion, and absorption of triglyceride in the upper small intestine. In addition bile salt facilitates the excretion of bilirubin and other waste products. PC is a component of bile and it functions together with conjugated bile acids for the solubilization of cholesterol in bile (11). Bile PC is composed of mainly the 1-palmitoyl, 2-linoleoyl species (12).

Acyl donor to cholesterol esters (1. 1. 2. 5.).

In the plasma, PC plays an important role in the metabolism of lipoprotein cholesterol. The enzyme lecithin:cholesterol acyltransferase (LCAT) (EC 2. 3. 1. 43), which is an important component of the plasma lipoprotein lipid transport system, is responsible for the formation of cholesterol esters by promoting the transfer of the acyl chain on the 2-position of PC to the 3-hydroxyl
group of unesterified cholesterol (13, 14). The normal substrates for this enzyme are nascent and mature high density lipoproteins which provide the PC and cholesterol reagents and contain the activator apolipoprotein A-I. The significance of this reaction is realised from studies on the plasma of patients with familial LCAT deficiency who have shown the presence of discoidal particles of HDL density, rich in phospholipids and apo E but low in cholesterol esters (15).

**Precursor for sphingomyelin synthesis (1. 1. 2. 6.).**

Sphingomyelin is the major phospholipid in the myelin sheath (16), which wraps around the neurones permitting rapid conduction of impulses. Sphingomyelin, together with PC, are also the main lipid components in the outer leaflet of the red cell membrane in humans. Sphingomyelin was initially thought to be produced from CDP-choline and ceramide (17), but recent studies, using pulse-chase experiments with $[^3\text{H}]$choline, have clearly identified PC as the donor of the phosphocholine moiety to the ceramide (18-21).

**Precursor for prostaglandin synthesis (1. 1. 2. 7.).**

Prostaglandins are lipophilic molecules which participate in a variety of physiological processes. This includes control of platelet aggregation, modulation of synaptic transmission, control of ion transport across membranes, inhibition of the hydroosmotic effect of antidiuretic hormone (ADH), and regulation of blood flow to particular organs. Arachidonic acid is the normal precursor fatty acid in the synthesis of prostaglandins. Arachidonic acid is the major fatty acid at the $sn$-2 position of PC and PI in platelets and may be formed
by the action of a specific phospholipase A$_2$ or phospholipase C and diglyceride lipase.
THE ENZYMES AND PATHWAYS OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS.

The CDP-choline (Kennedy) Pathway (1. 2. 1. 1.).

This is the major pathway for the biosynthesis of PC. This pathway was elucidated largely by Kennedy and co-workers (23). Initially, they noted that CTP, and not ATP, was the essential cofactor involved in the de novo synthesis of all phospholipids. The enzymatic synthesis of CDP-choline form phosphocholine and CTP was then described (24). This reaction was catalyzed by CTP:phosphocholine cytidylyltransferase, which was located both in the cytosolic and the microsomal fraction of guinea pig liver (24). The final reaction for the formation of PC was shown by Kennedy and Weiss (25) to involve the condensation of diglyceride with CDP-choline, a reaction catalyzed by a microsomal enzyme CDP-choline:diglyceride cholinephosphotransferase. The overall scheme of the pathway is shown in Figure 2.

Choline Kinase (1. 2. 1. 2.).

Choline kinase (CK) (EC 2. 7. 1. 32) catalyzes the phosphorylation of choline yielding cholinephosphate in the presence of ATP and Mg$^{2+}$. This is the first enzymatic step committing choline to the de novo (CDP-choline) pathway in all animal tissues leading to the biosynthesis of PC.

It is a general biochemical principle that the first committed reaction in a pathway is under metabolic control and is rate-limiting. In view of this principle, the role of CK as a regulatory step in PC
(CH$_3$)$_3$N—CH$_2$—CH$_2$OH + ATP $\xrightarrow{\text{choline kinase}}$ (CH$_3$)$_3$N—CH$_2$—CH$_2$—O—P—O$^-$ + ADP

**Phosphocholine**

(CH$_3$)$_3$N—CH$_2$—CH$_2$—O—P—O$^-$ + CTP $\xrightarrow{\text{CTP}:\text{phosphocholine cytidylyltransferase}}$ CDP-choline + PP$_i$

**Phosphocholine**

CDP-choline + R—C—O—C—H $\xrightarrow{\text{CDP-choline:1,2-diacylglycerol}}$ 1,2-diacylglycerol

**Diacylglycerol**

CMP + R—C—O—CH $\xrightarrow{\text{phosphocholinetransferase}}$

**Phosphatidylcholine**

Figure 2. **Phosphatidylcholine (PC) biosynthesis via the CDP-choline (de novo) pathway.**
biosynthesis has been proposed by several investigators (26-30). However, other approaches have identified the second reaction catalyzed by CTP:phosphocholine cytidylyltransferase is the rate-limiting step. Measurement of pool sizes have demonstrated the choline pool size to be 5-10 fold lower than phosphocholine in the liver (31), and HeLa cells (32) indicating a rapid conversion of choline to phosphocholine with the rate-limiting step subsequent to the CK reaction. Furthermore, pulse-chase experiments have demonstrated that labeled choline was rapidly converted to phosphocholine and the rate of PC biosynthesis was not accompanied by a corresponding change in CK activity (32,33-35). Therefore, there is an apparent discrepency between theoretical predictions and experimental evidence that the CK is the rate-limiting enzyme in PC biosynthesis.

CK is exclusively a cytosolic enzyme. Since the discovery of CK from yeast extract by Wittenberg and Kornberg (36) in 1953, many attempts have been made to purify this key enzyme. However, only recently has the complete purification of CK been reported. CK has been purified from rat kidney cytosol to homogeneity with respect to both native and SDS-PAGE (37). The purified enzyme has a molecular weight of 42Kd on SDS-PAGE. Gel filtration on Sephadex G-150 of the purified enzyme indicated that the native enzyme is most likely a dimer. Specific antibodies raised against the purified enzyme cross reacted with CK preparations from various rat tissues. The antiserum almost completely inhibited CK activity in crude preparations, not only from the kidney but also from lung, intestine, and liver cytosol
This indicated that the CK species in various rat tissues is similar in nature to that found in the kidney.

A lot of controversy has existed on whether the CK and ethanolamine kinase (EK) activity resides on the same protein. Earlier evidence had demonstrated the presence of different kinases for choline and ethanolamine in rat liver (38), lactating bovine mammary glands (39), and spinach leaves (40). However, Ishidate et al (37,41) demonstrated that the EK co-purified with the pure CK indicating that both activities reside on the same enzyme protein. This was supported by the inhibition of EK activity by CK-antiserum. Kinetic data with highly purified kidney enzyme further suggested that both kinase reactions are catalyzed on two distinct active sites on a single enzyme protein (41).

Several lines of evidence have indicated that CK exists in several different isoenzymic forms in various tissues (41,42). For example, three forms of CK (termed CK-I, -II, -III) have been identified in rat liver (42). These forms differ with respect to their isoelectric point and apparent molecular size. The physiological significance of the presence of more than one form of CK in rat tissues is not known.

Recently, the activity of CK in liver has been reported to be inducible in certain physiological states. Treatment of animals (or cells) with excess choline (43), insulin (44), essential fatty acid-deficient diet (27), and a synthetic steroid hormone, diethylstilbestrol (45, 46) led to induction of CK which was mediated via synthesis of new enzyme. Administration to rats of several hepatotoxic chemicals such as polycyclic aromatic
hydrocarbon carcinogens (47, 48) and carbon tetrachloride (CCl₄) (49) also caused a significant elevation of hepatic CK activity which was mediated by an increase in enzyme synthesis. Thus, CK is the only enzyme in the PC biosynthetic pathway that appears to be regulated at the level of gene expression.

**CTP:phosphocholine Cytidylyltransferase (1. 2. 1. 3.).**

CTP:phosphocholine cytidylyltransferase (CT) (EC 2. 7. 7. 15) catalyzes the synthesis of CDP-choline and pyrophosphate from CTP and phosphocholine. A number of different approaches have provided strong evidence that the reaction catalyzed by the CT is the key regulatory step in PC biosynthesis. An early indication for the rate-limiting role of CT came from studies of Sundler et al (31) when they demonstrated that the phosphocholine pool in rat liver (1.4 μmol/g liver) is 150 times higher than the CDP-choline pool. As CDP-choline is the only known metabolite of phosphocholine, these results suggest that once CDP-choline is formed it is quickly converted to PC or, to a lesser extent, sphingomyelin. Similarly, a ratio of 50:1 has been found for the relative pool sizes of phosphocholine and CDP-choline in HeLa cells (32). More definitive evidence for the rate-limiting role of CT have been obtained from pulse-chase experiments (50-57). These have shown that the radioactivity from exogenously added [³H]choline is quickly converted to phosphocholine (~97% at the end of a 1h pulse). Subsequently, as the radioactivity disappeared from the phosphocholine, it was transiently associated with CDP-choline and immediately converted into PC.
CT is ambiguous in that it is recovered in both the cytosolic and the microsomal fractions of rat liver homogenates (58, 59). The distribution of enzyme activity between the two compartments is dependent on the conditions of homogenization (59). Homogenization of rat liver in the presence of 0.15 M NaCl results in majority (~70-80%) of the CT activity residing in the cytosolic fraction, whereas homogenization in water or 0.25 M sucrose results in majority (~70-80%) of the CT activity residing in the microsomal fraction (60). Thus, the ionic strength of the homogenizing medium can determine, to some extent, the amount of CT associated with the cytosolic and the microsomal fractions.

The microsomal form of CT has been identified as the active enzyme form whereas the cytosolic form is inactive (61-64). The inactive cytosolic form can be activated by certain anionic phospholipids (including PS, PG, and PI) and by PC:fatty acid vesicles (22, 53, 65). Numerous studies have correlated the increase in PC synthesis with an increase in CT associated with the microsomal fraction (61, 63, 64, 66, 67). Thus, the CT in the cytosol seems to be a reservoir of inactive enzyme that can be translocated to the microsomes where the enzyme is stimulated by certain phospholipid species. There are several advantages to regulation of CT activity this way. Induction of CT protein synthesis is not required for increased availability of the enzyme, and the activation of the enzyme can be rapid and reversible. Also the product of the membrane-associated CT reaction, CDP-choline, is probably in close proximity to the last enzyme of the pathway, namely CDP-choline:diacylglycerol cholinephosphotransferase. Vance et al (68) have shown that the
microsomal CT is exposed on the cytoplasmic face of the sealed fragments of the endoplasmic reticulum (E. R.) that form during homogenization.

Initially it was thought that the CT existed in two forms in the cytosol. The CT in fresh rat liver cytosol was designated as the L-form (low molecular weight form) with a molecular weight of ~200,000 (69). Incubation of the cytosol at 4 °C for 5 days (69), or 20 °C for 8 h (70) resulted in elevation of CT activity by 7-fold and a corresponding increase in molecular weight to 0.5-13 x 10^6 daltons. This was designated as the H-form (high molecular weight form). It now appears that the H-form is an artifact and is formed by CT binding to membrane fragments, which are generated during homogenization and contaminate the cytosol (J. Sanghera and D. Vance, unpublished results). This observation is supported by the fact that both the H-form and the microsomal form are immunologically identical (71), and the H-form can be pelleted under conditions similar to those which will sediment the microsomes.

Considerable efforts have been made to purify this enzyme to homogeneity. Until recently, most of the attempts were unsuccessful. This may have been because the rat liver cytosol was used as the source of CT. Since the CT in the cytosol is inactive and requires lipids for activation, purification procedures resulted in separation of CT from contaminating membrane fragments which resulted in a rapid loss of enzyme activity. Thus, as the purification of CT increased, this was accompanied by an increase in enzyme inactivation. This problem was overcome to some extent by inclusion of detergents in the buffers. Recently, Weinhold et al (72) reported the purification of
CT to homogeneity. Their purification procedure included aggregation of CT with PC:fatty acid vesicles and then selective dissociation by detergents and purification by conventional chromatography. They reported initially that the CT existed of two subunits; a 48 Kd and a 39 Kd subunit as determined by SDS-PAGE (72). They then reported separation of the two subunits into a 45 Kd and a 38 Kd species (73). The 45 Kd subunit was reported to be the catalytic subunit whereas the 38 Kd subunit was thought to be regulatory. The purified enzyme had maximal activity at pH 7.0. The $K_m$ for CTP was 0.2 mM, while for phosphocholine it was 0.14 mM. These values are essentially the same as those previously reported by Choy et al (69) for partially purified CT from rat liver. The $K_m$ for CTP depends on the amount of phospholipid associated with CT. In the presence of phospholipid, the enzyme has a $K_m$ of 0.2-0.3 mM while the delipidated form has a $K_m$ of about 5 mM. The $K_m$ for phosphocholine does not depend on the amount of phospholipid present.

*In vivo* and *in vitro* work with CT has shown free fatty acids (63, 73) and phosphorylation/dephosphorylation (74, 75) as two mechanisms of short term regulation of CT activity. *In vitro* addition of 100 μM arachidonate, oleate, or palmitate stimulates rat liver cytosolic CT activity by 3-4 folds (63). In each case activation was only seen in the presence of phospholipid indicating that the fatty acid may enhance affinity of the enzyme for the phospholipid which in turn activates the enzyme. Long chain fatty acids also stimulated approximately 2-fold [methyl-$^3$H]choline incorporation from phosphocholine into PC by isolated Hepatocytes (63).
CAMP analogues and phosphodiesterase inhibitors have been shown to reduce the rate of PC synthesis via CDP-choline in cultured monolayers of rat hepatocytes (75). This inhibition could be correlated with reduced CT activity in microsomes recovered from hepatocytes treated with cAMP analogues. Similarly, in vitro experiments with magnesium and ATP led to inhibition of enzyme activity when cytosol from rat liver was incubated at 37 °C for 10 min (74). However incubation with protein kinase inhibitors prevented this inhibition (74). The CT could also be activated by phosphatase activity and this could be prevented with NaF.

CDP-choline:1,2-diacylglycerol cholinephosphotransferase (1. 2. 1. 4.).

The final step in the de novo pathway is the formation of PC and CMP from diglyceride and CDP-choline, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) (EC 2. 7. 8. 2). The CPT has been localized to the smooth and rough endoplasmic reticulum membranes in liver (59, 76, 77), and lung (78). The golgi, mitochondria, nuclear, and plasma membrane from rat liver contain negligible activity (76). However, Baker and Chang (79) reported high levels of CPT activity in the nuclear envelope fractions from cerebral cortex.

The transfer of cholinephosphate to diacylglycerol in isolated microsomes requires CDP-choline, magnesium or manganese, and is greatly stimulated by the addition of diacylglycerol. The CPT activity is dependent on the manner of diacylglycerol presentation. Sonicated preparations of diacylglycerol in Tween-20, which can stimulate CPT
activity (80), has become the most common way to add this substrate.

CPT activity in isolated sealed microsomes is trypsin (68), chymotrypsin and pronase (81) sensitive suggesting that its active site faces the cytoplasmic surface. The CPT is an integral membrane protein since it cannot be released from membranes by high ionic strength buffer (1 M KCl) or low concentrations of deoxycholate that render the vesicles leaky. CPT is only removed from the membrane by detergents at concentrations which solubilize the membrane lipid and protein (82). However, membrane solubilization by high detergent concentrations leads to > 90% inhibition of enzyme activity. This is probably due to disruption of the phospholipid environment by the detergent which is crucial for enzyme activity.

The sensitivity of CPT to detergents has posed a major block towards its purification. Kanoh and Ohno (83) purified CPT 4-fold from rat liver microsomes by a two-step sonication procedure in buffer containing 4 mM deoxycholate and 20% glycerol. The first sonication was done at pH 7.4 and the second at pH 8.5. The enzyme remained active in the presence of glycerol, but eluted in the void volume of a Sepharose 4B column indicating that the activity was part of a large aggregate. Further attempts at solubilization with Triton X-100 led to enzyme inactivation.

Recently Cornell and MacLennan reconstituted CPT activity from sarcoplasmic reticulum after solubilization and > 90% inactivation by cholate, deoxycholate, Triton X-100, or octylglucoside (82). Soybean phospholipid was added to the solubilized preparation and the detergent was removed by dialysis, gel filtration, or
adsorption to Biobeads SM-2. Recoveries of activity were up to 100% after treatment with Triton X-100, octylglucoside or deoxycholate, and up to 60% after cholate treatment. Inclusion of CDP-choline in the solubilization buffer improved the recovery from octylglucoside-solubilized samples but had no effect on recovery from deoxycholate-treated samples. At high detergent:protein weight ratios (> 10), the enzyme was irreversibly inactivated. Diacylglycerol and glycerol protected the enzyme from permanent denaturation if they were included in the solubilization medium. A combination of 40% glycerol and 2% diacylglycerol:soybean phospholipid (1:3) added to 2% cholate-solubilized preparation, restored 99% of the activity. With no additions, only 3% of the original activity was recovered.

CPT is a separate enzyme from the ethanolaminephosphotransferase (EPT). Evidence for distinct CPT and EPT has been obtained from studies with rat liver. Kanoh et al (83) cosolubilized both activities and found that CPT separated into a magnesium- and manganese-dependent component, while the EPT only associated with a manganese-dependent component. Additional evidence for distinct enzymes was provided by different specificities of these enzymes for the diglyceride substrate. CPT favours palmitate rather than stearate at position 1 of diglyceride, while the opposite holds true for EPT. Also EPT selects for eicosahexaenoate at position 2 of diglyceride, while CPT shows a slight preference for linoleate at this position.

The rate of PC biosynthesis does not seem to be influenced by CPT in all systems examined to date. Rather, there appears to be an
excess of CPT in cells and its activity appears to be controlled by the supply of CDP-choline via the cytidylyltransferase reaction.

Phosphatidylethanolamine N-Methylation Pathway (1. 2. 2. 1.).

The conversion of PE to PC by the sequential donation of methyl groups was first described in rat liver microsomes by Bremmer and Greenberg in 1961 (84). S-adenosyl methionine (AdoMet) is the methyl donor in each of the three reactions catalyzed by phosphatidylethanolamine methyltransferase (PEMT) (EC 2. 1. 1. 17). The conversion of PE to PC involves the stepwise transfer of methyl groups from AdoMet to PE to form the mono- (PMME) and dimethylated (PDME) intermediates and subsequently PC (Figure 3.). This pathway is only significant in the liver and accounts for 20-30% of hepatic PC synthesis (85).

Phosphatidylethanolamine N-methyltransferase (1. 2. 2. 2.).

Phosphatidylethanolamine N-methyltransferase (PEMT) is an integral membrane protein and is localized almost exclusively in a hepatic microsomal fraction (86). Audubert and Vance (87) reported that in sealed microsomes, the PEMT activity was susceptible to trypsin digestion, indicating a cytosolic orientation for the enzyme.

There have been many disagreements over the number of enzymes involved in the PE methylation pathway. Studies with microorganisms provided some of the first signs that PE methylation may involve at least two distinct methyltransferases. Clostridium butyricum can produce phosphatidyl-N-methylethanolamine (PMME), but not PC (88). Agrobacterium tumefaciens appears to
Figure 3. Phosphatidylcholine (PC) synthesis by successive N-methylation from Phosphatidylethanolamine (PE).
generate PC exclusively by transmethylation and a soluble methyltransferase purified 40-fold from this bacteria could only support methylation of PE, whereas the homogenate catalyzed all three methylation reactions (89). This indicated that one methyltransferase (PEMT 1) catalyzed the formation of PMME from PE, while the second methyltransferase (PEMT 2) converts PMME to PC by two successive methylations.

The existence of two methyltransferases has also been reported in animals. Two methyltransferases have been observed associated with microsomes from bovine adrenal medulla (90). The first acted on PE to form PMME. It had a relatively low specific activity \( (3.9 \times 10^{-6} \text{ unit/mg}) \) with a pH optimum of 6.5 and required \( \text{Mg}^{2+} \). The second methyltransferase converted PMME to PDME and PC and had a pH optimum of 10 but no requirement for \( \text{Mg}^{2+} \). The specific activity of the latter enzyme was not reported.

A similar system has been described for membranes from erythrocytes (91). The PEMT had considerably lower activity than observed in the adrenal microsomes (between \( 1.6 \times 10^{-7} \) and \( 7.6 \times 10^{-8} \text{ unit/mg} \)). The second transferase had similarly low activity \( (3.2 \times 10^{-7} \text{ unit/mg}) \). Evidence was presented that the initial methylation of PE occurs on the inside of the membrane and the subsequent methylation occurs on the outside of the membrane.

Similar conclusions based on kinetic data, pH, and \( \text{Mg}^{2+} \) dependence have been made for the existence of more than one methyltransferase in rat brain synaptosomes (92). However, the theory that two enzymes convert PE to PC, drawn from kinetic data that did not take into account the steady state nature of
intermediates in the methylation pathway, may be erroneous (87). That is, the synthesis of PMME quickly reaches a steady state value where the rate of PMME synthesis is equivalent to the subsequent conversion of PMME to PDME and PC. Hence, one must take into account the subsequent conversion of PMME and PDME to PC when the rate of formation of PMME, PDME, and PC is estimated. The formation of PMME is best estimated from the dpm in PMME plus 1/2 dpm in PDME plus 1/3 dpm in PC. Similarly, the synthesis of PDME from PMME is best estimated from 1/2 the dpm in PDME and 1/3 dpm in PC, and the formation of PC from PDME is estimated by 1/3 dpm in PC (87). Using these equations, it has been reported that all three methylation activities in rat liver microsomes have pH optima between 10 and 10.5 and similar $K_m$ values for AdoMet (87). This suggested that the methylation of PE may be catalyzed by a single enzyme.

Previous attempts at purification of PEMT, until recently, were unsuccessful. The main problem was enzyme instability in detergents. Pajares et al (93) reported purification of PEMT from rat liver to a final specific activity of 0.27 μmol/min/mg protein. They reported that the PEMT consisted of a 25 Kd monomer and a 50 Kd dimer. Phosphorylation of the 50 Kd protein by cAMP-dependent protein kinase (94) and protein kinase C (95) lead to inhibition of PEMT activity.

Recently, the PEMT has been purified to homogeneity using a 7-step purification scheme in the presence of a nonionic detergent Triton X-100 (96). The purified PEMT is a single isoprotein of molecular mass 18.3 Kd, as determined by SDS-PAGE. The enzyme is
very basic and passes unretained on anion exchange resin at pH 9.4. All three methylation activities of the purified enzyme have a pH optimum of 10.

The purified PEMT can catalyze the methylation of PE to PC indicating that a single enzyme is responsible for all three methylation steps in the conversion of PE to PC. This is further supported by the fact that when the purified PEMT is subjected to gel filtration on Sephacryl S-300 in the presence of Triton X-100, all three methylation activities co-elute(96).

The purified enzyme seems to be a substrate for cAMP-dependent protein kinase \textit{in vitro}, but the effects on enzyme activity are not clear (N. D. Ridgway and D. E. Vance, unpublished observation).

\textbf{Base Exchange Pathway (1. 2. 3. 1.).}

It is clear that the major pathway for the biosynthesis of PC is via the CDP-choline pathway. The conversion of PE to PC is of quantitative significance only in the liver. A third pathway in which choline exchanges with the headgroup of a preexisting phospholipid to yield PC has been demonstrated in microsomes (97, 98). This pathway appears to be active primarily in brain tissue and the quantitative significance of the exchange reaction needs to be established. The base exchange reaction requires calcium and appears to be energy-independent (99, 100). The choline exchange activity seems to be distinct from the exchange activity which makes PE and PS (101). The choline exchange activity in microsomes from rat brain was trypsin-sensitive and is presumably located on the
cytosolic side of these vesicles (102). In contrast to PC biosynthesis, the exchange of L-serine with the ethanolamine moiety of PE is believed to represent the principal route of PS synthesis in animal tissue (103).
The fatty acid and sterol components of triacylglycerols and membrane lipids can be derived either by interconversions of dietary fat, or by de novo synthesis. The pathway of conversion of citrate, derived from carbohydrates, into lipids in mammals is summarised in Figure 4, together with the key regulatory enzymes.

The liver plays the major role in lipid synthesis although adipose tissue is also very active in triacylglycerol synthesis and, in some species, fatty acid synthesis (1). Since the synthesis of one molecule of triacylglycerol from acetyl-CoA and glycerol-3-phosphate requires the consumption of around 50 molecules of ATP and 50 molecules of NADPH, it should come as no surprise that the pathway is stringently regulated. The lipogenic enzymes are subject to reversible control at the level of protein synthesis, a phenomenon which is well documented though poorly understood. However, since these enzymes generally have half-lives of 24 h or more, there is clearly a need for acute regulation of enzyme activity. The synthesis of fatty acids is stimulated within minutes by insulin, and inhibited by adrenaline or glucagon. These controls ensure that lipids are synthesized in times of plenty (when insulin levels are high) but not during times of stress or starvation (when catecholamines and/or glucagon are released). It is now clear that fatty acid synthesis, and the other pathways of lipid synthesis, are controlled by covalent enzyme modification as well as allosteric regulation. The covalent
Figure 4. Pathway of conversion of citrate into lipids in mammals
modification that we will discuss involves phosphorylation/dephosphorylation of key lipogenic enzymes.

The presence of phosphorus in proteins has been known for almost 100 years, but its importance has only been realised since the discovery of enzyme regulation by reversible phosphorylation. Initial work of Krebs, Fischer and Larner discovered that the neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the phosphorylation state of glycogen phosphorylase (104), phosphorylase kinase (105), and glycogen synthase (106). These three enzymes remained the only examples of this phenomenon until the late 1960s, but the situation changed rapidly following the discovery of cyclic AMP-dependent protein kinase (107). The past 10 to 15 years have seen an extraordinary and still accelerating growth in this area. It now appears that protein phosphorylation is the general mechanism by which intracellular events respond to external physiological stimuli (108).

REGULATION OF THE RATE-LIMITING ENZYMES FOR CHOLESTEROL, FATTY ACID, AND DIACYLGlycerol SYNTHESIS BY REVERSIBLE PHOSPHORYLATION

Hydroxymethylglutaryl-CoA reductase (1. 3. 1. 2.).

The reaction sequence leading to cholesterol formation is well known. The initial formation of acetoacetyl-CoA from two acetyl units is catalyzed by a specific thiolase. The reaction is driven to completion by the subsequent condensation of the reaction product with another acetyl-CoA unit, which forms hydroxymethylglutaryl-
CoA (HMG-CoA). HMG-CoA is then converted to mevalonic acid by the enzyme HMG-CoA reductase (EC 1.1.1.34) in the presence of NADPH. The reductase is a microsomal enzyme, of molecular weight 97 Kd, whose activity is generally rate limiting in cholesterol formation (109, 110).

Evidence from several laboratories has demonstrated that HMG-CoA reductase activity is inhibited by phosphorylation and activated by dephosphorylation (111-115). Phosphorylation of the reductase is catalyzed by HMG-CoA reductase kinase. Phosphorylation is on specific serine residues located on the cytosolic domain of the reductase (116). Tryptic peptide analysis suggest that there may be at least two distinct sites of phosphorylation on the reductase(117).

HMG-CoA reductase kinase, molecular weight 58 Kd, is also an interconvertible enzyme. Gibson and co-workers have shown that it can be inactivated by a phosphoprotein phosphatase and restored to full activity by a separate reductase kinase kinase present in liver cytosol (118, 119). Thus, two protein kinases, reductase kinase and reductase kinase kinase, are active in a bicyclic cascade responsible for modulating the catalytic efficiency of HMG-CoA reductase. HMG-CoA reductase is only active in the dephosphorylated state, whereas reductase kinase is inactive under these conditions. Both kinases in the bicyclic system do not depend on cAMP (119).

Ingebritsen et al examined the effects of glucagon and insulin on HMG-CoA reductase activity in hepatocytes (120). They found that the hormones regulated the activity of HMG-CoA reductase both by controlling the total amount of enzyme present and by the
proportion of the enzyme in an active state. Insulin and glucagon also had a pronounced effect on the fraction of reductase kinase in the active state. Insulin diminished, whereas glucagon enhanced, the expressed kinase activity.

Since both reductase kinase and reductase kinase kinase are cAMP-independent enzymes, the route for glucagon regulation of the bicyclic system is not clear. It has been proposed that glucagon regulation may occur through phosphoprotein phosphatase, which catalyzes dephosphorylation of both HMG-CoA reductase and reductase kinase (119). Reductase phosphatase activity was diminished after glucagon pretreatment of hepatocytes (121). It is possible that this action of glucagon is mediated through protein phosphatase inhibitor-1, which is active after being phosphorylated by cAMP-dependent protein kinase (122).

The mechanism by which insulin stimulates HMG-CoA reductase activity is even less apparent in terms of modulation of cAMP levels in hepatocytes. Insulin added alone to suspensions of hepatocytes did not significantly diminish the basal cAMP concentrations (123). Several studies suggest that insulin may regulate the level of phosphoprotein phosphatase inhibitor, and the activity of phosphoprotein phosphatase itself (124). The proposed model for regulation of HMG-CoA reductase activity through a bicyclic modulating system is represented in Figure 5.

Acetyl-CoA carboxylase (1. 3. 1. 3.).

Acetyl-CoA carboxylase (EC 6. 4. 1. 2) catalyzes the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA
Figure 5. Model for regulation of HMG-CoA reductase activity

- ATP, Mg\(^{2+}\)
- reductase kinase kinase (inactive) → reductase kinase (active) → Pi
- phosphatase (active) → P-inhibitor 1-phosphatase (inactive)
- inhibitor 1 ↔ cAMP-protein kinase
- HMG-CoA reductase (active) ↔ Pi
- HMG-CoA reductase (inactive)
- P-inhibitor 1
which provides all but two of the carbon atoms to be incorporated into fatty acid products by fatty acid synthetase. This enzyme is the rate-limiting enzyme in fatty acid synthesis. Acetyl-CoA carboxylase consists of an inactive dimer of molecular weight 500,000 Kd. This species can be dissociated into two apparent identical subunits of molecular weight 215-265 Kd. Each of these subunits contains 1 mol/mol of biotin and active sites both for ATP-dependent carboxylation of biotin and for the transfer of a carboxyl group to acetyl-CoA producing malonyl-CoA (125).

Acetyl-CoA carboxylase has been shown to be activated under conditions that promote the aggregation of dimers of the enzyme into linear, filamentous polymers (such as in the presence of citrate), and to be inactivated under conditions such as high salt, low temperature, long-chain acyl-CoA esters, or MgATP plus bicarbonate which promote depolymerization (126,127). With purified preparations of acetyl-CoA carboxylase, half-maximal effects of citrate are observed in the range 0.5-2.5 mM (126), and of palmitoyl-CoA and other fatty acyl-CoA esters at free concentrations of less than 10 nM (127). However, there have been few reports of parallel changes in fatty acid synthesis and changes in whole tissue or cytoplasmic concentrations of citrate and fatty acyl-CoA esters.

The first hint that reversible phosphorylation might play a role in the regulation of acetyl-CoA carboxylase came from the finding that the enzyme purified from rat liver contained about 2 mol of alkali-labile phosphate per mol of 215 Kd subunit (128). Subsequently, Carlson and Kim (129) found that incubation of ammonium sulfate fractions from rat liver with MgATP led to loss of
enzyme activity whereas incubation with Mg$^{2+}$ resulted in activation, which could be blocked by fluoride. These findings were consistent with the activity of acetyl-CoA carboxylase being inhibited by increasing degree of phosphorylation. Phosphorylation of acetyl-CoA carboxylase was then demonstrated within intact epididymal fat cells by Brownsey et al (130) who showed incorporation of $^{32}$P into a 230 Kd protein which comigrated with purified acetyl-CoA carboxylase on SDS-PAGE.

Tryptic digestion and analysis of $^{32}$P-labelled peptides in a two-dimensional system has shown acetyl-CoA carboxylase to be phosphorylated at multiple sites (131). Incorporation of $^{32}$P into one group of 2 or 3 peptides (designated A-peptides) was increased by epinephrine about two fold but was unaffected by insulin. In contrast, insulin increased incorporation into a different peptide (I-peptide) some fivefold. Neither hormone apparently affected phosphorylation of a group of other peptides (C-peptides) (132). In all cases, the phosphorylation was on serine residues. Thus, these studies showed that exposure of fat cells to insulin and epinephrine led essentially to the phosphorylation of different sites on acetyl-CoA carboxylase and suggested that these different patterns of phosphorylation might be associated with opposite changes in activity.

Acetyl-CoA carboxylase has been shown to be a substrate for a number of different protein kinases. Studies with cAMP-dependent protein kinase has shown inactivation of the acetyl-CoA carboxylase upon phosphorylation (133). Phosphorylation resulted in a decrease in $V_{\text{max}}$ and an increase in the apparent $K_a$ for citrate. A number of
studies have used phosphopeptide analysis to compare the sites exhibiting increased phosphorylation in cells exposed to hormones that increase cAMP to those phosphorylated in purified acetyl-CoA carboxylase treated with cAMP-dependent protein kinase (134, 135). In general, a good correlation has been found.

Other protein kinases which have been shown to catalyse phosphorylation of acetyl-CoA carboxylase include two different cyclic nucleotide independent protein kinases (136), and casein kinase I and II (137). The sites phosphorylated by the two cyclic nucleotide independent kinases are similar to those phosphorylated by cAMP dependent protein kinase, whereas those phosphorylated by casein kinase I and II are distinctly different.

Characterization of the protein phosphatases able to dephosphorylate acetyl-CoA carboxylase is still in its early stages. A number of phosphatase preparations have been found to be capable of dephosphorylating the sites on acetyl-CoA carboxylase phosphorylated by cAMP-dependent protein kinase and hence activating the enzyme. These include a number of phosphatases purified from rat liver (phosphatase 1, 2A₁, 2A₂, and 2C) (138, 139).

Glycerol phosphate acyltransferase (1. 3. 1. 4.).

The synthesis of diacylglycerol and triacylglycerol begins with the activation of fatty acids to acyl-CoA esters, which involves the input of energy from ATP. The main acceptor for acyl-CoA in most tissues is thought to be sn-glycerol-3-phosphate that is formed by glycolysis or by the phosphorylation of glycerol. This reaction is catalyzed by glycerolphosphate acyltransferase (EC 2. 3. 1. 15).
In the liver, the glycerolphosphate is divided almost equally between the mitochondrial and the microsomal fractions. By contrast, in heart, kidney, adrenal glands, and adipose tissue, the mitochondrial activity is only about 10% of the total activity, and the microsomal activity predominates (140). The microsomal activity is found on the cytosolic side of the endoplasmic reticulum, while the mitochondrial enzyme is on the inner surface of the outer mitochondrial membrane.

Glycerolphosphate acyltransferase may also be subject to short-term regulation by reversible phosphorylation. Earlier studies had demonstrated that administration of adrenaline to isolated rat fat cells causes a decrease in the activity of glycerolphosphate acyltransferase (141). Insulin specifically increased the activity of the mitochondrial glycerolphosphate acyltransferase within 30 min after its addition to a perfused liver system (142). Soler-Argilaga et al reported, in studies with the liver, that dibutyryl cAMP decreases the activity of the microsomal glycerolphosphate acyltransferase (143).

*In vitro* experiments involving the incubation of microsomal fractions from rat adipose tissue with cAMP dependent protein kinase resulted in 90% inhibition of enzyme activity (144). The enzyme was rapidly reactivated by addition of alkaline phosphatase. This represented convincing evidence that a protein phosphorylation inhibits the enzyme, although further work is required to show that the glycerolphosphate acyltransferase itself is modified.
REGULATION OF ENZYMES INVOLVED IN PC BIOSYNTHESIS BY REVERSIBLE PHOSPHORYLATION (1.3.2.1.).

The influence of glucagon on glycerolipid synthesis in isolated adult rat hepatocytes was initially examined by Geelen et al (145, 146). The effect of glucagon on PC synthesis in these studies was difficult to interpret because the results varied with the radioactive label used. The incorporation of [2-14C]glycerol, [methyl-3H]choline, and [32P]phosphate into PC was stimulated, unaffected and inhibited, respectively, in the presence of glucagon. Other investigators have reported a stimulation of PC biosynthesis in the lung by aminophylline, a specific inhibitor of cAMP phosphodiesterase, and cAMP analogues (147-149).

Choline transport and choline kinase (1.3.2.2.).

Choline is taken up by hepatocytes by a saturable and a non-saturable component (54). In certain cases, choline uptake has been shown to be rate-limiting in PC synthesis. For instance, in Novikoff hepatoma cells (150), choline uptake can serve as the rate-limiting step for PC formation.

The effect of glucagon and cAMP on choline uptake in rat hepatocytes was examined. Glucagon had no effect (151), while chlorophenylthio-cAMP resulted in a small inhibition of choline uptake. The inhibition by chlorophenylthio-cAMP was shown to be primarily due to a 3.6-fold reduction in the $V_{\text{max}}$ of the saturable component of choline uptake (152).

Since choline in hepatocytes can be metabolized to form phosphocholine or betaine, the effect of cAMP on formation of these
compounds was examined. It was concluded that the hepatic synthesis and metabolism of betaine is not regulated by cAMP. When isolated rat hepatocytes were incubated with cAMP analogues and phosphodiesterase inhibitors, the relative incorporation of \([\text{methyl-}^3\text{H}]\text{choline}\) into phosphocholine and betaine resembled untreated cells (152). Moreover, these compounds did not affect the rate at which betaine disappeared from the cells. Examination of the effect of cAMP analogues on choline kinase activity revealed a slight increase, even though the incorporation of \([\text{methyl-}^3\text{H}]\text{choline}\) into PC was decreased. Thus, it seems that the effects of cAMP on choline transport and on choline kinase activity are not clear.

**CTP:phosphocholine cytidylyltransferase** (1.3.2.3.).

Incubation of rat hepatocytes with glucagon (151) and cAMPS (adenosine 3', 5'-cyclic phosphorothioate) (63) resulted in inhibition of PC synthesis. The inhibition was evident within 30 min, consistent with rapid regulation by protein phosphorylation rather than an adaptive change in the amount of one of the enzymes of the de novo pathway.

The activities of the de novo PC biosynthetic enzymes from control and 0.5 mM chlorophenylthio-cAMP treated hepatocytes were determined in an attempt to correlate the reduced PC synthesis with an inhibition of one of the enzymes (152). After 90 min exposure to the cAMP analogue, the cells were subjected to subcellular fractionation in the presence of 20 mM NaF and 2.5 mM EDTA. These reagents were included as a precaution to preserve the state of phosphorylation of any enzymes which might have been
covalently modified by cAMP-dependent protein kinase. Choline kinase and phosphocholinetransferase activities were unchanged by the cAMP analogue. Cytidylyltransferase activity, on the other hand, was reduced 31% in microsomes from cAMP analogue treated hepatocytes. The cytosolic activity of cytidylyltransferase was decreased 33%, but when the soluble enzyme was activated by phospholipid liposomes, 15% more activity was detected in the cytosols from chlorophenylthio-cAMP treated cells. Thus, the finding that the cAMP-mediated inhibition of PC synthesis is exerted through cytidylyltransferase is not surprising since numerous studies have identified this as the key regulatory enzyme in PC biosynthesis via the de novo pathway (50-57).

One of the distinct features of cytidylyltransferase is the time-dependent activation of the cytosolic cytidylyltransferase upon incubation at 37 °C. The possibility that the activation might be the result of dephosphorylation of cytidylyltransferase by endogenous protein phosphatases was examined (74). The addition of Mg.ATP, a substrate for protein kinase, was found to prevent the time-dependent activation at 37 °C. Similarly, the presence of NaF, an inhibitor of protein phosphatases, retarded the rate at 20 °C. While preincubation of cytosol at 37 °C resulted in a 2-fold activation of cytidylyltransferase that was essentially complete after 6 min, incubation of the same cytosol at 20 °C for 5 h permitted a 4-fold activation of enzyme activity. Possibly increased protein kinase activity in cytosol at 37 °C may have prevented further activation of the cytidylyltransferase at this temperature. Hence, cytosol was incubated with specific protein kinase inhibitors from rabbit muscle,
beef heart, and pig heart. These are heat-stable proteins which inhibit cAMP-dependent protein kinases. All three of these inhibitor proteins prevented the Mg.ATP inhibition at 37 °C in a concentration-dependent manner (74). In addition, incubation with alkaline phosphatase from hog intestine produced a rapid activation of cytosolic cytidylyltransferase.

Incubation of cytosol at 37 °C for 10 min, results in a shift in the pH optimum from 5.6 to 6.2, while the apparent $K_m$ is reduced from 4-5 mM to 1.3 mM (74). However, incubation in the presence of Mg.ATP results in no alterations in these kinetic values. Inclusion of rabbit muscle protein kinase inhibitor at the start of incubation results in a decrease in the apparent $K_m$ for CTP to 0.35 mM.

The activity of cytidylyltransferase in Mg.ATP treated cytosol can be stimulated 8-10 fold when the enzyme is assayed in the presence of liposomes prepared from total rat liver phospholipid. But for cytidylyltransferase that is completely activated by preincubation with protein kinase inhibitor, phospholipid actually has an inhibitory effect (74). The $V_{max}$ of the Mg.ATP treated cytidylyltransferase in cytosol with phospholipid is 60% higher than measured for protein kinase inhibitor treated enzyme with phospholipid. The effect of phospholipid on Mg.ATP treated cytidylyltransferase is a 10-fold reduction in the apparent $K_m$ for CTP and a shift in the pH optimum to between 6.4 and 6.6.

Cytidylyltransferase activity in the post mitochondrial supernatant of rat liver is activated 4-fold in a time-dependent fashion at 20 °C, and the recovery of cytidylyltransferase in the microsomal fraction after ultracentrifugation increases 4-fold.
Inclusion of protein kinase inhibitor in the post mitochondrial supernatant accelerates the association of cytidylyltransferase with the microsomes, while Mg.ATP retards the interaction (153). Collectively, these findings suggest that conditions which promote protein dephosphorylation can induce the translocation of cytidylyltransferase to the microsomes. The observation that chlorophenylthio-cAMP treatment of rat hepatocytes produces a 31% reduction of the specific activity of cytidylyltransferase in the microsomes, and a marginal concomitant increase in the cytosolic enzyme activity, is consistent with the notion that protein phosphorylation results in release of cytidylyltransferase from the microsomes.

**CDP-choline:1,2-diacylglycerol cholinephosphotransferase** (1. 3. 2. 4.).

Gross and Rooney (149) found that aminophylline stimulated about 1.8-fold the incorporation of \([\text{methyl}^3\text{H}]\text{choline}\) into the phospholipid of explants of fetal rat lung in organ culture. However, the activity of cholinephosphotransferase was not significantly altered by the exposure to aminophylline.

Exposure of hepatocytes to cAMP analogues (152) resulted in a reduction in PC synthesis. Analysis of cholinephosphotransferase activity in these cells showed no significant change in the activity by the cAMP analogues. These results strongly suggest that cholinephosphotransferase activity is not subject to regulation by reversible phosphorylation.

**Phosphatidylethanolamine N-methyltransferase** (1. 3. 2. 5.).
Hepatic PE methyltransferase has been reported to be regulated by reversible phosphorylation. Castano et al (154) reported a 2-fold activation of PE methyltransferase in hepatocytes exposed to glucagon and cAMP analogues. The activation of the enzyme by glucagon reflected an increase in the $V_{\text{max}}$ of the reaction rather than a change in the apparent $K_m$ of the enzyme for $S$-adenosyl-$l$-methionine. Pritchard et al (155) also observed a stimulation in PE methyltransferase activity by glucagon and cAMP analogues, but found that the conversion of PE to PC was inhibited. Alemany et al (156) reported a stimulation of PE methyltransferase by calmodulin. The activation may have been through a calmodulin-dependent protein kinase since hydrolysis of ATP was required for the stimulation. Recent reports (94, 95) have suggested that partially purified PE methyltransferase can be phosphorylated and activated by cAMP-dependent protein kinase and protein kinase C. However, these reports showed phosphorylation of a 50 Kd protein which was thought to be the dimer of PE methyltransferase. The purification of PE methyltransferase by Ridgway and Vance (96) has clearly demonstrated that the 50 Kd protein is a contaminant and not part of the PE methyltransferase. In light of this finding, the role of phosphorylation was re-evaluated. Preliminary evidence (N. D. Ridgway and D. E. Vance, unpublished observations) has suggested that the PE methyltransferase can be phosphorylated on serine residues by cAMP-dependent protein kinase in vitro. However, no alteration in enzyme activity was detected as a consequence of this phosphorylation. Exposure of hepatocytes to $[^{32}\text{P}]}$phosphate in the presence of cAMP analogues, and subsequent isolation of PE
methyltransferase and detection by antibodies after 2 dimensional-PAGE, has revealed that the PE methyltransferase was not phosphorylated \textit{in vivo}. However, further studies are needed to establish whether the PE methyltransferase itself or whether a modifier protein, which regulates PE methyltransferase activity, is regulated by reversible phosphorylation.
Ca\(^{2+}\)-an important cellular second messenger (1.4.1.1.).

Ca\(^{2+}\) is involved in regulating a variety of cellular systems. The mechanism of action of Ca\(^{2+}\) is different from that of other metal ions. The role of Ca\(^{2+}\) seems to be more similar to the regulatory actions of cyclic nucleotides which produce multiple metabolic effects by binding to the regulatory subunit of cAMP-dependent protein kinase and thereby activating the enzyme. The specificity of the cyclic nucleotide system of any cell is determined at two levels. The first is by the receptors (for hormones and so on, that elevate the intracellular content of cAMP) the membrane is endowed with. The second is which substrate(s) for the protein kinase the cell possesses. By analogy to cAMP, it has been proposed that Ca\(^{2+}\) is an intracellular second messenger (157). Continuing this analogy, calmodulin may be considered an intracellular receptor for Ca\(^{2+}\). It is suggested that physiologically active cellular Ca\(^{2+}\) is calmodulin bound. Studies by Grand and Perry (158) have shown that each mammalian tissue possesses a distinct set of these Ca\(^{2+}\)-binding proteins, through which the specificity of Ca\(^{2+}\) action is presumably determined. Amplification of a Ca\(^{2+}\) signal in some cases, like a cAMP signal, can be accomplished through the activation of specific protein kinases which in turn may affect a number of additional enzyme activities.

The cytosolic concentrations of Ca\(^{2+}\) can be elevated in two ways. The first is by mobilization of Ca\(^{2+}\) from intracellular stores such as the endoplasmic reticulum and the mitochondria, and the second is by influx of extracellular Ca\(^{2+}\). The elevated Ca\(^{2+}\)
concentrations can be diminished by rapid extrusion from the cell by a plasma membrane \((\text{Ca}^{2+}\text{-Mg}^{2+})\text{ATPase}\). Mobilization of intracellular \text{Ca}^{2+} can be accomplished by \(\alpha_1\)-adrenergic agonists such as vasopressin and angiotensin II.

**Mode of action of vasopressin and other \(\alpha_1\)-adrenergic agonists** (1. 4. 1. 2.).

It was generally accepted that the stimulatory effects of \(\alpha_1\)-adrenergic agonists, vasopressin and angiotensin, on glycogen breakdown in rat liver required an increase in the cytoplasmic \text{Ca}^{2+} concentrations (159, 160). Further, a body of evidence suggested that a major source of this \text{Ca}^{2+} was the endoplasmic reticulum and possibly the mitochondria (161-163). A proposal was developed that, after binding of \(\alpha_1\)-adrenergic agonists or vasopressin to their receptors, a signal or intracellular mediator was generated which led to the release of \text{Ca}^{2+} from the endoplasmic reticulum.

It is now well established that hormone-induced \text{Ca}^{2+} mobilization in a wide variety of tissues is associated with enhanced phosphatidylinositol (PI) breakdown and subsequent resynthesis. Changes of inositol lipid metabolism induced by vasopressin, angiotensin II, and other \(\alpha_1\)-adrenergic agents in isolated hepatocytes was first demonstrated by the specific enhancement of \(^{32}\text{Pi}\) incorporation into PI (164-166). Further work showed that this increased labeling was preceded by a decrease of about 5% of total cellular PI (167, 168). These studies were extended by the findings of Michell *et al* (169) and Kirk *et al* (170) that there was a decrease in the \(^{32}\text{P}\) content of PI 4,5-bis-phosphate and PI 4-monophosphate.
after treatment of prelabeled hepatocytes with Ca\(^{2+}\)-mobilizing hormones.

It is now well accepted that binding of vasopressin and other \(\alpha_1\)-adrenergic agonists to their receptors leads to the activation of a PI-specific phospholipase C (171). G-protein(s), particularly a pertussis toxin-insensitive G-protein, Gp, is involved in coupling Ca\(^{2+}\)-mobilizing receptors to the activation of phospholipase C (172-174). Evidence for this was suggested by the fact that pertussis toxin had no effect on agonist-mediated PI response, and by the observation that GTP\(\gamma\)S (a nonhydrolyzable GTP analog) could stimulate inositol lipid breakdown in membrane fractions from most cells (175, 176). Activation of phospholipase C then leads to the breakdown of PI 4,5-bis-phosphate to form diacylglycerol and inositol 1,4,5-triphosphate (and also some inositol 1,3,4-triphosphate) (177). Diacylglycerol is an important cellular mediator which is responsible for the activation of protein kinase C (178) and the formation of arachidonic acid by the sequential actions of diacylglycerol lipase and monoacylglycerol lipase. On the other hand, inositol 1,4,5-triphosphate is able to increase the intracellular Ca\(^{2+}\) concentrations by triggering the release of Ca\(^{2+}\) from an intracellular source, particularly the endoplasmic reticulum, thus activating Ca\(^{2+}\)-dependent processes among which calmodulin is the best known. Removal of the phosphate at the 5-position of inositol 1,4,5-triphosphate, leads to inactivation of the Ca\(^{2+}\) mobilizing action of inositol 1,4,5-triphosphate (179). Inositol 1,4,5-triphosphate can be phosphorylated with ATP to form inositol 1,3,4,5-tetrakisphosphate which appears to act in concert with inositol 1,4,5-triphosphate to
mobilize Ca\(^{2+}\) (180). It has been shown that inositol 1,4,5-triphosphate recognizes and binds to specific receptors on the endoplasmic reticulum through which it initiates the release of Ca\(^{2+}\) into the cytosol (181).

**Regulation of PC synthesis by vasopressin and other α\(_1\)-adrenergic agonists** (1, 4, 1, 3).

There are several indications that the synthesis of hepatic PC may be regulated by Ca\(^{2+}\) and Ca\(^{2+}\)-dependent hormones. Earlier work of Soler Argilaga et al (182) showed that the synthesis of phosphatidate, diacylglycerol, and triacylglycerol from sn-glycerol-3-phosphate, was inhibited by Ca\(^{2+}\) in rat liver microsomes. The inhibition was dependent on the concentration of Ca\(^{2+}\) used. It was concluded that the enzymatic mechanisms participating in the microsomal biosynthesis of glycerolipids, was disrupted by Ca\(^{2+}\).

Alemany et al (183) showed that treatment of rat hepatocytes with vasopressin or angiotensin, stimulated 2-fold the incorporation of methyl-\(^3\)H groups into phospholipids. When Ca\(^{2+}\) were removed from the incubation medium, by addition of EGTA, none of the hormones had a significant effect on phospholipid methylation. Addition of Ca\(^{2+}\) ionophore A23187 also enhanced phospholipid methylation. The activation by the hormones was time-dependent with maximum effects occurring after 5 min. In addition, Ca\(^{2+}\) was shown to activate phospholipid methyltransferase in isolated rat liver microsomes (184).

More recent work of Alemany et al (185) has shown a 2-fold inhibition of [methyl-\(^3\)H]choline incorporation into PC by vasopressin
and angiotensin. This effect was transient and the rate of incorporation of radioactivity into phospholipids recovered control values about 4 min after the addition of the hormone. The addition of ionophore A23187 also induced a fast, partially reversible, dose-dependent inhibition of the incorporation of [methyl-3H]choline into PC. These results suggested that the inhibition of label incorporation into PC was secondary to an increase in the intracellular Ca\(^{2+}\) concentration. Results with isolated microsomes supported this view, whereby incubation of microsomes with Ca\(^{2+}\) inhibited PC synthesis (185). In these studies, Ca\(^{2+}\) was shown to change the kinetic properties of CDP:1,2-diacylglycerol cholinephosphotransferase by decreasing the \(V_{\text{max}}\) value of the enzyme, without affecting the \(K_m\) for CDP-choline.

Haagsman et al (186) showed that norepinephrine, in the presence of propanolol, inhibited the incorporation of [methyl-3H]choline into PC in a dose-dependent manner. Norepinephrine also decreased the incorporation rates of [1-14C]palmitic acid and [1-14C]oleic acid into PC. The effect of norepinephrine was mediated through \(\alpha\)-adrenergic receptors. Norepinephrine did not decrease uptake or phosphorylation rate of [methyl-3H]choline, but the reaction catalyzed by CTP:phosphocholine cytidylyltransferase was diminished.

Recent work by Pollard et al (187) however, has demonstrated an increase in [1-14C]oleic acid incorporation into triacylglycerol and phospholipid fraction by incubation of hepatocytes with vasopressin for 30 min. This effect could be mimicked or antagonized by using
ionophore A23187 or EGTA respectively. This suggested that the effects of vasopressin were being mediated by Ca$^{2+}$.

Work by Tijburg et al (188) has demonstrated the inhibition of PC synthesis and an increase in PE synthesis by Ca$^{2+}$-dependent hormones in isolated hepatocytes. The mechanism of inhibition of PC synthesis appears to be similar to the inhibition of PC synthesis by norepinephrine (186). Neither vasopressin, nor norepinephrine affects the uptake, phosphorylation or oxidation of choline. Results from pulse-chase studies with either of these hormones indicated that the inhibition of synthesis of PC might be due to an inhibition of CTP:phosphocholine cytidylyltransferase. This seems to be in direct contrast to the findings of Alemany et al (185) who showed that the inhibitory effect of vasopressin was secondary to an increase in intracellular Ca$^{2+}$, leading to an inhibition of CDP:1,2-diacylglycerol cholinephosphotransferase. Although inhibition of this enzyme could not be excluded, a direct decrease of the activity of CDP:1,2-diacylglycerol cholinephosphotransferase by vasopressin was not demonstrated by these authors.

More recent work by Graham et al (189) has shown inhibition of $[^{14}\text{C}]$choline incorporation into PC by chlorpromazine, verapamil (a Ca$^{2+}$ channel blocker), and EGTA. This suggested that Ca$^{2+}$ is required for active PC synthesis. Thus, it seems that although Ca$^{2+}$ is important in PC synthesis, the exact role it plays is not clear. Agents which regulate Ca$^{2+}$ concentrations have been shown to either stimulate or inhibit PC synthesis.
AGENTS WHICH ARE ACTIVATED BY Ca\(^{2+}\) (1. 4. 2. 1.).

Ca\(^{2+}\) is an important cellular second messenger. Elevation in cellular Ca\(^{2+}\) concentrations by external stimulants can lead to effects on a wide range of cellular components. Among the more notable effects of Ca\(^{2+}\) is the alteration of protein kinase C, Ca\(^{2+}\)-calmodulin-dependent protein kinases, and phospholipase A\(_2\) activity.

Protein kinase C (1. 4. 2. 2.).

Protein kinase C (PKC) was first found in 1977 as a proteolytically activated protein kinase that was capable of phosphorylating histones (190), and later found to be activated reversibly by association of membrane phospholipid in the presence of diacylglycerol and physiological concentrations of Ca\(^{2+}\) (191, 192). PKC is present ubiquitously in tissues, with the brain having the highest activity (193). In this tissue, a large quantity of the enzyme is associated with the synaptic membranes, whereas in most other tissues the enzyme is present mainly in the soluble fraction as an inactive form.

The brain enzyme shows a single band, with a molecular weight of 82,000 on SDS-PAGE (194). The isoelectric point of the enzyme is pH 5.6. The optimum pH range for activity is 7.5-8.0. Mg\(^{2+}\) is essential for the catalytic activity with the optimum range having about 5-10 mM, and can be replaced to some extent by Mn\(^{2+}\) or Co\(^{2+}\). ATP is the phosphate donor and PKC has little affinity for GTP. Heterogeneity of PKC in brain and other tissues has been described (195), and the enzyme is very susceptible to proteolysis (194). At
last count, seven subspecies of PKC had been identified. These differ in their response to phospholipid, diacylglycerol, and Ca^{2+} (196).

PKC in cell-free extracts is normally inactive, and depends on Ca^{2+} and phospholipid for activation. Among the phospholipids tested, phosphatidylserine is absolutely required for enzyme activation. Diacylglycerol, which is produced in membranes from inositol phospholipid breakdown in a signal-dependent fashion, dramatically increases the affinity of PKC for Ca^{2+}, thereby activating it at low Ca^{2+} concentrations (192). Although various diacylglycerols are capable of activating PKC, 1-stearoyl-2-arachidonyl glycerol is most likely the physiological activator since most of the inositol phospholipids contain this species (197).

PKC can be alternatively activated by proteolysis with Ca^{2+}-dependent protease or trypsin (198). When a Ca^{2+}-dependent protease is employed, a limited proteolysis takes place, and a smaller component with enzymatic activity is produced. The molecular weight is estimated to be about 51,000. The catalytically active component thus produced is totally independent of Ca^{2+}, phospholipid, and diacylglycerol. PKC, which is attached to the membrane, is more susceptible to limited proteolysis by Ca^{2+}-dependent protease. The PKC fully activated either proteolytically or non-proteolytically show similar kinetic and catalytic properties, and exhibit the same levels of enzyme activity. Although it has been proposed that such proteolytic activation of PKC may occur in intact cell systems (199), the physiological significance of this proteolysis has not been defined.
Although it is generally accepted that inositol phospholipid turnover is firmly linked to the activation of PKC, the evidence for this signal transduction has primarily come from experiments with platelets as a model system. Upon stimulation of platelets by thrombin or platelet-activating factor (PAF), two endogenous platelet proteins with approximate molecular weights of 47 Kd and 20 Kd are heavily phosphorylated, and this phosphorylation reaction is normally associated with the release of their constituents such as serotonin (200). The 20 Kd protein is myosin light chain, and a calmodulin-dependent protein kinase is responsible for this phosphorylation reaction (201). Although the function of 47 Kd protein is unknown, the phosphorylation of this protein may be used as a marker of the activation of PKC (202).

PKC is a receptor protein of tumor-promoting phorbol esters, such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA). These have been shown to activate PKC both in vitro and in vivo (203, 204), and there is an approximate correlation between the ability of individual phorbol esters to promote tumor development and to activate PKC. Kinetic analysis indicate that TPA, which has a diacylglycerol-like structure, is able to substitute for diacylglycerol and dramatically increase the affinity of PKC for Ca\(^{2+}\) to 10\(^{-7}\) M range, resulting in its full activation without detectable mobilization of Ca\(^{2+}\).

Involvement of protein kinase C in PC synthesis (1. 4. 2. 3.).

One of the earliest metabolic events that occurs concomitant with the initial stimulation of cell growth and proliferation by tumor-promoting phorbol esters, including TPA and several related
structures, is the increased synthesis of PC. Enhanced \([\text{methyl-}^{3}\text{H}]\text{choline}\) and \([^{32}\text{P}]\text{phosphate}\) labelling of PC occurs after TPA administration to mouse skin (205-207). Similarly, pulse-labelling experiments in cell culture have demonstrated that phorbol esters stimulate PC synthesis in HeLa cells (208-211), bovine lymphocytes (212), chick embryo myoblasts (213, 214), HL-60 promyelocytic leukemia cells (215), human neutrophils (216), GH3-pituitary cells (217, 218), and NG108-15 neuroblastoma x glioma hybrid cells (219). Continued occupation of the phorbol ester receptor appears essential for maintaining promotor-induced stimulation of PC synthesis (215, 220).

TPA and other phorbol esters exert their effects on PC anabolism within 15 min (213, 215), and as soon as 15 s as seen with human polymorphonuclear neutrophils exposed to TPA (216). The TPA-mediated stimulation of PC synthesis is due to the acceleration of the reaction catalyzed by CTP:phosphocholine cytidylyltransferase (208, 217-219). The response to TPA treatment is insensitive to actinomycin D and cycloheximide (209, 212, 214), indicating that the increased PC synthesis is not due to increased enzyme protein synthesis but due to translocation of the cytidylyltransferase from the cytosol to the microsomes (221).

One of actions of phorbol ester tumor-promoters is the activation of protein kinase C (PKC). PKC has been implicated as the phorbol ester receptor, and correlation between the ability of individual phorbol esters to promote tumors and to activate PKC has been shown (203, 204). Since TPA has been shown to activate PC synthesis in a variety of cells and PKC is a receptor for TPA, it would
seem that PKC may be involved in regulation of PC synthesis. Cook and Vance (222) examined the regulation of cytidylyltransferase activity, the rate-limiting enzyme in PC synthesis, by PKC in vitro. It was discovered that PKC did not directly affect cytidylyltransferase activity. However, since the incubations were carried out in the absence of membranes, it may be that PKC regulates activity of a modifier protein which in turn is responsible for activation of cytidylyltransferase. Thus, although the actions of TPA on PC synthesis would implicate a role for PKC-mediated regulation of PC synthesis, the direct effects of PKC on cytidylyltransferase are not established.

Ca\(^2+\)-calmodulin-dependent protein kinases (1. 4. 2. 4.).

The compelling evidence that Ca\(^2+\) was a second messenger led to investigations to identify mechanisms for Ca\(^2+\) selectivity to biological responses. In particular, proteins that detect and respond to a Ca\(^2+\) signal have been identified (223, 224). These Ca\(^2+\) binding proteins include calmodulin, troponin C, parvalbumin, intestinal Ca\(^2+\)-binding protein, S 100 protein, and the regulatory light chain of myosin. Calmodulin is unique because it is found in most, if not all, eukaryotic cells and mediates Ca\(^2+\) control of a large number of enzymes. These two general properties confer to calmodulin the role of mediator of many Ca\(^2+\)-dependent cellular processes.

The properties of vertebrate calmodulin have been studied in considerable detail. It is an acidic 148-residue protein that contains no tryptophan, cysteine, phosphate, or carbohydrate (225). Based upon a comparison of calmodulin’s amino acid sequence to other
Ca$^{2+}$-binding proteins, four Ca$^{2+}$-binding domains have been described. These Ca$^{2+}$-binding domains are separated by regions of α-helical structure. There is homology among the four Ca$^{2+}$-binding domains in calmodulin, with greater homology between domains 1 and 3 than between 2 and 4.

Calmodulin binds 4 Ca$^{2+}$ per mol of calmodulin with high affinity, and studies on the Ca$^{2+}$-binding properties of calmodulin indicate multiple classes of sites of negative cooperativity (226, 227). However, positive cooperativity at low Ca$^{2+}$ concentrations has also been described (228). Upon binding Ca$^{2+}$, calmodulin undergoes conformational changes as indicated by circular dichroism, optical rotary dispersion, nuclear magnetic resonance, and UV-difference spectroscopy (229). Low- and high-affinity Ca$^{2+}$-binding sites of calmodulin have been detected by these procedures. Based upon differences in binding properties of the different sites for Ca$^{2+}$, it has been suggested that various calmodulin-related enzymes might be activated by distinct conformers of calmodulin containing different amounts of Ca$^{2+}$ (229).

One of the principal actions of Ca$^{2+}$-calmodulin is to alter the phosphorylation states of intracellular proteins. A number of Ca$^{2+}$-calmodulin-dependent enzymes have been described that regulate intracellular protein phosphorylation in mammalian cells. These include a high K$_{m}$ cyclic nucleotide phosphodiesterase (230), brain adenylate cyclase (231), phosphorylase kinase (232), NAD kinases (233), guanylate cyclase (234), myosin light chain kinases (235), phospholamban kinase (236), and a multifunctional protein kinase (237, 238).
One of the most studied Ca\(^{2+}\)-calmodulin-dependent protein kinases is the myosin light chain kinase (MLCK). Ca\(^{2+}\)-calmodulin-dependent phosphorylation of myosin P-light chain by MLCK plays a central role in the regulation of smooth-muscle contraction (239). Studies with purified smooth-muscle actomyosin have demonstrated that phosphorylation of the myosin P-light chain increases actin activation of myosin Mg\(^{2+}\)-ATPase activity which is viewed as an \textit{in vitro} correlate of myosin cross-bridge cycling and smooth-muscle force generation \textit{in vivo}.

MLCKs have been purified from a number of different animal species. The enzyme is a single protein of molecular weight in the range 130-150 Kd. A number of studies indicate that the stoichiometry for activation of MLCK is 1 mol calmodulin per mol of MLCK (240, 241). Analysis of the stoichiometry and affinity of the \textit{Ca}\(_n\)^{2+}.calmodulin species which is in equilibrium with activated MLCK indicated that the activation is a sequential, fully reversible process, and the first step of the activation requires binding of Ca\(^{2+}\) to all four binding sites on calmodulin to form the complex, \textit{Ca}\(_4\)^{2+}.calmodulin. This complex then binds to and activates the inactive catalytic subunit of MLCK to form the active holoenzyme complex \textit{Ca}\(_4\)^{2+}.calmodulin.MLCK (242).

A feature of phosphotransferase reactions catalyzed by MLCK's is the specificity for myosin P-light chain. The narrow protein substrate specificity of MLCK's distinguishes them from other protein kinases which phosphorylate a broad spectrum of protein substrates. Serine is the amino acid phosphorylated by MLCK's, which also
undergo autophosphorylation with the incorporation of about 1 mol phosphate/mol enzyme (243).

Another Ca\(^{2+}\)-calmodulin-dependent protein kinase that has been extensively studied is phosphorylase kinase. This enzyme is responsible for the activation of glycogen phosphorylase and the inhibition of glycogen synthase. As a result of this, glycogenolysis is activated and glycogen synthesis is inhibited. This sequence of events occurs during muscle contraction and in response to adrenaline (244).

The rabbit skeletal-muscle phosphorylase kinase has a molecular weight of approximately 1.3 \( \times \) 10\(^6\) and is composed of four types of subunit in the stoichiometry \( \alpha(\alpha')_4\beta_4\gamma_4\delta_4 \) (245). Two isoenzymes have been identified that differ in the size of the largest subunit, designated \( \alpha \) or \( \alpha' \) (246). Phosphorylase kinase appears as a bilobal structure resembling two opposing parentheses held together by two short cross bridges (247). This butterfly-like, nonglobular structure has a molecular mass of the hexadecamer holoenzyme.

Several reports indicate that the \( \gamma \)-subunit has catalytic activity (248, 249), although some reports of \( \beta \)-subunit having catalytic activity have also been described (250). The \( \delta \)-subunit has been identified as the Ca\(^{2+}\)-binding domain and the amino acid sequence of this is identical to that of bovine uterus calmodulin (251). The \( \alpha \)- and \( \beta \)-subunit serve a regulatory function. Phosphorylation of the \( \alpha \)- and \( \beta \)-subunit by cAMP-dependent protein kinase (252), or by autophosphorylation (253) results in activation, as does limited proteolytic degradation of these subunits (254). It appears that phosphorylation of at least one \( \beta \)-subunit is required to produce a
conformational change that then permits \( \alpha \)-subunit phosphorylation. Activation of the enzyme also results from dissociation of the holoenzyme by LiBr (255), which has led to the suggestion that the activity of the enzyme is inhibited by the regulatory subunits (\( \alpha \) and \( \beta \)), and that this inhibition can be relieved by phosphorylation, limited proteolysis, or dissociation. Phosphorylase kinase can also be activated by extrinsic \( \text{Ca}^{2+} \)-calmodulin (256). Cohen (257) has termed this extrinsic calmodulin the "\( \delta' \)-subunit". Over and above the almost total requirement of holophosphorylase kinase for \( \text{Ca}^{2+} \), addition of extrinsic \( \text{Ca}^{2+} \)-calmodulin activates the enzyme, synergistically, a further 2- to 7-fold. The activation by exogenous calmodulin is very pH-dependent.

The major reaction that is catalyzed by phosphorylase kinase \textit{in vivo} is the phosphorylation of phosphorylase b. In this reaction, phosphorylase b, a dimer, is phosphorylated at each of two identical serine residues in the presence of \( \text{Mg}^{2+} \) and ATP. ADP, \( \text{Ca}^{2+} \), calmodulin, glycogen, and \( \text{Mg}^{2+} \) are all allosteric activators of the phosphorylase kinase (258). ADP stimulates both phosphorylase conversion and autophosphorylation and inhibits \( \beta \)-subunit dephosphorylation. Nonactivated phosphorylase kinase is only significantly active above pH 7.6, whereas the activated enzyme is fully functional at pH 6.8-7.0 (259).

\textbf{Involvement of \( \text{Ca}^{2+} \)-calmodulin-dependent protein kinases in PC synthesis} (1. 4. 2. 5.).

There is no direct evidence for the involvement of \( \text{Ca}^{2+} \)-calmodulin-dependent protein kinases in PC biosynthesis. However,
indirect evidence that Ca$^{2+}$-calmodulin-dependent protein kinases may be involved in the regulation of CTP:phosphocholine cytidylyltransferase, the rate limiting enzyme in PC synthesis, was shown by Pelech (153). It was observed that Ca$^{2+}$ inhibited cytosolic cytidylyltransferase and this inhibition could be reversed by the inclusion of cAMP-dependent protein kinase inhibitors. Calmodulin was also observed to inhibit cytosolic cytidylyltransferase (E. Sommerman unpublished data). However, anaesthetics such as trifluoperazine, chlorpromazine, and tetracaine, which are thought to inhibit calmodulin action, also inhibited cytosolic cytidylyltransferase (153). Thus, the role, if any, of Ca$^{2+}$-calmodulin-dependent protein kinases in PC synthesis is not clear.

**Phospholipase A$_2$ (1. 4. 2. 6.).**

Phospholipase A$_2$ is ubiquitous in nature and catalyzes the hydrolysis of 2-acyl chain of phospholipids, liberating the precursor of biologically active lipids, such as eicosanoids and platelet-activating factor. The most studied phospholipase A$_2$s are from the snake venom and pancreatic juice. These were initially identified when it was discovered that PC was degraded in the presence of pancreatic juice (260), while snake venom was shown to degrade membranes of erythrocytes (261). Phospholipase A$_2$ is extremely stable to a variety of manipulations which has allowed it to be easily purified.

The pancreatic phospholipases are synthesised as zymogens that are activated by the cleavage of a heptapeptide by trypsin. Both the zymogen and the processed enzyme (molecular weight 14,000)
are active on monomers of phospholipid. Verger et al (262) has postulated a model for the binding and activation of the pancreatic enzyme to monolayers of PC. The initial binding of the enzyme (E) in the subphase is a slow process that precedes the formation of the active enzyme (E*) adsorbed to the monolayer of phospholipid. The E* then forms the catalytically active complex (E*S) with the interfacial substrate (S).

There is clear evidence that the catalytic site is distinct from the lipid binding site in both the pancreatic and venom phospholipases, as has been demonstrated by chemical modification of the processed phospholipases and nuclear magnetic relaxation studies (263). Compounds such as bromophenacyl bromide that react with His-48 in the active site do not prevent binding of the substrate to the processed enzyme (263). Likewise, the presence of distinct catalytic and binding sites would account for both the zymogen and the processed enzyme acting on monomeric substrates, while only the processed enzyme binds and acts at interfaces. Dennis et al (264) has suggested that the snake venom enzyme may be thought of as a two-site dimer. One monomer of enzyme binds the activator molecule while the other monomer binds the substrate. Interaction of the two monomers then activates the catalytic monomer which promotes hydrolysis.

The venom and pancreatic phospholipase A2 have an absolute requirement for Ca$^{2+}$, which is bound adjacent to His-48, as shown by Ca$^{2+}$ blockage of the binding of bromophenacyl bromide to this residue (263). The binding of Ca$^{2+}$ involves an octahedral arrangement with seven oxygen bridges and lowers the pK of the
essential His-48 from 7 to 5.7. A proton relay system that employs a molecule of water as the nucleophile attacking the ester bond has been proposed as the mechanism of catalysis (265). Asp-99-His-48 pair removes a proton from bound water, producing the nucleophilic hydroxyl group. Ca\(^{2+}\) interacts with both the phosphate and the carbonyl groups of the ester undergoing hydrolysis as well as the carboxyl of Asp-49 and binds the free fatty acid formed until the fatty acid diffuses from the active site (265).

The mechanism of activation and regulation of cellular phospholipase A\(_2\) is not completely understood. In human platelets, phospholipase A\(_2\) activity is induced by physiological stimuli (266) as well as Ca\(^{2+}\)-ionophores (267). Halenda et al (268) demonstrated that phospholipase A\(_2\)-mediated liberation of arachidonic acid in response to ionophore A23187 was enhanced by activators of protein kinase C, including phorbol esters and 1-oleoyl-2-acetylglycerol. These results suggested that protein kinase C may be involved in phospholipase A\(_2\) activation. Dawson et al (269) has shown stimulation of phospholipase A\(_2\) by diacylglycerol. Kramer et al (270) also showed activation of a Ca\(^{2+}\)-activated human platelet phospholipase A\(_2\) by diacylglycerol.

Involvement of phospholipase A\(_2\) in PC synthesis (1. 4. 2. 7.).

The direct effect of phospholipase A\(_2\) on PC synthesis has not been demonstrated but the product of a phospholipase A\(_2\) reaction, namely fatty acid, has been shown to regulate PC synthesis in a variety of model systems. Studies with rat liver slices (271) and isolated hepatocytes (145) demonstrated that 1 mM concentration of
fatty acid in the medium can stimulate \(^{3}\text{H}\)glycerol incorporation into triacylglycerol and PC. Pulse-chase experiments with \([\textit{methyl-}^{3}\text{H}]\text{choline}\) have shown fatty acids to accelerate the appearance of radiolabel into PC in rat hepatocytes (63), rat alveolar type II cells (55), rabbit kidney cortex (272), Hela cells (273, 274), and lymphocytes (275).

The primary site of action of fatty acids in stimulating PC synthesis was at the CTP:phosphocholine cytidyltransferase catalyzed reaction (63, 273). Fatty acid were not true activators of the cytidyltransferase, but promoted binding of the cytidyltransferase to membranes which in turn resulted in stimulation of enzyme activity. For example, treatment of hepatocytes with 1 mM oleate (63) resulted in doubling of the cytidyltransferase associated with the microsomes with a corresponding reduction in cytosolic activity. This phenomenon was even more vividly demonstrated in HeLa cells (273) where treatment with oleate resulted in complete translocation of the cytidyltransferase to the microsomes and a 5-20 fold increase in PC synthesis.

Long-chain saturated or unsaturated fatty acids were found to be equally effective in stimulating PC synthesis in hepatocytes (63). However, in HeLa cells (273, 274) and lymphocytes (275), unsaturated fatty acids were much more effective in stimulating PC synthesis than saturated fatty acids. Cornell \textit{et al} (274) has examined the nature of the fatty acyl chain that acted as stimulator of cytidyltransferase and thus of PC synthesis in HeLa cells. It was discovered that acyl-chains with a free hydroxyl group that were not
-CoA derivatives of the fatty acid, acted as good stimulators of cytidylyltransferase activity. The effect of fatty acids was found to be reversible and removal of fatty acid or treatment with BSA, resulted in translocation of the cytidylyltransferase from the microsomes to the cytosol (274, 276).

The molecular details of how fatty acids stimulate PC synthesis are not clear. Pelech et al (63) found that the diacylglycerol pool size was doubled in rat hepatocytes supplemented with 1 mM oleate. This suggested that diacylglycerol may account for some of the translocation of cytidylyltransferase in these cells. Feldman et al (22) suggested that fatty acids altered the anionic charge at membrane sites which resulted in promotion of cytidylyltransferase binding to these membranes. However, Cornell et al (274) has shown that cytidylyltransferase binding to membrane is not an ionic interaction, since oleyl alcohol and monoolein promoted binding, while high ionic strength medium did not block translocation by fatty acids. Thus, the exact effect of fatty acid on cytidylyltransferase translocation is not clear.
AIM OF THESIS (1. 5. 1. 1.).

Earlier work by several investigators had implicated short term regulation of PC synthesis by reversible phosphorylation and free fatty acids. In each case, the effect was shown to be on the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase. However, these studies could not evaluate whether the effects of reversible phosphorylation and free fatty acids were directly on the cytidylyltransferase itself or on a modifier protein which in turn regulated the enzyme activity. Therefore, it was of paramount importance to purify the cytidylyltransferase to homogeneity. Purification procedures involving conventional and high performance chromatographic techniques were to be employed to achieve this goal. Using the purified enzyme, it would be established whether or not cytidylyltransferase is itself phosphorylated or is regulated by a phosphorylatable modulator protein. If the cytidylyltransferase were found to be a substrate for protein kinase, I would determine which protein kinase catalyzed the phosphorylation and which protein phosphatase catalyzed the dephosphorylation. Also we planned to establish at which residue(s) the phosphate is incorporated and whether it is a multisite phosphorylation by different kinases. The purified cytidylyltransferase would also be used to examine the specificity of lipid activation using commercial and microsomal lipid species.

The purified cytidylyltransferase would be used to raise polyclonal antibodies in a rabbit. These antibodies would be used to look at changes in mass of enzyme, by different physiological
stimulants, between the cytosolic and the microsomal compartments. These studies should clarify whether the translocation phenomenon involves mass changes between the two compartments or whether it was a selective activation in one compartment and inhibition in another. Antibodies would also be used to immunoprecipitate cytidylyltransferase from hepatocytes which have been incubated in the presence of cAMP analogues and $^{32}$Pi. The immunoprecipitated enzyme could then be analyzed to examine whether phosphate can be incorporated *in vivo*.

Since previous work had implicated Ca$^{2+}$-mobilizing and Ca$^{2+}$-activated agents in the regulation of PC synthesis, the effect of various concentrations of Ca$^{2+}$ in the medium on fresh hepatocytes would be examined. We would examine whether these effects of Ca$^{2+}$ are mediated through a Ca$^{2+}$-calmodulin-dependent protein kinase or whether it is some other agent(s) which is activated by Ca$^{2+}$. These studies should enlighten us on the role of Ca$^{2+}$ in PC synthesis.
EXPERIMENTAL PROCEDURES

MATERIALS

Animals (2. 1. 1. 1.).

Female Wistar rats (200-250 g) were obtained from the University of British Columbia animal unit or from Charles River Inc. Female Sprague-Dawley rats (50-75 g) were obtained from the University of Alberta animal unit.

Chemicals (2. 1. 1. 2.).

The commercial source of the chemicals used during this investigation are listed in Table 1. All other chemicals used were of reagent grade.

GENERAL PREPARATIVE AND ANALYTICAL PROCEDURES

Preparation of rat hepatocytes (2. 2. 1. 1.).

Materials

1. A peristaltic pump with cannula tubing and a needle, speed set at about 5ml/min.
2. A water bath (37 °C).
3. A bottle of Hanks-EGTA solution (80 ml) with 0.8 ml insulin (1 mg/ml), prewarmed to 37 °C.
4. A bottle of Hanks-collagenase solution (80 ml) with 0.8 ml insulin (1 mg/ml) and 45-50 mg collagenase, prewarmed to 37 °C. Transfer about 15 ml of the solution to a capped tube.
5. Two bottles of Dulbecco's Modified Eagle Medium (MEM) (500 ml) with 5 ml insulin. To one bottle add 17% (g/vol) fetal bovine serum. Prewarm to 37 °C.

6. A dissecting tray, a stop watch, a pair of middle size dissecting scissors, a pair of fine surgical scissors, a pair of fine forceps, a clamp, 4 pieces of thread (in different colours), culture dishes.

7. Pentobarbitone solution (500 mg in 5 ml ethanol and 5 ml ethylene glycol).

8. 1-cc syringe with needle, diethyl ether, a haemocytometer, trypan blue solution, a coarse cell filter and a fine cell filter.

Procedure

1. Anesthetize the rat with diethyl ether in a box, and inject intraperitoneally with pentobarbitone (0.22 ml/50 g body weight).

2. After anesthetizing, cut the abdominal skin by mid-line incision with a pair of dissecting scissors. Separate the outer skin and cut abdominal wall (U-shape) with a pair of dissecting scissors.

3. Move intestine to the right side to expose the portal vein and lower vena cava. Make a loop of thread around lower vena cava above the renal vein. Make two loops of thread around portal vein.

4. Clamp off portal vein and make a small cut with a pair of fine surgical scissors on portal vein between the clamp and the loops.

5. Start the peristaltic pump (5 ml/min) and insert cannula needle quickly into the cut and fix the needle by fastening the loops. Cut the lower vena cava below the renal vein and perfuse liver for 4 min with Hanks-EGTA solution.
6. Meanwhile, remove thorax plus diaphragm and make a loop of thread around the upper vena cava.

7. Tie off the lower vena cava and quickly cut the heart and let the perfusate run out until the blood is drained.

8. Tie off upper vena cava, and let the liver swell. Snip edges of the liver and continue perfusion for 4 min. Stop the pump and change the cannula tubing to Hanks-collagenase solution. Continue perfusion with Hanks-collagenase for 10-11 min, until the liver tissue is loosened.

9. Smash the liver with scissors and transfer cells to a capped plastic centrifuge tube containing 15 ml of Hanks-collagenase solution. Shake the tube for about 5 min in a 37 °C water bath.

10. Stop the enzymic reaction by adding 70 ml of MEM containing serum and filter cells through a coarse filter. Transfer cells into 2 capped plastic centrifuge tubes and centrifuge.

11. Remove the supernatant by suction, and wash pellet with MEM without serum and spin as before. Repeat twice.

12. Resuspend cells in MEM containing serum. Take an aliquot of cell suspension and count the cell number. Adjust the cell concentration to 3 x 10^6 cells/3 ml. (The # of cells in 16 squares x 10^4 = # of cells/ml). Plate out 3 ml of cell suspension/50-mm culture dish and incubate cells in an incubator with an atmosphere of 5% CO_2/air at 37 °C for 3-4 h.

Subcellular fractionation of whole rat liver or isolated hepatocytes (2. 2. 1. 2.).
Female Wistar rats were anesthetized and the liver perfused with ice-cold isotonic saline (0.15 M NaCl) for 2-3 min at a flow rate of 10 ml/min. The liver was removed and placed in a beaker containing ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM DTT, 1 mM PMSF, 0.025% sodium azide, and 1 mM EDTA). The liver was cut into small pieces and homogenized (5 ml buffer A/g liver) with 7 strokes up and down in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant (post mitochondrial supernatant) was subsequently centrifuged at 120,000 x g for 60 min at 4 °C. The resulting supernatant was designated the cytosolic fraction. The microsomal pellet was resuspended in buffer A with 20 strokes of a glass-teflon homogenizer.

For preparation of microsomal and cytosolic fractions from isolated rat hepatocytes, the cells from 3 dishes were scraped in 2 ml of buffer A using a rubber policeman. The cells were pelleted by centrifugation at 2,000 x g for 5 min at 4 °C. The pellet was resuspended in 2 ml of buffer A and the cells broken with 40 strokes of a tight-fitting glass dounce homogenizer. The homogenate was centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was removed and the pellet rehomogenized and centrifuged as before. The combined 10,000 x g supernatant was centrifuged at 120,000 x g for 1 h at 4 °C. The supernatant was designated the cytosolic fraction. The microsomal pellet was resuspended in 2 ml of buffer A with 20 strokes of a glass-teflon homogenizer.

**PURIFICATION OF CTP:PHOSPHOCHOLINE CYTIDYLTRANSFERASE**
Purification by aggregation/deaggregation of cytosolic form (2. 2. 2. 1.).

The procedure used was adopted from the procedure reported by Choy et al (69) with some modifications. Rat liver cytosol (100 ml) was incubated at 37 °C for 4 h in the presence of protease inhibitors PMSF (1 mM), bacitracin (1 mM), pepstatin A (0.2 μg/ml), and benzamidine (1 mM). The cytosol was adjusted to 20% ammonium sulfate saturation with stirring at 4 °C, and the precipitate removed by centrifugation at 10,000 x g for 10 min. The supernatant was readjusted to 30% ammonium sulfate saturation and the precipitate formed after 2 h storage at 4 °C was collected by centrifugation at 10,000 x g for 10 min. The pellet was resuspended in 5 ml of 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% glycerol, and 2 mM DTT buffer. The sample was loaded, at a flow rate of 1 ml/min, onto a Sepharose 6B gel filtration column (2.5 x 40 cm) which had been initially equilibrated with the same buffer. Cytidylyltransferase activity was eluted in the void volume and the active fractions pooled. Triton X-100 (5%) was added to the pooled fractions to a final concentration of 0.05% and the sample stirred at 4 °C for 2 h. The sample was reapplied to a second Sepharose 6B column which had been equilibrated with the above buffer containing 0.05% Triton X-100. Cytidylyltransferase was separated into two peaks. The second peak was pooled and concentrated by ultrafiltration using an Amicon PM 30 membrane.
Purification by conventional and high performance techniques of cytosolic form (2.2.2.2.).

To 300 ml of cytosol, Triton X-100 (5%) was added so as to give a final concentration of 0.05%. The cytosol was adjusted to 30% ammonium sulfate saturation with stirring at 4 °C. After 15 min, the sample was centrifuged at 15,000 x g for 15 min. The supernatant was readjusted to 40% ammonium sulfate saturation with stirring. The precipitate formed after 30 min was collected by centrifugation at 15,000 x g for 15 min. The pellet was left overnight at -70 °C. Next day, the pellet was resuspended in 325 ml buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The sample was loaded, at a flow rate of 2 ml/min, onto a DEAE-cellulose column (2.5 x 40 cm), which had been equilibrated with the same buffer. The column was eluted, at 2 ml/min, with 100 ml of Tris-succinate buffer containing 75 mM NaCl before starting a 200 ml gradient from 75-200 mM NaCl in Tris-succinate buffer. The cytidylyltransferase activity was eluted into 9 ml fractions above 100 mM NaCl concentration. The most active fractions were pooled and applied, at a flow rate of 1 ml/min, onto a phospho-cellulose column (2.5 x 10 cm) which had been equilibrated with Tris-succinate buffer. The column was washed, at the same flow rate, with 75 ml each of 100 mM and 200 mM NaCl in Tris-succinate buffer. The cytidylyltransferase activity was eluted into 5 ml fractions with 75 ml of 300 mM NaCl in Tris-succinate buffer. The most active fractions were pooled (~45 ml) and concentrated to 10 ml by ultrafiltration using an Amicon PM 30 membrane. The concentrated sample was subsequently diluted to 30 ml and loaded onto a Mono-Q
column (1 ml bed volume) on the FPLC at a flow rate of 1 ml/min. The column was washed with 10 ml of Tris-succinate buffer containing 100 mM NaCl at the same flow rate. The cytidylyltransferase was eluted into 1 ml fractions with a 25 ml 100-250 mM NaCl gradient in Tris-succinate buffer. The most active fractions were pooled (~12 ml) and concentrated to 4 ml by ultrafiltration as described above. The concentrated sample was diluted to 12 ml with buffer (20 mM MOPS-HCl, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The diluted sample was loaded, at a flow rate of 1 ml/min, onto a Mono-S column (1 ml bed volume), which had been equilibrated with the same buffer. After washing the column with 5 ml of buffer, the cytidylyltransferase activity was eluted into 1 ml fractions with a 20 ml gradient of 0-500 mM NaCl in MOPS-HCl buffer.

Purification of cytosolic form by lipid-protein aggregation and resolution on FPLC (2. 2. 2. 3.).

The purification procedure reported by Weinhold et al (72) was adopted with some modifications. Rat liver cytosol (400 ml) was mixed with PC-oleic acid vesicles (5 mM PC: 10 mM oleic acid; vesicles prepared by evaporating solvent, adding buffer A and then sonicating) to give a final PC-oleic acid to protein ratio of 80 nmol/mg protein. The mixture was stirred at room temperature for 20 min and then the pH of the cytosol-lipid mixture reduced to 5.0 by the addition of 1.0 M acetic acid. The mixture was placed on ice for 10 min and then centrifuged at 20,000 x g for 20 min. The pellet was resuspended in 250 ml of buffer A (50 mM Tris-HCl, pH 7.4, 0.15
M NaCl, 2 mM DTT, 1 mM PMSF, 1 mM EDTA, and 0.025% sodium azide) by homogenization with a Potter-Elvehjem homogenizer. The mixture was centrifuged at 20,000 x g for 20 min, and the supernatant subsequently discarded. The pellet was resuspended, by homogenization, in buffer A containing 20 mM octyl glucoside (7 ml of octyl glucoside solution/100 mg of protein in the original cytosol). The mixture was stirred for 20 min at room temperature and then centrifuged at 20,000 x g for 20 min. The supernatant was removed and loaded onto a DEAE Sepharose CL-6B column (2.6 x 14.5 cm) which had been equilibrated with 500 ml buffer A at 50 ml/h. The octyl glucoside extract was passed through the column at a flow rate of 50 ml/h. The rest of the procedure was done with the Pharmacia FPLC system. The column was washed sequentially at the same flow rate with 50 ml buffer A, and a 300 ml gradient of 0.15 M NaCl to 0.3 M NaCl. The cytidylyltransferase was eluted with a 150 ml gradient of 0.3 M NaCl-50 mM octyl glucoside to 0.4 M NaCl-50 mM octyl glucoside in 8 ml fractions. The active fractions were pooled and diluted with buffer A to 20 mM octyl glucoside. This was applied onto a column of hydroxylapatite (1.7 x 12 cm), which had been equilibrated with 300 ml of buffer A at 30 ml/h. The column was then washed, at the same flow rate, sequentially with 50 ml 0.2 M potassium phosphate in buffer A, pH 7.5, and a 200 ml gradient of 0-100 mM octyl glucoside in 0.2 M potassium phosphate buffer, pH 7.5. The cytidylyltransferase activity was eluted, in 4 ml fractions, with 110 ml of 0-0.03% Triton X100-0.2 M phosphate buffer gradient. The active fractions were pooled and diluted 4 fold with buffer B (50 mM Tris-HCl, 50 mM potassium phosphate, 1 mM EDTA, 2 mM DTT,
0.025% sodium azide, and 0.03% Triton X100, pH 7.4). The extract was applied onto a Mono-Q column (1 ml bed volume) at a flow rate of 1 ml/min. The column was washed, at the same flow rate, with a 50 ml gradient of 0-0.5 M NaCl in buffer B. Cytidylyltransferase was eluted, in 1 ml fractions, between 0.15-0.3 M NaCl.

PREPARATION OF SUBSTRATE AND LIPID ACTIVATORS OF CTP:PHOSPHOCHOLINE CYTIDYLTRANSFERASE

Synthesis of \[^{3}H\]phosphocholine (2. 2. 3. 1.).

Reaction mixture for the synthesis of phosphocholine;

- 2 mCi \([methy-{^3}H]\)choline chloride (1 mCi/ml)
- 25 µl 1 M Tris-HCl, pH 8.0
- 25 µl 0.1 M MgCl\(_2\)
- 25 µl 0.1 M ATP
- 175 µl dialyzed choline kinase (0.25 units)

0.5 ml aliquots of \[^{3}H\]choline chloride in ethanol were dried under N\(_2\) in a plastic test tube until 2 ml had been dried. To this tube was added the above components, except the choline kinase. The mixture was vortexed and incubated at 37 °C for 5 min. Then the choline kinase was added and the mixture incubated at 37 °C for 1 h. The reaction was stopped by placing the tube in boiling water bath for 2 min. The mixture was centrifuged at 5,000 x g for 5 min, and then the supernatant applied as a single streak 2.5 cm from the
bottom on a TLC plastic silica plate (1 x 1 cm square sections were marked with a pencil in the middle of the plate). The plate was developed in CH$_3$OH/0.6% NaCl/NH$_4$OH (10/10/1) for 3 h. Individual squares (1 x 1 cm) were scraped into tubes containing 2 ml water, vortexed and then removed 20 µl aliquots for counting radioactivity. The lane where abundant radioactivity was located was scraped and the silica extracted with 2 ml aliquots of water. Extraction with water was repeated until most of the radioactivity from the silica had been eluted. The combined extract was diluted with water so as to give a specific activity of 8-9 µCi/µmol. "Cold" phosphorylcholine was added to a final concentration of 15 mM.

**Lipid extraction (2.2.3.2.).**

Lipid was extracted from whole rat livers and microsomes by the method of Folch *et al.* (277). The tissue was homogenized in water using the Potter-Elvehjem homogenizer. Homogenate (1 ml) was removed and added to this 12 ml of chloroform (containing antioxidant 2,6 di-t-butyl-4-methylphenol; 50 mg/l) and 6 ml of methanol. The extract was rehomogenized, left on ice for 2 h, and then filtered through filter paper. Water (3.8 ml) was added to the extract so as to give a final ratio of chloroform/CH$_3$OH/water of 2/1/0.8 (by vol). The mixture was centrifuged at 1,000 x g for 15 min, and then the upper layer removed and discarded. The lower layer (chloroform phase) was washed twice with 3 ml of theoretical upper phase (CH$_3$OH/water/chloroform 48/47/3; by vol). The lower phase containing the lipids was dried using a rotary evaporator and then resuspended in 2 ml of chloroform.
Extraction of lipid from isolated hepatocytes was accomplished by a similar method except the cells were suspended in 50% CH₃OH, and sonicated for 30 seconds, at an output setting of 4, with an Ultrasonic processor W-385, prior to the addition of chloroform.

**Preparation of PC:oleic acid vesicles (2. 2. 3. 3.).**

PC:oleic acid vesicles (1/1 or 1/2 molar ratio) were prepared by mixing the appropriate volumes of stock solutions of PC and oleic acid in a round bottomed flask. The solvent was evaporated under vacuum in a rotary evaporator. Buffer A (50 mM Tris-HCl, pH 7.4, 0.15M NaCl, 2 mM DTT, 1 mM EDTA, and 0.025% sodium azide) was added and the mixture sonicated with a large probe sonicator for 60 min at 4 °C.

**Separation of lipids (2. 2. 3. 4.).**

The dried lipid was suspended in 2 ml of chloroform and then a 50 µl aliquot removed and spotted onto a silica-gel 60 TLC plate (in the analysis of radiolabelled lipids, appropriate lipid standards were also applied as carriers for identification purposes). The plate was developed half way in chloroform/CH₃OH/acetic acid/formic acid/water (70/30/12/4/2; by vol) (1st solvent; separates phospholipids). The plate was dried in an oven for 30 min, and then chromatographed to the top in hexane/diisopropyl ether/acetic acid (65/35/2; by vol) (2nd solvent; separates neutral lipids). The plate was dried, and placed in an iodine tank for 2 min. Bands corresponding to lipids were scraped and extracted 3 times with 2 ml of chloroform/CH₃OH/acetic acid/water (50/39/1/10; by vol). The
extracts were combined and 1 ml of water added to make two phases. After vortexing and centrifugation, the upper layer was discarded and the lower layer used as the source of lipid.

Assay of CTP:phosphocholine cytidylyltransferase (2. 2. 4. 1.).

Assay mixture;
10 μl cytidylyltransferase sample
50 μl water
10 μl 2 mM PC:oleic acid vesicles (1/1 molar ratio) or 10 μl TRLP (100 μg)
10 μl 15 mM $[^3]$H phosphocholine
7.5 μl 100 mM magnesium acetate
7.5 μl 1 M Tris-HCl, pH 6.5
5 μl 40 mM CTP
Total assay volume is 100 μl.

The assay mixture, except the phosphocholine substrate, was incubated at 37 °C for 5 min. Then the substrate was added to start the reaction. The test tube was incubated for 15 min at 37 °C before stopping the reaction by placing the test tube in a boiling water bath for 2 min. The reaction mixture was centrifuged at 1,000 x g for 5 min and the supernatant containing choline metabolites spotted on TLC and analyzed.

Analysis of choline-containing metabolites by TLC (2. 2. 4. 2.).

Assay supernatant (15 μl) was applied as a streak 1.5 cm from the bottom on a silica-gel plastic plate. Standard (5 μl) (contains 15 mg/ml choline, 60 mg/ml phosphocholine, and 10 mg/ml CDP-
choline) was applied just below the sample. The plate was dried, and then developed in CH$_3$OH/0.6% NaCl/NH$_4$OH (10/10/1; by vol) for 45 min. After drying the plate, the choline-containing metabolites were visualized by exposure to iodine vapour for 5 min, or by examination under UV-light. The bands were scraped into plastic scintillation vials and 0.5 ml of water added. After vortexing, 5 ml of ACS was added and the samples counted.

The specific activity (S.A.; in nmol/min/ml) of the cytidylyltransferase was calculated from the equation:

\[
\text{S.A.} = \frac{(\text{dpm} - \text{blank dpm}) \times 100}{\text{assay time (min)} \times \text{vol spotted (ml)} \times \text{vol of cytidylyltransferase phosphocholine (μl)} \times \text{used in assay (ml)}}
\]

**Liquid scintillation counting (2.2.4.3.).**

Water (0.5 ml) was added to plastic vials containing the lipid or aqueous sample. The mixture was vortexed and then 5 ml of ACS (aqueous counting scintillant) added. The samples were counted in a scintillation counter immediately, in the case of aqueous metabolites, or the next day, in the case of lipid metabolites. The counting efficiency was determined from the channel-ratios of standards.

**Estimation of protein (2.2.5.1.).**

Protein was determined with the Bio-Rad protein assay based on the method of Bradford (278). Bovine serum albumin was used as the standard. Standards were prepared with the same concentration of the enzyme buffer ingredients that were in the sample.
For samples with protein content above 50 µg/ml, the diluted assay method was used. Basically, to 0.1 ml of sample was added 5 ml of filtered Bio-Rad reagent (diluted 1:5 by volume with water). The absorbance at 595 nm after 10 min was measured. For samples with protein content less than 50 µg/ml, the micro-assay was used. This involved adding 0.2 ml of concentrated reagent to 0.8 ml of sample and then measuring the absorbance after 10 min at 595 nm.

**ANALYSIS OF CTP-PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE BY POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)**

**Preparation of sample for SDS-PAGE (2.2.6.1.).**

Samples which contained less than 50 µg/ml of protein were prepared for analysis by SDS-PAGE by TCA precipitation. Sodium deoxycholate was added to the sample to give a final concentration of 5 µg/ml. The sample was placed in an ice-bath and one half the volume of cold 20% TCA added (4 °C). The protein was allowed to precipitate for 30 min at 4 °C, before centrifugation at full speed for 20 min in a microfuge. The supernatant was removed carefully and the pellet washed with 1 ml of cold acetone (-20 °C). The protein was repelleted by centrifugation at full speed for 10 min in a microfuge. The washing procedure was repeated and then the pellet resuspended in a small volume of water. After vortexing, an equal volume of SDS-disruption buffer was added and the sample boiled for 5 min before analyzing by SDS-PAGE.
**SDS-PAGE (2. 2. 6. 2.).**

SDS-PAGE was performed on 1.5 mm thick gels using the buffer system described by Laemmli (279). A 5-20% separating gel was poured by placing 16 ml of heavy solution (30% acrylamide made with 75% glycerol) in the mixing chamber, and 16 ml of light solution (5% acrylamide in water) in the reservoir in a gradient maker. After polymerization, a 3% stacking gel was poured on top of the separating gel. Electrophoresis, at constant current, was done at 15 mA for 7 h or 9 mA for 14 h. The separating gel was silver stained using the method of Merril et al (28) or was stained with 0.04% coomassie blue R-250 in water/isopropanol/acetic acid (325/125/75; by vol) and destained with 7% acetic acid.

**2-D-PAGE (2. 2. 6. 3.).**

2-D-PAGE was carried out essentially as described by O'Farrell (281). The first dimension was IEF (isoelectric focusing) using Pharmacia ampholytes to make a pH gradient from 3-10. Electrophoresis was carried out in tube gels. Enzyme, which had been TCA precipitated, was mixed with 40 µl lysis buffer and then overlayed with 10 µl of overlay buffer. Electrophoresis was at 400 V for 10 h. After electrophoresis, the gel was removed from the tube with a syringe full of water and placed in a petri-dish containing equilibration buffer. After 15 min, the gel was laid on top of a SDS-slab gel (2nd dimension) apparatus and sealed with hot agarose made in equilibration buffer. After the agarose had solidified, electrophoresis was done at 25 mA for 7 h. The gel was subsequently removed and silver stained.
IEF- and native-PAGE (2. 2. 6. 4.).

IEF-(isoelectric focusing) and native-PAGE was done using the Pharmacia Phast system. Electrophoresis was done using preformed gels and the whole procedure of running and staining gels was completed within 1 h. For IEF-PAGE, gels with a pH gradient from 3-9 were used. Native-PAGE was done using 5% separating gels.

IMMUNOLOGICAL STUDIES

Preparation of polyclonal antibody in rabbits (2. 2. 7. 1.).

Purified CTP:phosphocholine cytidylyltransferase (~50 μg) was diluted 4-fold and passed through a Mono-Q column on the FPLC. The enzyme was eluted in 1 ml with 5 ml of 0-0.5 M NaCl gradient. The concentrated enzyme was placed in a syringe and mixed, using a double-hub connector, with 2 ml of Freund's complete adjuvant. Mixing was carried out by squirting the aqueous phase into the oil as vigorously as possible and then squirting the total content to- and fro- from one syringe to the other at least 20 times. After the enzyme sample had been completely mixed with the adjuvant (total ~3 ml), 0.5 ml of the emulsion was injected into 6 different sites under the skin, at the back of the neck of a rabbit (this is the priming injection). Injection was done by pinching the skin and then gently inserting the needle under the skin. This route of injection is less painful to the animal and bleeding is not a problem.
After 4 weeks, a booster injection was given to the rabbit. Cytidylyltransferase (~30 μg in 1 ml) was mixed with 2 ml of Freund's incomplete adjuvant, as described before, and injected into 6 different sites under the skin at the back of the neck. Four weeks later, a second booster injection, of similar magnitude as the first booster, was given. Two weeks after the second booster injection, the rabbit was placed in a clamp cage and a blood sample (~5 ml) from the large vein in the ear removed with a syringe.

Separation of serum from blood (2. 2. 7. 2.).

Blood was collected from a rabbit in a clean capped tube and incubated at 37 °C for 1 h (no shaking), to allow clotting. The tube was then allowed to sit in the cold room for ~2 h. The blood clot which had formed, was retracted from the walls of the tube with a sterile glass rod, and the serum removed into another clean capped tube. The serum was centrifuged at 2,000 x g for 20 min, and the supernatant removed and used as the source of antibodies. The serum was stored in 0.5 ml aliquots at -20 °C.

Detection of CTP:phosphocholine cytidylyltransferase with antibody using the western-blot technique (2. 2. 7. 3.).

Reagents:
1. TBS (Tris-buffered saline); 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl.
2. TTBS (Tween-20 TBS); 0.5 ml Tween-20/litre of TBS.
3. Blocking solution; 3% gelatin in TBS.
4. Antibody buffer; 1% gelatin in TTBS.
5. First antibody solution; 1:500 dilution of antiserum in antibody buffer.
7. Colour reagents; 30 mg NBT in 1 ml of 70% DMF, 15 mg BCIP in 1 ml of DMF, carbonate buffer (0.1 M NaHCO₃, 10 mM MgCl₂.6H₂O, pH 9.8). Mix BCIP and NBT just before use and add to 100 ml of carbonate buffer.
8. Transfer buffer (25 mM Tris, 192 mM glycine in 20% MeOH).

Procedure:
1. Separated cytidylyltransferase on SDS-PAGE (12 % separating gel).
2. After electrophoresis, soaked gel in 150 ml of transfer buffer for 15 min.
3. Soaked a piece of nitrocellulose paper and two pieces of thick filter paper and sandwich pads in transfer buffer for a short while.
4. Assembled the gel and membrane sandwich between two thick filter papers and sandwich pads. The gel was placed on black side of the perforated sandwich apparatus and the membrane on colourless side. Care was taken not to trap any air bubbles.
5. Inserted the sandwich in the middle slot of the transblot cell. Transferred at constant current of 0.2 A for 14 h.
6. After transfer, incubated membrane with blocking solution for 2 h. Rinsed membrane with 150 ml TTBS (2 x 5 min). Incubated
membrane with first antibody for 5 h. Rinsed membrane with TTBS (2 x 5 min).

7. Incubated membrane with second antibody solution for 2 h. Rinsed membrane with TTBS (2 x 5 min).

8. Incubated membrane with colour reagent for 5-15 min. After colour development, rinsed membrane with water and dried between filter paper.

EXPERIMENTS WITH HEPATOCYTES

Digitonin-mediated release of CTP:phosphocholine cytidylyltransferase from hepatocytes (2. 2. 8. 1.).

Cytidylyltransferase was released from cultured hepatocytes by digitonin treatment as described by Mackall et al (282). Digitonin (0.5 mg/ml) was dissolved in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25 M sucrose, and 0.5 mM PMSF. After treatment of cells with Ca\(^{2+}\) or Ca\(^{2+}\)-mobilizing agents, the medium was removed and the cells washed with 2 ml/dish of ice-cold PBS. This was subsequently removed and the dishes placed on an ice-cold glass plate in an ice bath. Digitonin solution (1 ml) was added to each dish and after 2, 4, and 8 min, the digitonin solution was removed with a pipet and assayed for cytidylyltransferase activity.

Radiolabelling studies with hepatocytes (2. 2. 8. 2.).

Incorporation of radiolabel into PC was examined by incubating hepatocytes with [methyl-\(^{3}\)H]choline chloride (10-15 μCi/dish) for
30 min in choline- and methionine-free medium. After the pulse, the medium was removed and the cells chased with choline- and methionine-supplemented medium.

To study the effects on PC degradation, hepatocytes from choline deficient rats (maintained on choline-free diet for 3 days) were pulsed for 2 h with 15 μCi/dish of [methyl-3H]choline chloride in choline- and methionine-free medium. After this interval, the medium was removed and the cells chased with choline- and methionine-supplemented medium.

PHOSPHORYLATION STUDIES

Phosphorylation of CTP:phosphocholine cytidylyltransferase in vitro (2. 2. 9. 1.).

The preincubation mixture for phosphorylating conditions contained, in a volume of 3 ml, 20 mM Tris-HCl, pH 7.4, 0-150 units cAMP-dependent protein kinase, 4 mM MgCl₂, 2 mM DTT, 2 μg pure cytidylyltransferase, 1 mg washed microsomal protein (prepared by rehomogenizing microsomes in buffer A and centrifuging at 130,000 x g for 1h) and either 0.1 mM ATP or 0.1 mM [γ-32P]ATP (400 cpm/pmol). The cytidylyltransferase was initially "dephosphorylated" by incubation with 4 mM MgCl₂, and 40 units of alkaline phosphatase bound to agarose at 4 °C with continuous shaking. After 1 h, the alkaline phosphatase was separated from the cytidylyltransferase by centrifugation at 2,000 x g for 5 min at 4 °C. The preincubation commenced with the addition of microsomes at 37
°C. After 30 min, the incubation mixture was centrifuged at 130,000 x g for 1h. The fractions which contained the "cold" ATP were used to assay for cytidylyltransferase activity. The pellet was resuspended in 1 ml of buffer A and then 20 μl used for enzyme assay. The supernatant (20 μl) was assayed in the presence of exogenous lipid. The supernatant from the incubation mixture which contained [γ-32P]ATP was used to determine the incorporation of 32P into the cytidylyltransferase. The supernatant was diluted 4-fold with 20 mM Tris-HCl, pH 7.4, buffer and passed through a Q-Sepharose column (1 ml bed volume). The column was washed with 10 ml of 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl buffer to remove the cAMP-dependent protein kinase. The cytidylyltransferase was eluted with 5 ml of 20 mM Tris-HCl, pH 7.4, containing 0.3 M NaCl. The pooled cytidylyltransferase was precipitated by addition of 5 μg/ml sodium deoxycholate and 2.5 ml of 20% trichloroacetic acid at 0 °C for 30 min. The precipitate was washed with cold acetone and analyzed on SDS-PAGE as described previously. After electrophoresis, the gel was stained with 0.04% Commassie blue R-250, 10% acetic acid, and 25% isopropanol for 2 h, and destained with 7% acetic acid overnight. The gel was dried on filter paper under a vacuum, covered with Saran wrap, and exposed for 24 h to Kodak XAR-5 film at -70 °C.

For some experiments, the preincubation mixture for phosphorylating conditions also contained 250 nM pure synthetic peptide inhibitor for cAMP-dependent protein kinase (283).

For dephosphorylating conditions, pure cytidylyltransferase was first "phosphorylated", as described above, with either 0.1 mM ATP or 0.1 mM [γ-32P]ATP for 30 min at 30 °C. After this time
interval, the mixture was passed through a Q-Sepharose column to remove the cAMP-dependent protein kinase as described above. The cytidylyltransferase (2 µg) was eluted from the column and incubated, with continuous shaking at 4 °C, with 4 mM MgCl₂, and 0-40 units of alkaline phosphatase bound to agarose. After 1 h, the alkaline phosphatase was separated from the cytidylyltransferase by centrifugation at 2,000 x g for 5 min at 4 °C. The supernatant was incubated with 20 mM Tris-HCl, pH 7.4, 2 mM DTT, and 1 mg washed microsomal protein. The preincubation and assay was as for phosphorylating conditions described above.

**Identification of the amino acid phosphorylated on the cytidylyltransferase (2. 2. 9. 2.).**

1 µg of cytidylyltransferase was phosphorylated with [γ-³²P]ATP as described previously. After trichloroacetic acid precipitation and acetone washing, the dried sample was hydrolyzed in 6 N HCl (250 µl) for 2 h at 110 °C under N₂. HCl was evaporated under N₂, and the sample dissolved in 10 µl of thin layer electrophoresis buffer (acetic acid: 88% formic acid: water, 78/25/897, v/v/v) and applied to a cellulose thin-layer plate (0.1 mm thickness). Phosphoamino acids were separated by electrophoresis towards the anode for 3 h at 500 V. The plate was sprayed with 0.2% ninhydrin in 95% ethanol to identify the position of the phosphoamino acid standards, and then exposed to Kodak XAR-5 film for 24 h at -70 °C.
Phosphorylation of CTP:phosphocholine cytidylyltransferase \textit{in vivo} (2. 2. 9. 3.).

Hepatocytes were incubated in phosphate-free medium for 2 h. The medium was removed, and the cells incubated for 2 h with phosphate-free medium containing 100 $\mu$Ci/dish $[^{32}\text{P}]$inorganic phosphate. The medium was subsequently removed and the cells chased with label-free medium containing 0.5 mM chlorophenylthiocAMP for 30-120 min. After the chase, the cells were suspended in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl buffer, and homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 120,000 x $g$ for 1 h to isolate the cytosolic fraction. The cytidylyltransferase in the cytosolic fraction was partially purified by precipitation and extraction with octyl glucoside as described before. The partially purified enzyme was then analyzed by 2-D-PAGE and transferred onto nitrocellulose by the Western-blot technique. Antibody was used to detect the cytidylyltransferase. The nitrocellulose membrane was subsequently exposed to Kodak XAR-5 film for 24 h at -70 °C.

Preparation of aminophenylphosphorylcholine affinity column (2. 2. 10. 1.).

Procedures
1. Wash 50 ml Sepharose 4B with 100 ml of 1 M NaCl followed by 100 ml of water.
2. Resuspend 30 ml of washed gel in 30 ml of water and stir gently.
3. Add 8 g (266 mg/ml) of cyanogen bromide and quickly raise pH to 11 with 2 M NaOH (work in fumehood).
4. Maintain pH at 11 and temperature at 20 °C of the mixture.
5. After 12 min, add quickly large amount of ice and filter mixture on a buckner funnel. Wash gel in the funnel with 150 ml of ice-cold 0.1 M sodium carbonate buffer, pH 9.5.
6. Quickly add gel to 150 μmoles of glycyl L-tyrosine (150 μmole in 20 ml 0.1 M sodium carbonate, pH 9.5).
7. Stir overnight for 16 h at 4 °C.
8. Wash with 100 ml each of sodium carbonate buffer, water, and sodium carbonate buffer.
9. Resuspend gel in 20 ml sodium carbonate buffer and add diazotized compound.
10. Stir mixture gently for 4 h at 4 °C.
11. Wash gel with 100 ml each of sodium carbonate buffer, water, and 20 mM Tris-succinate, pH 6.5. Resuspend gel in Tris-succinate buffer.

**Preparation of diazo compound (2. 2. 10. 2.).**
1. Dissolve 150 μmole aminophenylphosphorylcholine in 1.5 ml of 1.5 N HCl.
2. Place solution at 4 °C and stir. Add over 1 min period sodium nitrite solution (800 μmole in 0.5 ml water).
3. Stir for 8 min at 4 °C.
4. Quickly add to tyrosyl-Sepharose solution and adjust pH to 9.5 with NaOH.
Table 1. Commercial Source Of Research Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS fluid - Amersham</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (type XVIII) - Sigma</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase linked to agarose - Sigma</td>
<td></td>
</tr>
<tr>
<td>Aminophenylphosphorylcholine - Sigma</td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate - BDH</td>
<td></td>
</tr>
<tr>
<td>[γ-32P]ATP - Amersham</td>
<td></td>
</tr>
<tr>
<td>ATP - Sigma</td>
<td></td>
</tr>
<tr>
<td>BSA - Calbiochem</td>
<td></td>
</tr>
<tr>
<td>cAMP-dependent protein kinase - Sigma</td>
<td></td>
</tr>
<tr>
<td>cAMP-dependent protein kinase (pure) - Dr. M. Walsh's Lab, Calgary</td>
<td></td>
</tr>
<tr>
<td>cAMP-dependent protein kinase inhibitor - Dr. S. Pelech's Lab, U.B.C</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride - Fisher</td>
<td></td>
</tr>
<tr>
<td>Carboxymethyl cellulose - Whatman</td>
<td></td>
</tr>
<tr>
<td>CDP-choline - Sigma</td>
<td></td>
</tr>
<tr>
<td>CTP-agarose - Sigma</td>
<td></td>
</tr>
<tr>
<td>Chlorophenylthio-cAMP - Sigma</td>
<td></td>
</tr>
<tr>
<td>Choline - Sigma</td>
<td></td>
</tr>
<tr>
<td>[methyl-3H]choline chloride - Amersham</td>
<td></td>
</tr>
<tr>
<td>Choline kinase - Sigma</td>
<td></td>
</tr>
<tr>
<td>Chromatofocusing PB 94 column - Pharmacia</td>
<td></td>
</tr>
<tr>
<td>Collagenase - Sigma</td>
<td></td>
</tr>
<tr>
<td>Culture dishes - Falcon</td>
<td></td>
</tr>
<tr>
<td>CTP - Sigma</td>
<td></td>
</tr>
<tr>
<td>Cyanogen bromide - Sigma</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B - Pharmacia</td>
<td></td>
</tr>
<tr>
<td>Digitonin - Sigma</td>
<td></td>
</tr>
<tr>
<td>Dulbecco's Modified Eagles medium - Gibco</td>
<td></td>
</tr>
<tr>
<td>Dyematrix Green A resin - Amicon</td>
<td></td>
</tr>
<tr>
<td>EDTA - BDH</td>
<td></td>
</tr>
<tr>
<td>EGTA - Sigma</td>
<td></td>
</tr>
<tr>
<td>Fetal calf serum - Wildlife serums</td>
<td></td>
</tr>
<tr>
<td>Gels for Phast system - Pharmacia</td>
<td></td>
</tr>
<tr>
<td>Glycyl L-tyrosine</td>
<td></td>
</tr>
<tr>
<td>Hexyl-agarose - Sigma</td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite - Bio-Rad</td>
<td></td>
</tr>
<tr>
<td>[32P]inorganic phosphate - Amersham</td>
<td></td>
</tr>
<tr>
<td>Ionophore A23187 - Sigma</td>
<td></td>
</tr>
<tr>
<td>Kodak XAR-5 film - Sigma</td>
<td></td>
</tr>
<tr>
<td>Mono-Q - Pharmacia</td>
<td></td>
</tr>
</tbody>
</table>
Mono-S - Pharmacia
Octylglucoside - Sigma
Octyl-Sepharose - Pharmacia
Oleic acid - Sigma
Phosphatidylcholine (type XI-E) - Sigma
Phosphocellulose - Whatman
Phospholipase A2 and C - Sigma
Phospholipids - Serdary
PMSF - Sigma
Protein kinase inhibitor - Sigma
Rat chow - Purina
Sepharose 4B - Pharmacia
Sepharose 6B - Pharmacia
Sodium fluoride - Fisher
Tris - Sigma
Triton X-100 - Sigma
Vasopressin - Sigma
Verapamil - Sigma
RESULTS

PURIFICATION OF CTP:PHOSPHOCHOLINE CYTIDYLylTRANSFERASE

Purification by aggregation/deaggregation of cytosolic form (3. 1. 1. 1.).

Choy et al (69) reported the first successful partial purification of cytidylyltransferase. Their procedure exploited the tendency of cytosolic cytidylyltransferase to aggregate by incubation for 5 days at 4 °C. The aggregated enzyme was concentrated by ammonium sulfate precipitation and applied to a Sepharose 6B column. The void volume material from this column was treated with 0.05% SDS at 4 °C for 2 h and reapplied to the gel filtration column. The cytidylyltransferase was shown to be a single protein with a molecular weight of ~200,000 Kd on Sepharose 4B column. However, attempts to reproduce the procedure were not successful. The main problem was that aggregation and dissociation by SDS were variable and SDS inhibited cytidylyltransferase activity and interfered with protein determinations.

We tried to overcome some of the problems posed by Choy's procedure by using Triton X-100 for dissociation of aggregated enzyme instead of SDS, and by incubating the cytosol at 37 °C for 4 h instead of 5 days at 4 °C. Fig. 6 shows the comparison between Choy's procedure and our procedure.

The cytosolic cytidylyltransferase, which had been aggregated at 37 °C for 4 h in the presence of protease inhibitors, was precipitated with ammonium sulfate. Initially, we characterized the
Figure 6. Comparison of purification schemes for cytidylyltransferase

<table>
<thead>
<tr>
<th>Choy's procedure</th>
<th>Our procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>4 h, 37 °C</td>
</tr>
<tr>
<td>5 days, 4 °C</td>
<td></td>
</tr>
<tr>
<td>Aged cytosol</td>
<td></td>
</tr>
<tr>
<td>20-25% Ammonium sulfate Precipitation</td>
<td>20-30%</td>
</tr>
<tr>
<td>1st Sepharose 6B</td>
<td></td>
</tr>
<tr>
<td>Void volume</td>
<td></td>
</tr>
<tr>
<td>0.05% SDS, 4 °C, 2 h Dissociation</td>
<td>0.05% Triton X-100</td>
</tr>
<tr>
<td></td>
<td>4 °C, 2 h</td>
</tr>
<tr>
<td>2nd Sepharose 6B</td>
<td></td>
</tr>
</tbody>
</table>
ammonium sulfate saturation at which the aggregated and non-aggregated cytidylyltransferase precipitated. Fig. 7 shows that majority of the aggregated cytidylyltransferase is precipitated between 20-30% saturation, while the majority of the non-aggregated enzyme is precipitated between 30-40% ammonium sulfate saturation. Therefore, a 20-30% ammonium sulfate saturation cut was used to precipitate the aggregated form of the enzyme. Fig. 7 also shows that a large amount of the aggregated cytidylyltransferase is precipitated between 25-30% ammonium sulfate saturation. Since Choy et al only used a 20-25% ammonium sulfate saturation cut to precipitate the aggregated form, a large amount of the enzyme could have potentially been lost.

The 20-30% ammonium sulfate precipitated cytidylyltransferase was resuspended in a small volume of buffer containing 1% glycerol and loaded onto a Sepharose 6B column. Fig. 8 shows that the majority of the cytidylyltransferase is eluted in the void volume. This is to be expected since the enzyme was aggregated, with contaminating lipid membrane fragments, to form a large molecular weight complex which would not be fractionated by the column. This purification step also seems to be very useful since most of the contaminating proteins are eluted in the fractionation range after the cytidylyltransferase.

The aggregated cytidylyltransferase was treated with 0.05% Triton X-100 to dissociate the cytidylyltransferase from the lipid complex. Triton X-100 was found to have no inhibitory effect on cytidylyltransferase activity, but was able to dissociate (~70%) the aggregated enzyme when used at a concentration around its c.m.c
Figure 7. Ammonium sulfate precipitation of aggregated and non-aggregated cytidylyltransferase. Cytosol (50 ml) was incubated at 37 °C for 1 h. Ammonium sulfate (20% saturation) was added to the cytosol with continuous stirring, over a 10 min period and then the cytosol centrifuged at 10,000 × g for 10 min and the pellet removed. The supernatant was readjusted to 25% ammonium sulfate saturation with stirring. The precipitate was again removed and the supernatant readjusted to a higher ammonium sulfate saturation (increase by 5% increments). This procedure was repeated until 45% ammonium sulfate saturation had been achieved. The precipitate formed at each ammonium sulfate saturation step was resuspended in 5 ml of buffer A (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% glycerol, and 2 mM DTT) and the cytidylyltransferase activity measured in the presence or absence of TRLP.
Figure 8. Chromatography of aggregated cytidylyltransferase on 1st Sepharose 6B column. The protein precipitated with ammonium sulfate (20-30% saturation) was resuspended in 5 ml of buffer A (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% glycerol, and 2 mM DTT) and loaded, at 1 ml/min, onto a Sepharose 6B column (2.5 x 40 cm) which had been equilibrated with the same buffer. The column was eluted at 1 ml/min with buffer A and 7 ml fractions collected. The cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 280 nm (□) of each fraction was measured. The void volume of the column was ~67 ml.
value (~0.03%) (data not shown). Also since Triton X-100 is an uncharged detergent, this may provide a better hydrophobic environment for the binding and stabilization of cytidylyltransferase than SDS. The partially dissociated cytidylyltransferase was again chromatographed on Sepharose 6B (Fig. 9). Cytidylyltransferase now eluted from the column in two peaks, with the enzyme in the broad second peak requiring phospholipid for activation, indicating that it was now not contaminated with lipid. It was also noticed that Triton X-100 dissociated the aggregated enzyme since the majority of the cytidylyltransferase now appeared in the second peak. However, since the enzyme eluted as a broad peak, this may indicate variability in the amount of lipid still associated with the enzyme.

A summary of the purification is given in Table 2. Although the initial purification steps resulted in enrichment of the enzyme, the last step did not further enhance the purification. The overall recovery of the enzyme was poor (3.7%) with only a ~17-fold purification. The last step was not very useful since a lot of enzyme activity was lost with little removal of contaminating proteins. The rapid loss of enzyme activity was probably associated with the removal of lipid from the cytidylyltransferase. The enzyme sample obtained after the end of the purification procedure was concentrated and analyzed on SDS-PAGE (Fig. 10). As expected, the enzyme is still contaminated with a large variety of proteins and required further purification.

Purification by conventional and FPLC techniques (3. 1. 2. 1.)
Figure 9. Chromatography of dissociated cytidylyltransferase on 2nd Sepharose 6B column. The cytidylyltransferase activity eluted in the void volume (fraction #5-11) from the 1st Sepharose 6B column was incubated with 0.05% Triton X-100 for 2 h at 4 °C. The mixture was adjusted to 40% ammonium sulfate saturation with stirring, and the precipitate formed removed by centrifugation at 10,000 x g for 10 min. The pellet was resuspended in 5 ml of buffer B (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% glycerol, 0.05% Triton X-100, and 2 mM DTT) and loaded, at 1 ml/min, onto a Sepharose 6B column (2.5 x 40 cm) which had been equilibrated with the same buffer. The column was eluted at 1 ml/min with buffer B and 7 ml fractions collected. The cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 280 nm (□) of each fraction was measured.
Table 2. Purification of cytidylyltransferase by aggregation/deaggregation. The results are the average of five preparation using 100 ml of rat liver cytosol for each.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume (ml)</th>
<th>Total Activity (nmol/min)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>% Recovery</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>100</td>
<td>2916</td>
<td>3840</td>
<td>0.7</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Aged cytosol</td>
<td>100</td>
<td>3450</td>
<td>3800</td>
<td>0.9</td>
<td>118</td>
<td>1.3</td>
</tr>
<tr>
<td>20-30% AmSO₄</td>
<td>5</td>
<td>1680</td>
<td>311</td>
<td>5.4</td>
<td>58</td>
<td>7.7</td>
</tr>
<tr>
<td>1st Sepharose 6B</td>
<td>49</td>
<td>1080</td>
<td>50</td>
<td>21.6</td>
<td>37</td>
<td>30.1</td>
</tr>
<tr>
<td>2nd Sepharose 6B</td>
<td>105</td>
<td>110</td>
<td>9</td>
<td>12.2</td>
<td>3.7</td>
<td>17.5</td>
</tr>
</tbody>
</table>
Figure 10. SDS-PAGE of sample after purification by aggregation/deaggregation. Lane 1 cytosol; 2, sample after 20-30% ammonium sulfate saturation cut; 3, 1st Sepharose 6B; 4, 2nd Sepharose 6B; 5, molecular weight standards (phosphorylase B, 94000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; soyabean trypsin inhibitor, 20000; and lactalbumin, 14400). The gel was stained with silver.
Since the recovery and purification of cytidylyltransferase by aggregation/deaggregation was very poor, alternative purification procedures were examined. We tried to use non-aggregated enzyme from fresh cytosol as the starting source of cytidylyltransferase, since a lot of problems were encountered when aggregated cytidylyltransferase was used. By using non-aggregated enzyme, the problem of dissociation prior to further purification was eliminated.

At the start of the purification, Triton X-100 (0.05%) was added to fresh cytosol. This protocol was adopted to prevent aggregation of cytidylyltransferase during purification and to increase the amount of enzyme precipitated by a 30-40% ammonium sulfate saturation cut. Also, the addition of Triton X-100 would provide a hydrophobic environment which may be important in enzyme stabilization.

The cytidylyltransferase, precipitated by ammonium sulfate (30-40% saturation), was resuspended in Tris-succinate buffer and loaded onto a DEAE-cellulose column (Fig. 11). Many contaminating proteins were removed with a low salt (75 mM NaCl) wash prior to elution of the cytidylyltransferase. The cytidylyltransferase was eluted with a gradient, above 100 mM NaCl concentration, as a broad peak (volume of peak ~81 ml).

The most active fractions containing cytidylyltransferase activity from DEAE-cellulose were pooled and loaded onto a phosphocellulose column (Fig. 12). Again contaminating proteins were removed by washing with buffer containing 200 mM NaCl prior to elution of the cytidylyltransferase. The cytidylyltransferase was eluted with 300 mM NaCl. This step seems to give very good...
Figure 11. Chromatography of cytidylyltransferase on DEAE-cellulose column. The precipitate from a 30-40% ammonium sulfate saturation cut was resuspended in 325 ml of buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100) and loaded, at 2 ml/min, onto a DEAE-cellulose column (2.5 x 40 cm) which had been equilibrated with the same buffer. The column was eluted, at the same flow rate, with 100 ml buffer containing 75 mM NaCl (A) before starting a 200 ml gradient from 75-200 mM NaCl (B) in Tris-succinate buffer. The cytidylyltransferase was eluted into 9 ml fractions and the enzyme activity (■) in the presence of TRLP and the absorbance at 595 nm (□) measured.
Figure 12. Chromatography of cytidylyltransferase, eluted from DEAE-cellulose, on phospho-cellulose column. The most active fractions containing cytidylyltransferase activity from DEAE-cellulose (fraction #19-27) were pooled (~81 ml) and loaded, at 1 ml/min, onto a phospho-cellulose column (2.5 x 10 cm) which had been equilibrated with buffer containing 20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100. The column was eluted, at the same flow rate, with 75 ml each of 100 mM and 200 mM NaCl (A) in Tris-succinate buffer. The cytidylyltransferase was eluted into 5 ml fractions with 75 ml of 300 mM NaCl (B) in buffer. The cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction were measured.
purification since the enzyme is eluted on the trailing side of the major protein peak.

Fractions containing cytidylyltransferase from phosphocellulose were then applied to Mono-Q, which is an anion exchanger with a quarternary amino group, and an elution profile is shown in Fig. 13. Again this step seems to give good purification since many contaminating proteins are removed before and after the cytidylyltransferase is eluted. The cytidylyltransferase is eluted as a sharp peak between 150-250 mM NaCl.

The active fractions containing cytidylyltransferase activity from Mono-Q were chromatographed on Mono-S (Fig. 14), which has a sulfonyl group as the active ligand and is a cation exchanger. Many proteins do not seem to bind to the column and are eluted in the wash with buffer. The cytidylyltransferase is eluted as a sharp peak with a 0-500 mM NaCl gradient.

A summary of the purification is given in Table 3. The precipitation of cytidylyltransferase with 30-40% ammonium sulfate saturation cut gives good purification with a high recovery. This indicated that the inclusion of Triton X-100 in the cytosol may have enhanced the recovery of cytidylyltransferase in the 30-40% ammonium sulfate saturation cut while a lot of proteins are not precipitated at this concentration. Although the steps after ammonium sulfate precipitation did enhance enrichment of the cytidylyltransferase, the recovery of the enzyme dropped rapidly. The enzyme was purified, prior to Mono-S, 5- or more fold with each purification step but the recovery after each step was only 50% or less. The loss of enzyme activity with increasing purification may be
Figure 13. Chromatography of cytidylyltransferase, eluted from phospho-cellulose, on Mono-Q column. The most active fractions containing cytidylyltransferase activity from phospho-cellulose (fraction #16-24) were pooled (~45 ml) and concentrated to 10 ml by ultrafiltration using an Amicon PM 30 membrane. The concentrated sample was diluted to 30 ml with buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100) and loaded, at 1 ml/min, onto a Mono-Q column (1 ml bed volume) which had been equilibrated with the same buffer. The column was eluted sequentially, at the flow rate, with 10 ml of 100 mM NaCl (A), 25 ml gradient of 100-250 mM NaCl (B), and 5 ml of 500 mM NaCl (C) in Tris-succinate buffer. One ml fractions were collected and the cytidylyltransferase activity ( ■ ) in the presence of TRLP and the absorbance at 595 nm ( □ ) of each fraction measured.
Figure 14. Chromatography of cytidylyltransferase, eluted from Mono-Q, on Mono-S column. The most active fractions containing cytidylyltransferase activity from Mono-Q (fraction #16-25) were pooled (~10 ml) and concentrated to 4 ml by ultrafiltration. The concentrated sample was diluted to 12 ml with buffer (20 mM MOPS-HCl, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The diluted sample was loaded, at 1 ml/min, onto a Mono-S column (1 ml bed volume) which had been equilibrated with the same buffer. The column was eluted with 5 ml of buffer (A) before starting a 20 ml gradient of 0-500 mM NaCl (B) in MOPS-HCl buffer. One ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
Table 3. Purification of cytidylyltransferase by conventional and FPLC techniques. The results are the average of three preparations using 300 ml of rat liver cytosol for each.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume (ml)</th>
<th>Total Activity (nmol/min)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>% Recovery</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>300</td>
<td>10219</td>
<td>9438</td>
<td>1.1</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>30-40%AmSO₄</td>
<td>320</td>
<td>6366</td>
<td>1868</td>
<td>3.4</td>
<td>62</td>
<td>3</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>81</td>
<td>2836</td>
<td>110</td>
<td>25.8</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>P-cellulose</td>
<td>45</td>
<td>1425</td>
<td>10</td>
<td>143</td>
<td>14</td>
<td>130</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>10</td>
<td>958</td>
<td>1.4</td>
<td>684</td>
<td>9.4</td>
<td>621</td>
</tr>
<tr>
<td>Mono-S</td>
<td>6</td>
<td>305</td>
<td>0.35</td>
<td>871</td>
<td>2.8</td>
<td>792</td>
</tr>
</tbody>
</table>
Figure 15. SDS-PAGE of sample after purification by conventional and FPLC techniques. Lane 1, cytosol; 2, DEAE-cellulose; 3, phospho-cellulose wash; 4, phospho-cellulose peak; 5, Mono-Q wash; 6 and 7, Mono-Q peak; 8, Mono-S wash; 9, Mono-S peak; 10, molecular weight standards (phosphorylase B, 94000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; soyabean trypsin inhibitor, 20000; and lactalbumin, 14400).
indicative of the removal of a factor(s) which stabilizes the enzyme. Table 3 also shows that the Mono-S step did not significantly enhance purification of the cytidylyltransferase, as a result of rapid loss of enzyme activity. The sulfonyl side groups on Mono-S may be involved in the loss of enzyme activity or a factor(s) may have been resolved from the enzyme on this column.

Analysis of the sample on SDS-PAGE after purification is shown in Figure 15. Although contaminating proteins had been resolved from the cytidylyltransferase, the sample after Mono-S is still not homogenous. Thus, further purification of cytidylyltransferase was required to obtain homogeneity.

**Stabilization of cytidylyltransferase after Mono-Q column (3. 1. 2. 2.).**

It was discovered that the cytidylyltransferase, after purification on Mono-Q, was rapidly inactivated upon storage at 4 °C (loss of 50% activity within 4 h). This may be the reason why no further purification was achieved by Mono-S chromatography. We examined whether the cytidylyltransferase could be stabilized by various agents after recovery from the Mono-Q column. This in turn would indicate if a factor(s) could have been removed during purification which was essential for enzyme stabilization. The cytidylyltransferase was purified with Tris-succinate buffer using the above procedure. After Mono-Q, the enzyme was incubated with various agents for different time intervals at 4 °C (Fig. 16). In the control (only buffer added), the enzyme activity decreased by 45% within 3 h. Thereafter, the cytidylyltransferase activity gradually decreased and by 45 h, only 15% of the starting activity remained.
Incubation of cytidylyltransferase with TRLP and oleate prevented the rapid loss of enzyme activity and even after 45 h, over 90% of the activity still remained. This confirmed the hypothesis that a lipid environment was essential for the maintenance of enzyme activity. It is most likely that as the purification of the enzyme increased, the enzyme was separated from the endogenous lipid and this resulted in rapid loss of enzyme activity and no further increase in purification. This is partially confirmed by the finding that incubation of the Mono-Q wash with cytidylyltransferase partially stabilized the enzyme. Since boiling of the Mono-Q wash did not prevent the stabilization, this indicated that a small factor or a non-protein compound (most likely lipid) was present in the wash which is required for enzyme stabilization. The presence of Triton X-100 also partially stabilized the enzyme indicating that a hydrophobic environment can also stabilize the enzyme. Since protease inhibitors and anti-oxidizing agents also partially stabilized the enzyme, this indicated that the purified enzyme was very susceptible to inactivation by proteolysis and oxidation. It is of interest to note that the substrates of the cytidylyltransferase also partially stabilized the enzyme activity. Presence of these may have allowed the active site on the enzyme to stay in its active configuration. Addition of BSA, ethylene glycol, and high salt failed to prevent inactivation. Therefore, we routinely used protease inhibitors, anti-oxidants, and detergent Triton X-100 in buffers used during purification, to help maintain enzyme activity. Triton X-100 was selected, instead of TRLP, to provide a suitable hydrophobic environment, similar to that provided by TRLP's, without actually aggregating the enzyme. This
Figure 16. Stabilization of partially purified cytidylyltransferase activity. Cytidylyltransferase was partially purified by conventional and FPLC techniques using Tris-succinate buffer which contained no Triton X-100, DTT, or PMSF. The partially purified cytidylyltransferase was incubated for 3, 21, and 45 h at 4 °C in the presence of an equal volume of Tris-succinate buffer, pH 6.5 (1); buffer containing 0.025% Triton (2); 0.5 mM PMSF, 1 mM bacitracin, and 1 mM benzamidine (3); 1M NaCl (4); Mono-Q wash (5); boiled Mono-Q wash (6); 200 μg TRLP, 100 μM oleate (7); 2 mg/ml BSA (8); 2 mM DTT (9); 0.5 mM CTP, 5 mM magnesium acetate, and 0.5 mM phosphocholine (10); 0.025% Tween-20 (11); 20 mM potassium phosphate (12); 10% ethylene glycol (13); 20% glycerol (14); 10 mM glutathione (15). After the incubation, an aliquot was removed and assayed for cytidylyltransferase activity in the presence of TRLP. The cytidylyltransferase activity at the start of experiment was 4.5 nmol/min/ml.
ensured partial stabilization of enzyme activity without having to dissociate the enzyme during purification.

Therefore, cytidylyltransferase was purified by the above described procedure up to the Mono-Q step. Thereafter, the sample was analyzed by other purification steps instead of Mono-S. In each case, after eluting the column with high salt, a small amount of the gel was removed from the top of the column and assayed for enzyme activity to see if any enzyme remained on the column.

Chromatography of cytidylyltransferase, after Mono-Q, on CTP-agarose (3. 1. 2. 3.).

Affinity chromatography is a very selective purification technique and thus is very useful because only a small number of proteins will recognise and bind to the affinity ligand. For this reason, we used CTP-agarose, which has a CTP moiety coupled to agarose, as an affinity column. The active fractions containing cytidylyltransferase activity from Mono-Q were pooled and after dilution, loaded onto a CTP-agarose column (Fig. 17A). Contaminating protein did not bind to the column and was removed in the wash with buffer. This was expected since only a small number of proteins will recognise and bind to the ligand. The majority (~80%) of the cytidylyltransferase was eluted with 0.2 M NaCl in a peak which coincided with the protein peak. A smaller cytidylyltransferase peak was also observed when the column was eluted with 0.5 M NaCl. It may be that the first peak represents cytidylyltransferase binding through electrostatic interactions to the column while the second peak represents affinity binding of the cytidylyltransferase to its
Figure 17A. Chromatography of cytidylyltransferase, eluted from Mono-Q, on CTP-agarose column. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~12 ml) and diluted 3-fold with buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, 1 mM magnesium acetate, and 0.025% Triton X-100). The diluted sample was loaded, at 0.2 ml/min, onto a CTP-agarose column (5 ml bed volume) which had been equilibrated with the same buffer. The column was eluted sequentially, at the same flow rate, with 10 ml 0 mM NaCl (A), 30 ml of 200 mM NaCl (B), and 20 ml of 500 mM NaCl (C) in Tris-succinate buffer. One ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
substrate CTP. This may explain why 0.2 M NaCl, a concentration high enough to disrupt electrostatic interactions, can elute the cytidylyltransferase while a much higher concentration of NaCl is required to disrupt affinity interactions.

Table 4. shows the purification of cytidylyltransferase after CTP-agarose. Although the enrichment of cytidylyltransferase was increased by almost 1.8-fold over the sample from Mono-Q, this was by no means high enough to give a homogenous preparation of cytidylyltransferase as demonstrated by contaminating proteins detected by SDS-PAGE (Fig. 17B).

Since the CTP-agarose column is not behaving as a true affinity column (cytidylyltransferase is eluted at relatively low salt concentrations together with other proteins), and the recovery of enzyme activity was poor, we examined alternative steps for purification.

**Chromatography of cytidylyltransferase, after Mono-Q, on aminophenylphosphorylcholine affinity column (3. 1. 2. 4.).**

The active fractions containing cytidylyltransferase activity from Mono-Q were pooled and after dilution, loaded onto an aminophenylphosphorylcholine affinity column (Fig. 18). Again contaminating protein did not bind to the column and was eluted in the wash. However, a small (~5%) amount of enzyme activity was also eluted in the wash. This could have been the result of overloading the column since the majority of cytidylyltransferase did bind to the column. As noticed with chromatography on CTP-agarose, cytidylyltransferase was eluted with a low and a high salt wash. This
Figure 17B. SDS-PAGE of sample after purification on CTP-agarose. Lane 1, molecular weight standards (phosphorylase B, 94000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; soyabean trypsin inhibitor, 20000; and lactalbumin, 14400); 2, fraction #4; 3, #16; 4, #18 (peak); 5, #22; 6, #26; 7, #30; 8, #14.
Figure 18. Chromatography of cytidylyltransferase, eluted from Mono-Q, on aminophenylphosphorylcholine affinity column. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~10 ml) and diluted 3-fold with buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, 0.8 mM magnesium acetate, 0.16 mM CTP, and 0.025% Triton X-100). The diluted sample was loaded, at 0.2 ml/min, onto a aminophenylphosphorylcholine affinity column (5 ml bed volume) which had been equilibrated with the same buffer. The column was eluted sequentially, at the same flow rate, with 5 ml 0 mM NaCl (A), 15 ml of 200 mM NaCl (B), and 5 ml of 500 mM NaCl (C) in Tris-succinate buffer. One ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
again probably means that the cytidylyltransferase was interacting in two different ways with the column. The first peak probably represents electostatic interaction while the second peak may represent affinity binding with the phosphorylcholine moiety.

Since a large amount (~80%) of the cytidylyltransferase was eluted in the first peak coinciding with the protein peak, this indicated that the column is not behaving as a true affinity column but as an ion-exchange column. Table 4 shows that this step was not of significant value in the overall purification of the cytidylyltransferase. Therefore, other purification procedures were examined.

Chromatography of cytidylyltransferase, after Mono-Q, on Superose 12 HR 10/30 column (3.1.2.5.).

The active fractions containing cytidylyltransferase activity from Mono-Q were pooled and after concentration, loaded onto a Superose 12 column (Fig. 19). Superose 12 is a gel filtration column with a fractionation range from 1,000 to 300,000. The cytidylyltransferase was eluted in the fractionation range as a sharp peak which coincided with the major protein peak. However, a large amount of enzyme activity was lost during this step which resulted in no further purification of the sample after Mono-Q. Therefore, this step is of little significant value in terms of further purification of the cytidylyltransferase.

Chromatography of cytidylyltransferase, after Mono-Q, on hydroxylapatite column (3.1.2.6.).
Figure 19. Chromatography of cytidylyltransferase, eluted from Mono-Q, on Superose 12 HR 10/30 column. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~10 ml) and concentrated by ultrafiltration to 0.5 ml. The concentrated sample was loaded, at 0.5 ml/min, onto a Superose 12 HR 10/30 column (~ 24 ml bed volume) which had been equilibrated with buffer (20 mM Tris-succinate, pH 6.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The column was eluted, at the same flow rate, with buffer. One ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
The active fractions containing cytidylyltransferase activity from Mono-Q were pooled and loaded onto a hydroxylapatite column. This column was selected because the gel not only has an anionic and cationic surface, but also the phosphate group may be recognized and bound by the active site on the enzyme since the two substrates for the cytidylyltransferase also contain a phosphate moiety. Fig. 20 shows that the cytidylyltransferase is eluted at the trailing end of the major protein peak indicating that the cytidylyltransferase is slightly more retarded by the column than the other proteins. However, the purification of cytidylyltransferase by this column was hampered because the recovery of the enzyme was relatively low as indicated in Table 4. Thus, other alternative purification procedures were examined.

Chromatography of cytidylyltransferase, after Mono-Q, on hexyl-agarose column (3. 1. 2. 7.).

Since it was apparent that the cytidylyltransferase contains a hydrophobic domain (because of its interaction and activation by lipids), we examined purification of the cytidylyltransferase by hydrophobic chromatography. Hexyl-agarose, which contains a six carbon alkyl side chain with an amino group at the terminal end, was selected for this purpose. The active fractions containing cytidylyltransferase activity from Mono-Q were pooled and after dilution, loaded onto a hexyl-agarose column (Fig. 21). Typically in hydrophobic chromatography, the sample is loaded in high salt buffers and then eluted with buffers containing detergents and low salt. However, when the cytidylyltransferase was loaded onto the
Figure 20. Chromatography of cytidylyltransferase, eluted from Mono-Q on hydroxylapatite column. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~12 ml) and diluted 3-fold with buffer (20 mM Tris-succinate, pH 6.5, 20 mM potassium phosphate (dibasic), 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The diluted sample was loaded, at 0.2 ml/min, onto a hydroxylapatite column (4 ml bed volume) which had been equilibrated with the same buffer. The column was eluted sequentially, at the same flow rate, with 5 ml of 20 mM potassium phosphate (A), before starting a 20 ml gradient of 20-300 mM potassium phosphate (B) in Tris-succinate buffer. One ml fractions were collected and the cytidylyltransferase activity ( ■ ) in the presence of TRLP and the absorbance at 595 nm ( □ ) of each fraction measured.
Figure 21. Chromatography of cytidylyltransferase, eluted from Mono-Q, on hexyl-agarose column. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~13 ml) and diluted 3-fold with buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The diluted sample was loaded, at 0.2 ml/min, onto a hexyl-agarose column (5 ml bed volume) which had been equilibrated with the same buffer. The column was eluted sequentially, at the same flow rate, with 10 ml of 0 mM NaCl (A), before starting a 30 ml gradient of 0-500 mM NaCl (B) in Tris-succinate buffer. One ml fractions were collected and the cytidylyltransferase activity ( ■ ) in the presence of TRLP and the absorbance at 595 nm ( □ ) of each fraction measured.
hexyl-agarose, the opposite occurred. The enzyme was bound to the column in a low salt buffer and subsequently eluted in high salt buffer. This indicated that the column was not behaving as a true hydrophobic column but as an ion-exchange column. It may be the terminal amino group is responsible for this ionic interaction. Although the cytidylyltransferase did elute as a sharp peak after the major protein peak, this step did not significantly increase the purification over the Mono-Q step. Therefore, other alternative purification steps were examined.

**Chromatography of cytidylyltransferase, after Mono-Q, on Green A column** (3.1.2.8.).

Amicon dyematrix gels are useful in purification because they contain both hydrophilic and hydrophobic side groups. Therefore, the interaction of any given protein with both hydrophilic and hydrophobic groups at the same time is quite selective. Chromatography of cytidylyltransferase on Green A was examined. The active fractions containing cytidylyltransferase activity from Mono-Q were pooled and loaded onto Green A column (Fig. 22). The cytidylyltransferase was eluted as a broad peak (~10 ml) coinciding with the protein peak. Table 4. shows that no significant purification was achieved by this step.

**Aggregation and isolation of cytidylyltransferase by density gradient centrifugation** (3.1.2.9.).

We tried to aggregate selectively cytidylyltransferase with TRLP, in the presence of oleate, and then isolate the aggregated
Figure 22. Chromatography of cytidylyltransferase, eluted from Mono-Q, on Green-A column. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~12 ml) and diluted 3-fold with buffer (20 mM Tris-succinate, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100). The diluted sample was loaded, at 0.2 ml/min, onto a dyematrix Green-A column (5 ml bed volume) which had been equilibrated with the same buffer. The column was eluted sequentially, at the same flow rate, with 5 ml of 0 mM NaCl (A), before starting a 25 ml gradient of 0-500 mM NaCl (B) in Tris-succinate buffer. One ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
enzyme by density gradient centrifugation. The idea was that only cytidylyltransferase would bind to the lipid and then isolate the lipid-cytidylyltransferase complex by centrifugation. The aggregated enzyme should float to the top of the gradient (since lipids have a lower density than proteins) while the majority of the proteins will remain at the bottom of the gradient.

The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled and after concentration, placed at the bottom of a 15-40% sucrose gradient (Fig. 23). Although the majority of the cytidylyltransferase did float to the top of the gradient together with the lipid, contaminating proteins also migrated to the top of the gradient. Thus the aggregation and isolation of the cytidylyltransferase was not as selective as we had hoped. Also the degree of aggregation of the enzyme was variable as indicated by the presence of the enzyme throughout the gradient. Thus, although this step has a potential of giving good purification, this was not the case in our hands.

Other purification steps tried (3. 1. 2. 10).

A number of other purification steps were examined. These included precipitation with ice-cold solvents (acetone and ethanol), chromatography on octyl-Sepharose (hydrophobic column), and chromatofocusing on PB 94 column. However, in each case either the enzyme bound so strongly to the column that it was not eluted, as in the case with octyl-Sepharose and PB 94 column, or the enzyme was rapidly denatured as in the case of precipitation with solvents. We assayed the octyl-Sepharose and PB 94 gels to see whether the
Figure 23. Isolation by density gradient centrifugation of aggregated cytidylyltransferase. The most active fractions containing cytidylyltransferase activity from Mono-Q were pooled (~12 ml) and concentrated to 3 ml by ultrafiltration. The concentrated sample was incubated with 8 mg of TRLP and 800 µl of 1 mM oleate for 1 h at 37 °C. The aggregated sample (~3.7 ml) was mixed with an equal volume of 80% sucrose (in 20 mM Tris-succinate, pH 6.5, 150 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, and 0.025% Triton X-100) and placed in a cellulose nitrate tube (capacity ~37 ml). Over this was gently layered 7.4 ml each of 30%, 25%, 20%, and 15% sucrose. The tube was centrifuged, without braking, at 150,000 x g for 17 h. A small hole was punctured at the bottom of the tube and the content allowed to drain into 3 ml fractions. The cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction was measured.
Table 4. Summary of purification of cytidylyltransferase after Mono-Q by various techniques.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume (ml)</th>
<th>Total Activity (nmol/min)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-Q</td>
<td>12</td>
<td>800</td>
<td>1.5</td>
<td>533</td>
<td>8.5</td>
<td>529</td>
</tr>
<tr>
<td>CTP-agarose</td>
<td>11</td>
<td>295</td>
<td>0.3</td>
<td>983</td>
<td>3.1</td>
<td>975</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>10</td>
<td>755</td>
<td>1.5</td>
<td>503</td>
<td>5.1</td>
<td>529</td>
</tr>
<tr>
<td>Aminophenyl-phosphorylcholine</td>
<td>10</td>
<td>120</td>
<td>0.15</td>
<td>240</td>
<td>1.6</td>
<td>252</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>10</td>
<td>780</td>
<td>1.4</td>
<td>557</td>
<td>7.9</td>
<td>506</td>
</tr>
<tr>
<td>Superose 12</td>
<td>6</td>
<td>105</td>
<td>0.2</td>
<td>525</td>
<td>1.1</td>
<td>476</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>12</td>
<td>875</td>
<td>2</td>
<td>437</td>
<td>8.9</td>
<td>460</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>7</td>
<td>254</td>
<td>0.3</td>
<td>846</td>
<td>2.6</td>
<td>890</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>13</td>
<td>950</td>
<td>2.1</td>
<td>452</td>
<td>9.1</td>
<td>475</td>
</tr>
<tr>
<td>Hexyl-agarose</td>
<td>12</td>
<td>350</td>
<td>0.4</td>
<td>875</td>
<td>3.3</td>
<td>919</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>12</td>
<td>914</td>
<td>2</td>
<td>457</td>
<td>8.7</td>
<td>415</td>
</tr>
<tr>
<td>Green-A</td>
<td>10</td>
<td>279</td>
<td>0.3</td>
<td>930</td>
<td>2.6</td>
<td>844</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>10</td>
<td>710</td>
<td>1.6</td>
<td>443</td>
<td>8.8</td>
<td>407</td>
</tr>
<tr>
<td>Density gradient</td>
<td>6</td>
<td>348</td>
<td>0.4</td>
<td>870</td>
<td>4.3</td>
<td>799</td>
</tr>
</tbody>
</table>
enzyme remained bound on the top of the gel after elution but no enzyme activity was detected.

**Purification by lipid-protein aggregation and resolution on FPLC (3.1. 3.1.).**

Weinhold *et al* (72) reported the purification of the cytidylyltransferase from rat liver cytosol. The procedure involved aggregation of the cytidylyltransferase with PC-oleate vesicles, acid precipitation, and subsequent extraction of the precipitate with octyl glucoside. The extract was passed through a DEAE-Sepharose CL 6B column before being chromatographed on a hydroxylapatite column. The cytidylyltransferase was eluted from the hydroxylapatite column with buffer containing Triton X-100. The purified enzyme was shown to exist with two subunits of molecular weight 39 and 48 Kd as analyzed on SDS-PAGE. However, we could not consistently reproduce the procedure. The main problem was that the enzyme after chromatography on hydroxylapatite was still contaminated with a large number of proteins. Also we were unable to detect the 48 Kd subunit in the preparation purified after hydroxylapatite, even though enzyme activity was still present. Thus, it seemed that the preparation reported by Weinhold *et al* was, in our hands, still not homogenous. Therefore, we attempted to modify Weinhold's procedure in order to obtain a homogenous preparation of enzyme. This involved Mono-Q chromatography after hydroxylapatite, and the use of linear gradients instead of step gradients to elute the enzyme.
The cytidylyltransferase was aggregated with sonicated PC-oleate vesicles and subsequently acid precipitated. The inclusion of PC-oleate was shown, by Weinhold et al., to increase markedly the recovery of enzyme after each purification step. The precipitate was extracted with octyl glucoside and the supernatant loaded on a DEAE-Sepharose CL 6B column (Fig. 24). Many contaminating proteins were eluted with high salt (300 mM NaCl), prior to the elution of the cytidylyltransferase. The cytidylyltransferase was eluted with buffer containing high salt (400 mM NaCl) and octyl-glucoside (100 mM). The aggregated enzyme was strongly bound to the column (since the non-aggregated enzyme elutes at about 200 mM NaCl), and only eluted by a combination of high salt and detergent. The use of a linear gradient, instead of a step gradient, to elute the cytidylyltransferase allowed the removal of contaminating proteins prior to the elution of the enzyme. If a step gradient was used for elution of the cytidylyltransferase, the major protein peak coincided with the enzyme activity peak. The DEAE-Sepharose step proved to be of significant value in terms of purification of cytidylyltransferase, since almost 70% of protein was removed prior to the elution of the enzyme.

The most active fractions (#53-60) containing cytidylyltransferase activity from DEAE-Sepharose Cl 6B column were pooled and after dilution, loaded onto a hydroxylapatite column (Fig. 25A). Again a large amount of contaminating proteins were removed prior to the elution of the cytidylyltransferase. The use of a linear 0-0.03% Triton X-100 gradient allowed further removal of contaminating proteins before the enzyme was eluted. The presence
Figure 24. Chromatography of cytidylyltransferase, after aggregation with lipid and acid precipitation, on DEAE-Sepharose CL-6B column. Cytidylyltransferase was aggregated with PC:oleate vesicles and acid precipitated. The precipitate was extracted with 20 mM octyl glucoside in buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM DTT, 1 mM EDTA, 1 mM PMSF, and 0.025% sodium azide). After centrifugation at 20,000 \( x \) g for 20 min, the supernatant was removed and loaded, at 50 ml/h, onto a DEAE-Sepharose CL-6B column (2.6 x 14.5 cm) which had been equilibrated with buffer A. The column was eluted sequentially, at the same flow rate, with 50 ml of 150 mM NaCl (A), before starting a 300 ml gradient of 150-300 mM NaCl (B) in Tris-HCl buffer. The cytidylyltransferase was eluted with a 150 ml gradient of 300 mM NaCl-50 mM octyl glucoside to 400 mM NaCl-50 mM octyl glucoside (C) in Tris-HCl buffer. Eight ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
Figure 25A. Chromatography of cytidylyltransferase after DEAE-Sepharose CL-6B, on hydroxylapatite column. The most active fractions containing cytidylyltransferase activity from DEAE-Sepharose CL-6B column (fractions #53-60) were pooled (~64 ml) and diluted 2.5-fold with buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM DTT, 1 mM EDTA, 1 mM PMSF, and 0.025% sodium azide). The diluted sample was loaded, at 30 ml/h, onto a hydroxylapatite column (1.7 x 12 cm) which had been equilibrated with the same buffer. The column was eluted sequentially, at the same flow rate, with 50 ml of 200 mM potassium phosphate in buffer A, pH 7.5 (A), before starting a 200 ml gradient of 0-100 mM octyl glucoside in 200 mM potassium phosphate-buffer A (B). The cytidylyltransferase was eluted with a 110 ml gradient of 0-0.03% Triton X-100 in 200 mM potassium phosphate-buffer A (C). Four ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
of octyl-glucoside in the buffer did not elute the cytidylyltransferase but eluted a large number of contaminating proteins. This proved to be very significant in the overall purification of cytidylyltransferase. Thus, proteins with weak hydrophobic interactions were eluted with a mild detergent before eluting the cytidylyltransferase with a stronger detergent.

The purity of the enzyme preparation after hydroxylapatite was checked by SDS-PAGE (Fig. 25B). The preparation was still contaminated by a number of proteins and thus required further purification. The absence of the 48 Kd subunit reported by Weinhold et al (72) is also evident.

The most active fractions (#77-88) containing cytidylyltransferase activity from hydroxylapatite column were pooled and after dilution, loaded onto a Mono-Q column (Fig. 26A). The cytidylyltransferase was eluted with a linear 0-500 mM NaCl gradient at a concentration above 150 mM NaCl. A number of proteins were eluted on either side of the enzyme activity peak. The cytidylyltransferase is eluted as a sharp peak coincident with a protein peak.

The purity of the preparation after chromatography on Mono-Q column was checked by SDS-PAGE (Fig. 26B). Only one subunit of molecular weight ~42 Kd is present. The intensity of the band on the gel corresponds directly to the cytidylyltransferase activity present in the fraction. Thus, the pure enzyme is a single subunit of molecular weight ~42 Kd on SDS-PAGE.

The overall summary of the purification is outlined in Table 5. A final purification of 9775-fold of cytidylyltransferase over rat liver
Figure 25B. SDS-PAGE of sample after purification on DEAE-Sepharose CL-6B and hydroxylapatite. Lane 1, low molecular weight standards (phosphorylase B, 94000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; soyabean trypsin inhibitor, 20000; and lactalbumin, 14400); 2, DEAE-Sepharose fraction #62; 3, DEAE-Sepharose #59; 4, DEAE-Sepharose #54; 5, hydroxylapatite #80.
Figure 26A. Chromatography of cytidylyltransferase. after hydroxylapatite, on Mono-Q column. The most active fractions containing cytidylyltransferase activity from hydroxylapatite column (fractions #77-88) were pooled (~48 ml) and diluted 4-fold with buffer B (50 mM Tris-HCl, pH 7.4, 50 mM potassium phosphate, 2 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.03% Triton X-100, and 0.025% sodium azide). The diluted sample was loaded, at 1 ml/min, onto a Mono-Q column (1 ml bed volume) which had been equilibrated with the same buffer. The column was eluted, at the same flow rate, with a 50 ml gradient of 0-500 mM NaCl in buffer B. One ml fractions were collected and the cytidylyltransferase activity (■) in the presence of TRLP and the absorbance at 595 nm (□) of each fraction measured.
Figure 26B. SDS-PAGE of sample after purification on Mono-Q column. Lane 1, low molecular weight standards (phosphorylase B, 94000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; soyabean trypsin inhibitor, 20000; and lactalbumin, 14400); 2, Mono-Q fraction #10; 3, #14; 4, #18; 5, #19; 6, #21; 7, #36; 8, #46. The gel was stained with silver.
Table 5. Summary of cytidylyltransferase purification by lipid-protein aggregation and resolution on FPLC. The results are the average of three preparations using 175-180 g of rat liver for each.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume (ml)</th>
<th>Total Activity (nmol/min)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>% Recovery</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>610</td>
<td>28454</td>
<td>8066</td>
<td>3.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE</td>
<td>64</td>
<td>12649</td>
<td>45.1</td>
<td>280</td>
<td>44.4</td>
<td>80</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>48</td>
<td>2814</td>
<td>.22</td>
<td>12506</td>
<td>9.9</td>
<td>3573</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>20</td>
<td>2395</td>
<td>.07</td>
<td>34214</td>
<td>8.4</td>
<td>9775</td>
</tr>
</tbody>
</table>
cytosol was achieved with a recovery of 8.4%. The use of a Mono-Q column proved to be critical, since a 3-fold purification of the enzyme after hydroxylapatite was achieved with little loss of enzyme activity.
CHARACTERIZATION OF PURIFIED CTP-PHOSPHOCHOLINE CYTIDYLTRANSFERASE

**Native-PAGE** (4.1.1.1.).

Native-PAGE of the purified enzyme was performed, on a 5% separating gel, using the Pharmacia Phast system (Fig. 27). The cytidyltransferase migrated as a single band at a molecular weight of ~90 Kd. Since the cytidyltransferase on SDS-PAGE gave a single band of molecular weight ~42 Kd, this implied that the native form of the enzyme may be a dimer of identical subunits.

**IEF-PAGE** (4.1.1.2.).

IEF-PAGE of the purified enzyme was performed using the Pharmacia Phast system on a pH 3-9 gradient gel (Fig. 28). The cytidyltransferase migrated as a single protein with a pI of ~5.8. This implied that the enzyme had a net negative charge in our preparations. The top band in lane 3 is a smear where the sample was applied.

**2D-PAGE** (4.1.1.3.).

2D-PAGE (IEF-PAGE in 1st dimension and SDS-PAGE in 2nd dimension) was performed on the purified enzyme as described by O'Farrell (281). Fig. 29 shows the cytidyltransferase to exist of at least two isoforms. Whether the two isoforms differ in their content of sugar residues or of other residues, such as phosphate, is not clear.

**Generation of polyclonal antibodies in rabbits** (4.1.2.1.).
Figure 27. Native-PAGE of purified cytidyltransferase. Native-PAGE of purified cytidyltransferase was carried out on a 5% non-denaturing gel using the Pharmacia Phast system. After electrophoresis, the gel was silver stained. Lane 1, cytidyltransferase (2 ng); 2, cytidyltransferase (0.5 ng); 3, catalase (molecular weight 214,000) (2 ng); 4, low molecular weight standards ((phosphorylase B, 94000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; soyabean trypsin inhibitor, 20000; and lactalbumin, 14400).
Figure 28. IEF-PAGE of purified cytidylyltransferase. IEF-PAGE of purified cytidylyltransferase was carried out on a pH 3-9 gradient gel using the Pharmacia Phast system. After electrophoresis, the gel was silver stained. Lane 1, buffer; 2, cytidylyltransferase (1 ng); 3, cytidylyltransferase (2 ng); 4, IEF standards (amyloglucosidase, pI 3.5; methyl red, pI 3.75; soyabean trypsin inhibitor, pI 4.55; b-lactoglobulin A, pI 5.2; bovine carbonic anhydrase B, pI 5.85; human carbonic anhydrase B, pI 6.55; horse myoglobin acidic band, pI 6.85; horse myoglobin basic band, pI 7.35; lentil lectin acidic band, pI 8.15; lentil lectin middle band, pI 8.45; lentil lectin basic band, pI 8.65; trypsinogen, pI 9.3)
Figure 29. **2D-PAGE of purified cytidylyltransferase.** 2D-PAGE of purified cytidylyltransferase (~2 μg) was carried out as described by O’Farrell (281). IEF-PAGE (1st dimension) was carried out using 16 cm tube gels. The cytidylyltransferase sample was loaded onto a pH 3-10 gradient gel and electrophoresis carried out for 10 h at 400 V. The gel was removed and equilibrated with equilibration buffer for 15 min. The gel was overlayed onto a SDS-PAGE slab gel (2nd dimension), without stacking gel, and electrophoresis performed for 17 h at 25 mA. The gel was removed and silver stained.
A primary injection of \(~50~\mu\text{g}\) of pure cytidylyltransferase in Freund's complete adjuvant was given at the back of the neck in at least six different sites in a rabbit. Four weeks later, a booster injection containing \(~30~\mu\text{g}\) of pure cytidylyltransferase in Freund's incomplete adjuvant was given. This process was subsequently repeated after another 4 weeks. Two weeks after the 2nd booster injection, the rabbit was bled from the ear, and the serum used to test for antibody production by the Western-blot technique. Pure cytidylyltransferase was transferred to a nitrocellulose membrane, which was subsequently incubated with serum (1:500 dilution). A second antibody (goat anti-rabbit IgG antibody), which had alkaline phosphatase coupled to it, was used to detect the first antibody in the presence of colour reagents (BCIP and NBT). Fig. 30 shows that although the presence of antibody to cytidylyltransferase was detected, the antibody titre was very weak at the dilution tested. A number of contaminating bands are also present, although the major contaminating band seems to be an artifact since it is also present in the lane with pre-stained standards. Thus, the fact that the antibody titre generated was weak and the antibody was non-specific for cytidylyltransferase, suggested that the generation of polyclonal antibodies in another rabbit be tried with at least 3 boosts of a higher magnitude. However, such attempts proved to be fruitless and a weak titre of non-specific antibody was still generated. This implied that either the cytidylyltransferase was not a good antigen or that the choice of injection of antigen under the epidermis in a rabbit was not sufficient enough to generate a marked antibody response.
Figure 30. Detection of antibody against cytidylyltransferase in the serum from a rabbit, using the Western-blot technique. Cytidylyltransferase (~1 μg) was analyzed by SDS-PAGE and then transferred to a nitrocellulose membrane by the Western-blot technique at 0.2 A for 14 h. The nitrocellulose membrane was removed, blocked with blocking solution and incubated with antiserum from a rabbit (primed and then boosted twice before removal of blood) for 5 h. The membrane was washed and subsequently incubated with an anti-rabbit antibody for 2 h. After washing, the membrane was developed using BCIP and NBT colour reagents. Lane 1 and 2, pure cytidylyltransferase; 3, pre-stained standards (phosphorylase, 130000; BSA, 75000; ovalbumin, 50000; carbonic anhydrase 39000; soyabean trypsin inhibitor, 27000; lysozyme, 17000).
2D-PAGE and detection by antibody (4. 1. 2. 2.).

Pure cytidylyltransferase was analyzed on 2D-PAGE and then transferred to a nitrocellulose membrane by Western-blot. The membrane was incubated with anti-serum from chicken (obtained from Dr. H. Jamil) and anti-chicken antibody, with conjugated alkaline phosphatase, added to detect the chicken antibody-cytidylyltransferase complex. Fig. 31 shows that at least 8-9 isoforms of cytidylyltransferase are present, with 2 isoforms present in a greater abundance than the other forms.

Whether these different isoforms may be the result of varying sugar residues associated with the the same protein, or whether the amino acid composition of the isoforms is different, is not clear. It will be interesting to see if the different isoforms are interconvertible and whether this is one of the modes of regulation of enzyme activity.

KINETICS
Determination of $K_m$ for CTP and phosphocholine (4. 1. 3. 1.).

The $K_m$ of the purified cytidylyltransferase with respect to CTP and phosphocholine was determined by measuring the reaction velocity at constant phosphocholine and varying CTP concentrations and vice versa (Fig. 32). The $K_m$ for CTP was established to be 0.31 mM while that for phosphocholine was 0.15 mM. These values are similar to the values reported by Choy et al (69) and by Weinhold et al (72).
Figure 31. 2D-PAGE of cytidylyltransferase and detection by antibody from chicken. Cytidylyltransferase (~2 μg) was analyzed by 2D-PAGE and then transferred to a nitrocellulose membrane by the Western-blot technique. The nitrocellulose membrane was removed, blocked with blocking solution and incubated with anti-serum from a chicken (primed and then boosted once before removal of blood) for 5 h. The membrane was washed and subsequently incubated with an anti-chicken antibody for 2 h. After washing, the membrane was developed using BCIP and NBT colour reagents. The pre-stained standards (phosphorylase, 130000; BSA, 75000; ovalbumin, 50000; carbonic anhydrase 39000; soyabean trypsin inhibitor, 27000; lysozyme, 17000) are shown on the right hand side.
Dependence of rate of reaction on the amount of protein (4. 1. 3. 2.).

Different amounts of purified cytidylyltransferase were incubated with the assay mixture for 15 min at 37 °C and the enzyme activity determined (Fig. 33). The reaction rate was essentially linear up to the addition of 50 µl of enzyme (~0.2 µg protein) to the assay mixture. Thereafter, the reaction became non-linear probably as a result of increased interference of Triton X-100 with the binding of substrates by the enzyme or by the activation of enzyme by lipids.

Dependence of rate of reaction on time (4. 1. 3. 3.).

The assay of cytidylyltransferase was carried out at 37 °C for various time intervals in the presence of 40 µl (~0.16 µg protein) of purified enzyme (Fig. 34). The reaction rate was linear up to 25 min. After this time interval, the reaction became non-linear probably as a result of substrate depletion.

Dependence of rate of reaction on temperature (4. 1. 3. 4.).

The assay of cytidylyltransferase was carried out at various temperatures in the presence of 40 µl (~0.16 µg protein) of purified cytidylyltransferase for 25 min (Fig. 35). The highest reaction rate is achieved at 37 °C with relatively low activity on either side of this temperature.

Activation of CTP:phosphocholine cytidylyltransferase with commercial lipids (4. 1. 4. 1.).
Figure 32. Determination of Km of the purified cytidylyltransferase for CTP and phosphocholine. The activity of purified cytidylyltransferase was measured at 37 °C for 15 min in the presence of 1 mM phosphocholine and varying concentrations of CTP (A), or 1 mM CTP and varying concentrations of phosphocholine (B). PC-oleate vesicles (200 μM, 1:1 molar ratio) were used as the activator of cytidylyltransferase. Each point represents the means of three separate experiments.
Figure 33. Linearity of the cytidylyltransferase catalyzed reaction with varying amounts of cytidylyltransferase. Cytidylyltransferase activity was measured in the presence of varying amounts of purified cytidylyltransferase at 37 °C for 15 min. PC-oleate vesicles (200 μM, 1:1 molar ratio) were used as the activator of cytidylyltransferase. Fifty μl of cytidylyltransferase correspond to ~ 0.2 μg of protein.

Figure 34. Linearity of the cytidylyltransferase catalyzed reaction at various time intervals. Cytidylyltransferase activity was measured at various time intervals in the presence of 40 μl (~0.16 μg protein) of purified cytidylyltransferase at 37 °C. PC-oleate vesicles (200 μM, 1:1 molar ratio) were used as the activator of cytidylyltransferase.
Assay of cytidylyltransferase was done under normal conditions in the presence of various concentrations of commercially available lipids (Fig. 36). PC-oleate, which had been shown to activate CT (22, 53, 65) gave the highest activation of enzyme activity, while PC and oleate alone only gave 42 and 20% respectively of the activation with PC-oleate. PS and PI both activated cytidylyltransferase to ~70% of the activation seen with PC-oleate and the presence of oleate did not further enhance the activation by these lipids. Lyso-lipids such as LPC and LPE, which had been previously shown by Choy et al (284) to stimulate markedly the partially purified cytidylyltransferase, only stimulated cytidylyltransferase to 45% of the value with PC-oleate. Diglyceride only gave 38% of the activation of cytidylyltransferase seen with PC-oleate.

Activation with microsomal lipids (4. 1. 4. 2.).

Lipids were extracted from microsomes by the Folch method (277) and separated by TLC. The cytidylyltransferase activity was determined in the presence of these lipids at 37 °C for 15 min. Fig. 37 shows the % activation of cytidylyltransferase by the different lipids when compared to the activation by commercial PC-oleate vesicles (which were assumed to give 100% activation). PC, LPC, DG+cholesterol, FFA, TG, and CE all gave 35-60% activation of cytidylyltransferase when compared to activation by PC-oleate. PS+PI, together with total lipid, gave almost 100% activation of the cytidylyltransferase. It was interesting to note that at low concentrations, PE stimulated cytidylyltransferase to almost 85% of
Figure 35. Rate of cytidylyltransferase catalyzed reaction at various temperatures. Cytidylyltransferase activity was measured at various temperatures in the presence of 40 μl (~0.16 μg protein) of purified cytidylyltransferase for 25 min. PC-oleate vesicles (200 μM, 1:1 molar ratio) were used as the activator of cytidylyltransferase.
Figure 36. Activation of cytidylyltransferase with commercial lipids. Assay of cytidylyltransferase (20 μl, ~0.08 μg protein) was carried out at 37 °C for 15 min in the presence of various concentrations of commercial lipids from Serdary and Sigma.
Figure 37. Activation of cytidylyltransferase with microsomal lipids. Assay of cytidylyltransferase (20 μl, ~0.08 μg protein) was carried out at 37 °C for 15 min in the presence of various concentrations of lipids extracted from microsomes.
the activation seen with PC-oleate. Spingomyelin did not activate the cytidylyltransferase.

Association of CTP:phosphocholine cytidylyltransferase with various membrane fractions in vitro (4.1.5.1.).

Membrane fractions corresponding to plasma membrane, mitochondrial membrane, rough ER, smooth ER, and golgi, which were isolated by differential centrifugation using sucrose gradients (79), were provided by Dr. Jean Vance. The cytidylyltransferase was incubated, with or without 200 μM oleate for 15 min at 37 °C in the presence of various membrane fractions (200 μg membrane protein/μg cytidylyltransferase). The mixture was centrifuged at 150,000 x g for 1 h and the pellet and the supernatant assayed for cytidylyltransferase activity. Fig. 38 shows that, in the absence of oleate, very little cytidylyltransferase is bound to the different membrane fractions (although plasma membrane fraction seems to bind almost twice as much cytidylyltransferase as other membranes) and the majority of the enzyme remains in the supernatant. In the presence of oleate, the amount of cytidylyltransferase associated with the plasma membrane increases almost 1.6-fold while with the other membranes, the association increases by almost 3-fold. Thus, oleate causes association of cytidylyltransferase with a variety of membranes in vitro.

Gel filtration of purified cytidylyltransferase and molecular weight estimation (4.1.6.1.).
Figure 38. Association of cytidylyltransferase with various membranes in the presence or absence of oleate. Various membrane fractions were isolated by differential centrifugation using sucrose gradients and incubated with cytidylyltransferase, with (+) or without (-) 200 μM oleate, for 15 min at 37 °C (200 μg membrane protein/μg cytidylyltransferase). The mixture was centrifuged at 150,000 x g for 1 h and the pellet and the supernatant (in presence of 200 μM PC-oleate) assayed for cytidylyltransferase activity.
Gel filtration of the purified cytidylyltransferase was carried out on a Sephacryl S-300 column (fraction range 10,000 to 1.5 x 10^6). Initially the void volume of the column was determined, using dextran blue 2000, and used to calculate the $K_{av}$ of each fraction. Fig. 39A shows the peak of cytidylyltransferase eluted from the column at a $K_{av}$ value of 0.44. Using this value, the molecular weight of the cytidylyltransferase is estimated to be ~195,000 (Fig. 39B). Since the cytidylyltransferase sample contained 0.03% Triton X-100, the actual molecular weight of the cytidylyltransferase is probably only about ~129,000 because the Triton X-100 micelle itself has a molecular weight of ~66,000 (72).
Figure 39. Chromatography of cytidylyltransferase on Sephacryl S-300 and molecular weight estimation. The void volume of a Sephacryl S-300 column (55 x 2 cm) was determined using dextran blue 2000 ($V_0$ was ~37 ml). The column was equilibrated with buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT, 150 mM NaCl, and 1 mM PMSF) before loading on 1 ml of concentrated purified cytidylyltransferase sample (~10 µg). The column was eluted with the same buffer and 1.5 ml fractions collected. The $K_{av}$ value and the enzyme activity of each fraction was determined (panel A). In a similar experiment, known molecular weight standards (ovalbumin, 45000; aldolase, 158000; catalase, 232000; ferritin, 440000; thyroglobulin, 669000) were chromatographed on the column and the $K_{av}$ determined. The $K_{av}$ of each standard was plotted against the log molecular weight (Mr) (panel B). Arrow indicates the position of cytidylyltransferase.
REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLTRANSFERASE BY REVERSIBLE PHOSPHORYLATION.

In Post Mitochondrial Supernatant

Effect of treatment of post mitochondrial supernatant with oleate (5. 1. 1. 1.).

Incubation of post mitochondrial supernatant with various concentrations of oleate (0-200 μM) resulted in an increase in cytidylyltransferase activity (Fig. 40). With 200 μM oleate in the incubation, the cytidylyltransferase activity increased to 3.22 ± 0.05 nmol/min/mg from 0.87 ± 0.02 nmol/min/mg in the control. The result is from four different preparations. These results are in agreement to those reported previously (73, 276).

Effect of treatment of post mitochondrial supernatant with BSA (5. 1. 1. 2.).

Incubation of post mitochondrial supernatant with various concentrations of fatty acid-poor BSA (0-0.2 mM) resulted in a decrease in cytidylyltransferase activity (Fig. 41). With 0.2 mM BSA in the incubation, the cytidylyltransferase activity decreased by ~73 % compared to control. Initially, increasing concentrations of BSA led to a rapid decrease in cytidylyltransferase activity after which the response starts to plateau.

Effect of treatment of post mitochondrial supernatant with oleate ± BSA (5. 1. 1. 3.).
Figure 40. Treatment of post mitochondrial supernatant with oleate. Post mitochondrial supernatant (5 mg protein) was incubated, in the presence of 10 mM NaF and 200 µg of protein kinase inhibitor, with various concentrations of oleate at 37 °C. After 15 min, 20 µl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. The results are the average of three separate experiments ± S.D.

Figure 41. Treatment of post mitochondrial supernatant with BSA. Post mitochondrial supernatant (5 mg protein) was incubated, in the presence of 10 mM NaF and 200 µg of protein kinase inhibitor, with various concentrations of BSA at 37 °C. After 15 min, 20 µl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. This experiment was repeated twice with similar results.
Incubation of post mitochondrial supernatant with increasing concentrations of oleate (0-200 μM) in the absence of BSA led to a rapid activation of cytidylyltransferase activity (Fig. 42). With 200 μM oleate in the incubation, the cytidylyltransferase increased by 2-fold compared to control. Incubation with oleate in the presence of BSA resulted in an abolition of the activation caused by oleate. Only when the oleate concentration is raised above 150 μM do we start to once again see activation by oleate. Thus, BSA at the concentration used can partially mask the effects of oleate.

Treatment of post mitochondrial supernatant with oleate under reversible phosphorylating conditions (5.1.1.4.).

Incubation of post mitochondrial supernatant with increasing concentrations of oleate (0-200 μM) led to an activation of cytidylyltransferase activity (Fig. 43). If the post mitochondrial supernatant is incubated with increasing oleate concentrations under phosphorylating conditions using cAMP-dependent protein kinase, the activation of cytidylyltransferase at low oleate concentrations is blocked and the activation is only seen when the oleate concentration is raised above 125 μM. Similarly, incubation with oleate under dephosphorylating conditions led to an elevation in the cytidylyltransferase activity at low oleate concentrations. However, at higher (above 150 μM oleate) concentrations the activation was the same as with oleate alone. Thus, it seems that phosphorylating conditions inhibit while dephosphorylating conditions stimulate cytidylyltransferase activity at low oleate concentrations. At higher oleate concentrations, these effects are abolished.
Figure 42. **Treatment of post mitochondrial supernatant with oleate in the presence or absence of BSA.** Post mitochondrial supernatant (1 mg protein) was incubated, in the presence of 10 mM NaF and 200 µg of protein kinase inhibitor, with various concentrations of oleate in the presence or absence of 0.75 mM BSA at 37 °C. After 15 min, 20 µl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. This experiment was repeated twice with similar results.

Figure 43. **Treatment of post mitochondrial supernatant with oleate under reversible phosphorylating conditions.** Post mitochondrial supernatant (1 mg protein) was incubated with various concentrations of oleate in the presence of buffer + 10 mM NaF + 100 µg protein kinase inhibitor (■), 100 units cAMP-dependent protein kinase + 4 mM MgCl₂ + 0.1 mM ATP + 10mM NaF (△), or 50 units porcine intestinal alkaline phosphatase + 100 µg protein kinase inhibitor (□) at 37 °C. After 15 min, 20 µl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. This experiment was repeated twice with similar results.
Treatment of post mitochondrial supernatant with Mg and ATP (5.1.2.1.).

Incubation of post mitochondrial supernatant with MgCl$_2$ (10 mM) led to a slight inhibition of cytidylyltransferase activity after 30 min compared to control (Fig. 44). Incubation of post mitochondrial supernatant with ATP (0.5 mM) led to a higher inhibition of cytidylyltransferase than that seen with MgCl$_2$. The effect is more pronounced after 30 min than after 15 min. However, incubation of post mitochondrial supernatant with MgCl$_2$ and ATP led to an even greater inhibition of cytidylyltransferase activity. Since Mg-ATP is a substrate for protein kinases, this suggested that cytidylyltransferase activity could be inhibited by phosphorylating conditions. It was not determined whether the ATP concentrations became rate-limiting, due to ATPase activity, after prolonged incubations.

Treatment of post mitochondrial supernatant with protein kinase inhibitor (5.1.2.2.).

Incubation of post mitochondrial supernatant with various concentrations of protein kinase inhibitor, in the presence of Mg-ATP, led to an activation of cytidylyltransferase activity (Fig. 45). The cytidylyltransferase activity increases with protein kinase inhibitor up to 50 μg, after which the effect plateaus. This result supported the previous result that phosphorylating conditions led to an inhibition of cytidylyltransferase activity.
Figure 44. Treatment of post mitochondrial supernatant with Mg and ATP. Post mitochondrial supernatant (2 mg protein) was incubated with buffer (control), 10 mM MgCl₂, 0.5 mM ATP, or 10 mM MgCl₂ + 0.5 mM ATP at 37 °C. After 15 min and 30 min, 20 μl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. This experiment was repeated twice with similar results.

Figure 45. Treatment of post mitochondrial supernatant with protein kinase inhibitor. Post mitochondrial supernatant (3 mg protein) was incubated with various concentrations of protein kinase inhibitor in the presence of 10 mM MgCl₂ + 0.5 mM ATP. After 15 min, 20 μl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. This experiment was repeated twice with similar results.
Treatment of post mitochondrial supernatant with cAMP-dependent protein kinase (5.1.2.3.).

Incubation of post mitochondrial supernatant with various concentrations of cAMP-dependent protein kinase (0-50 units) resulted in an almost 40% decrease of the cytidylyltransferase activity from $1.99 \pm 0.05$ nmol/min/mg to $1.30 \pm 0.02$ nmol/min/mg (Fig. 46). The incubation was in the presence of NaF to inhibit endogenous phosphatases which may counteract the effect of protein kinase and reduce phosphorylation of the cytidylyltransferase. Thus, it was demonstrated yet again that phosphorylating conditions can inhibit cytidylyltransferase activity.

Treatment of cytosol with cAMP-dependent protein kinase and chromatography on Sepharose 6B (5.1.2.4.).

The effect of phosphorylating conditions on the interconversion between the low molecular weight (non-aggregated) and the high molecular weight (aggregated) form of cytidylyltransferase was examined (Fig. 47). It was established that, after incubation of cytosol at 37 °C and room temperature, the majority of the cytidylyltransferase eluted in the void volume when chromatographed on Sepharose 6B suggesting that the enzyme was a high molecular weight complex. However, incubation under phosphorylating conditions resulted in most of the enzyme now being eluted in the fractionation range suggesting the enzyme was no longer aggregated. Thus, it seems that phosphorylating conditions can reverse aggregation and interconversion of the cytidylyltransferase to a high molecular weight form.
Figure 46. Effect of cAMP-dependent protein kinase on cytidylyltransferase activity in post mitochondrial supernatant. Post mitochondrial supernatant (5 mg) was incubated at 37 °C in the presence of 4 mM MgCl₂, 0.1 mM ATP, 10 mM NaF and various concentrations (0-50 units) of cAMP dependent protein kinase (cAMP PK). After 15 min, 20 µl aliquot was removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. The results are the average of three separate experiments ± S.D.

Figure 47. Treatment of cytosol with cAMP-dependent protein kinase and chromatography on Sepharose 6B. Cytosol (10 mg protein) was incubated in the presence or absence of 100 units cAMP-dependent protein kinase, 4 mM MgCl₂, 0.1 mM ATP, and 10 mM NaF for 30 min at 37 °C and then 2 h at room temperature. The mixture was loaded onto a Sepharose 6B column (40 x 2.5 cm). The column was eluted with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2 mM DTT buffer and 9 ml fractions collected. The cytidylyltransferase activity was determined in each fraction in the presence of TRLP.
Treatment of post mitochondrial supernatant with alkaline phosphatase (5. 1. 3. 1.).

Incubation of post mitochondrial supernatant with various concentrations of alkaline phosphatase (0-20 units) resulted in an increase in the cytidylyltransferase activity from $0.87 \pm 0.02$ nmol/min/mg to $1.88 \pm 0.03$ nmol/min/mg (Fig. 48). The incubation was done in the presence of protein kinase inhibitor to inhibit endogenous protein kinases.

Treatment of post mitochondrial supernatant with NaF (5. 1. 3. 2.).

Incubation of post mitochondrial supernatant with various concentrations of NaF (0-20 mM) resulted in a decrease in the cytidylyltransferase activity (Fig. 49). In the presence of 20 mM NaF, the cytidylyltransferase activity decreased by $\sim 27\%$ compared to control. The presence of NaF had no direct effect on cytidylyltransferase assay. Thus, the presence of protein phosphatase inhibitor can lead to inhibition of cytidylyltransferase activity. This result supports the previous result in that dephosphorylating conditions can activate cytidylyltransferase.

Fractionation of post mitochondrial supernatant after reversible phosphorylation (5. 1. 4. 1.).

We wished to determine whether the changes in cytidylyltransferase activity caused by phosphorylating/dephosphorylating conditions were due to the translocation of the enzyme between the microsomal (active) and the
Figure 48. Treatment of post mitochondrial supernatant with alkaline phosphatase. Post mitochondrial supernatant (5 mg) was incubated at 37 °C in the presence of various concentrations of alkaline phosphatase and 200 μg protein kinase inhibitor. After 15 min, 20 mM NaF was added to the incubation mixture to stop the alkaline phosphatase reaction and then 20 μl aliquot was removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. The results are the average of three separate experiments ± S.D.

Figure 49. Treatment of post mitochondrial supernatant with NaF. Post mitochondrial supernatant (3 mg protein) was incubated with various concentrations of NaF. After 15 min, 20 μl were removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. This experiment was repeated twice with similar results.
cytosolic (inactive) fractions. After incubations with buffer, cAMP-dependent protein kinase, or alkaline phosphatase, the post mitochondrial supernatant was fractionated into the cytosolic and the microsomal components by centrifugation at 130,000 x g for 1 h. The results are represented in Fig. 50. Incubation of post mitochondrial supernatant with cAMP-dependent protein kinase resulted in an increase (28%) in cytidylyltransferase activity in the cytosol compared to control. There was a corresponding decrease (21%) in microsomal cytidylyltransferase activity. Incubation of post mitochondrial supernatant with alkaline phosphatase resulted in a decrease in the cytosolic cytidylyltransferase activity (49%) and a corresponding increase in the microsomal activity (61%) compared to control. Therefore conditions favoring phosphorylation tended to inactivate the cytidylyltransferase in the microsomes, resulting in an increased recovery of activity in the cytosol. Whereas conditions favoring dephosphorylation resulted in more enzyme activity being recovered in the microsomal fraction.

Fractionation of post mitochondrial supernatant after treatment with BSA (5. 1. 4. 2.).

We examined the effects of BSA on cytidylyltransferase translocation between the cytosolic and the microsomal compartment (Fig. 51). As the BSA concentration increased, the amount of cytidylyltransferase associated with the microsomal fraction decreased with a corresponding increase in the cytosolic fraction. Therefore, the decrease in cytidylyltransferase activity in post
Figure 50. Fractionation of post mitochondrial supernatant after phosphorylating or dephosphorylating conditions. Post mitochondrial supernatant (5 mg) was incubated at 37 °C in the presence of 20 mM Tris-HCl, pH 7.4 buffer containing 0.1 mM ATP, 4 mM MgCl₂, and 50 units cAMP dependent protein kinase (PK), 20 units alkaline phosphatase (AP), or buffer (C). After 30 min, the post mitochondrial supernatant was fractionated into the cytosolic (supernatant) and microsomal (pellet) component by centrifugation at 130,000 x g for 1h. The microsomes were resuspended in 20 mM Tris-HCl buffer to a concentration of 2 mg/ml. The cytosolic activity was assayed in the presence of exogenous lipid. The results are the average of three separate experiments ± S.D.

Figure 51. Fractionation of Post Mitochondrial Supernatant after treatment with BSA. Post mitochondrial supernatant (3 mg) was incubated at 37 °C in the presence of various concentrations of BSA. After 30 min, the post mitochondrial supernatant was fractionated into the cytosolic (supernatant) and microsomal (pellet) component by centrifugation at 130,000 x g for 1h. The microsomes were resuspended in 20 mM Tris-HCl buffer to a concentration of 2 mg/ml before assaying for cytidylyltransferase. The cytosolic activity was assayed in the presence of exogenous lipid.
mitochondrial supernatant caused by BSA is due to translocation of the enzyme from the microsomes to the cytosol.

**Pure Cytidylyltransferase With Washed Microsomes**

**Incubation of pure cytidylyltransferase with oleate in the presence of washed microsomes (5. 2. 1. 1.).**

Oleate and phosphorylation/dephosphorylation may affect the cytidylyltransferase activity directly or affect activity of a cytosolic modifier protein which in turn regulates cytidylyltransferase activity. The cytidylyltransferase was purified to homogeneity and incubated with washed microsomes in the presence of oleate. After incubation, the soluble cytidylyltransferase (supernatant) was separated from the cytidylyltransferase bound to the microsomes (pellet) by centrifugation at 130,000 x g for 1 h.

Increasing concentrations of oleate resulted in an increase in the cytidylyltransferase activity in the pellet (microsomal) fraction with a corresponding decrease in the supernatant (cytosolic fraction) (Fig. 52). With 200 μM oleate in the incubation, the cytidylyltransferase activity in the pellet increased from 0.85 to 2.05 nmol/min/ml while the activity in the supernatant fraction decreased from 3.55 to 1.81 nmol/min/ml. Thus, oleate increases cytidylyltransferase activity in the microsomes and this effect is not through a cytosolic modifier protein.

**Incubation of pure cytidylyltransferase under phosphorylating conditions in the presence of washed microsomes (5. 2. 2. 1.).**
Figure 52. Incubation of pure cytidylyltransferase with oleate in the presence of washed microsomes. 2 μg of pure cytidylyltransferase was incubated at 37 °C with 20 mM Tris-HCl, pH 7.4 buffer containing 0-200 μM oleate, 2 mM DTT and 1 mg washed microsomes (prepared by rehomogenizing microsomes in Tris-saline buffer and centrifuging at 130,000 x g for 1 h). After 30 min, the incubation mixture was fractionated by centrifugation at 130,000 x g for 1 h into the supernatant (square), or the pellet (solid). The supernatant was assayed in the presence of exogenous lipid while the pellet was resuspended in Tris-saline buffer and assayed in the absence of lipid.
We tried to determine if the effects of phosphorylation in the post mitochondrial supernatant were directly or indirectly on the cytidylyltransferase activity. Pure cytidylyltransferase was incubated with washed microsomes in the presence of various concentrations of cAMP-dependent protein kinase and then fractionated into the supernatant (cytosolic) and the pellet (microsomal) fraction. Incubation with cAMP-dependent protein kinase resulted in a small increase in the cytidylyltransferase activity in the supernatant (3.12±0.02 to 3.77±0.03 nmol/min/ml) (Fig. 53). The cytidylyltransferase activity at the start of the incubation was very much higher in the supernatant compared to the pellet. The microsomal cytidylyltransferase activity at the start of the experiment was less than 0.2 nmol/min/ml. Therefore, the change in the cytidylyltransferase activity in the pellet was used as a positive indicator of the degree of inhibition of enzyme activity by cAMP-dependent protein kinase. As shown in Fig. 53, the cytidylyltransferase activity in the pellet decreased by 2.5 fold (1.36±0.01 to 0.56±0.05 nmol/min/ml) in the presence of 133 units of cAMP dependent protein kinase. If buffer, without cytidylyltransferase, were incubated with the cAMP-dependent protein kinase in the presence of washed microsomes, there was negligible change in the amount of cytidylyltransferase activity associated with the microsomes (data not shown). This indicated that the cAMP-dependent protein kinase affected binding of the exogenously added cytidylyltransferase to the microsomes and did not cause detectable release of the small amount of endogenous cytidylyltransferase.
Figure 53. Incubation of pure cytidylyltransferase with cAMP-dependent protein kinase in the presence of washed microsomes. 2 μg of pure cytidylyltransferase (initially "dephosphorylated" at 4 °C with 40 units alkaline phosphatase bound to agarose) was incubated at 37 °C with 20 mM Tris-HCl, pH 7.4 buffer containing 0-150 units cAMP-dependent protein kinase, 0.1 mM ATP, 4 mM MgCl₂, 2 mM DTT and 1 mg washed microsomes (prepared by rehomogenizing microsomes in Tris-saline buffer and centrifuging at 130,000 x g for 1 h). After 30 min, the incubation mixture was fractionated by centrifugation at 130,000 x g for 1 h into the supernatant (□), or the pellet (◆). The supernatant was assayed in the presence of exogenous lipid. The results are the average of three separate experiments ± S.D.
Incorporation of $^{32}$P into cytidyltransferase *in vitro* (5. 2. 2. 2.).

The ability of cAMP-dependent protein kinase to phosphorylate pure cytidyltransferase was initially examined. Purified cytidyltransferase was incubated in the presence of [$\gamma$-$^{32}$P]ATP and cAMP-dependent protein kinase (150 units) for 60 min. The incubation mixture was passed over Q-Sepharose to separate the cAMP-dependent protein kinase and the cytidyltransferase. This step was necessary due to the auto-phosphorylation of the cAMP-dependent protein kinase. As seen in Fig. 54, the cytidyltransferase was phosphorylated by cAMP-dependent protein kinase *in vitro*. In the absence of cAMP-dependent protein kinase, $^{32}$P was not incorporated into cytidyltransferase indicating that the cytidyltransferase is only phosphorylated by cAMP-dependent protein kinase and not by other endogenous protein kinases. Next, we examined whether the increase in cytidyltransferase activity in the supernatant with increasing cAMP-dependent protein kinase concentrations seen above, corresponded to an increase in $^{32}$P incorporation into the cytidyltransferase. The experiment in Fig. 53 was repeated with either unlabeled ATP or [$\gamma$-$^{32}$P]ATP. The results with unlabeled ATP are reported in Fig. 53. The results with labeled ATP are seen in Fig. 55. The amount of $^{32}$P incorporated into the cytidyltransferase in the supernatant increased with increasing cAMP-dependent protein kinase concentrations. This indicates that the increase in cytidyltransferase activity in the supernatant with increasing cAMP-dependent protein kinase concentrations is directly related to an increase in the phosphate content of
Figure 54. Phosphorylation of cytidylyltransferase with [γ-32P]ATP. 1 μg cytidylyltransferase (initially "dephosphorylated" with alkaline phosphatase) was incubated with [γ-32P]ATP in the absence (lane 1) and/or the presence of cAMP dependent protein kinase (150 units) for 60 min at 30 °C. The mixture was passed through a Q-Sepharose column to separate the cAMP dependent protein kinase (lane 2) from cytidylyltransferase (lane 3). The proteins were then analyzed on SDS-PAGE and the gel exposed to autoradiography. 2 μg of histones (lane 4) and casein (lane 5) as standards were phosphorylated under the same conditions as cytidylyltransferase with cAMP dependent protein kinase. These were not separated on Q-Sepharose.
Figure 55. Incorporation of $^{32}$P into cytidylyltransferase under phosphorylating conditions in the presence of washed microsomes. 2 μg of cytidylyltransferase (initially "dephosphorylated" with alkaline phosphatase) were incubated at 37 °C under phosphorylating conditions with 0-200 units of cAMP dependent protein kinase, 0.1 mM [$\gamma$-$^{32}$P]ATP, 4 mM MgCl$_2$, and 1 mg washed microsomal protein. After 30 min, the incubation mixture was fractionated into the supernatant and pellet fractions by centrifugation at 130,000 x g for 1h. The supernatant was passed through a Q-Sepharose column to separate the cAMP dependent protein kinase. The cytidylyltransferase was eluted, precipitated with 20% trichloroacetic acid and then analyzed on SDS-PAGE and the gel exposed to autoradiography.
cytidylyltransferase. Therefore, as more phosphate is incorporated into cytidylyltransferase by cAMP-dependent protein kinase, this leads to a decrease in cytidylyltransferase binding to microsomes resulting in more enzyme being recovered in the supernatant.

To ensure that the cAMP-dependent protein kinase was completely resolved from the cytidylyltransferase fraction after passing through the Q-Sepharose column, the cytidylyltransferase was incubated with cAMP-dependent protein kinase in the absence of $[\gamma^{-32}\text{P}]$ATP. The mixture was passed through a Q-Sepharose column to separate the cytidylyltransferase from the kinase. The cytidylyltransferase fraction was then incubated with $[\gamma^{-32}\text{P}]$ATP. No $^{32}\text{P}$ was incorporated into the cytidylyltransferase confirming that the kinase had been completely resolved from the cytidylyltransferase.

Incubation of pure cytidylyltransferase under dephosphorylating conditions in the presence of washed microsomes (5.2.3.1.).

We tried to determine if the effects of dephosphorylation in the post mitochondrial supernatant were directly or indirectly on the cytidylyltransferase activity. Cytidylyltransferase (which had been initially "phosphorylated" with cAMP-dependent protein kinase) was "dephosphorylated" with alkaline phosphatase bound to agarose. Incubation of "dephosphorylated" cytidylyltransferase with washed microsomes resulted in an increase in the cytidylyltransferase activity in the pellet fraction and a corresponding decrease in the supernatant fraction (3.61±0.08 to 2.88±0.07 nmol/min/ml) (Fig. 56). The cytidylyltransferase activity in the pellet increased by 2
Figure 56. Effect of alkaline phosphatase on pure cytidylyltransferase in the presence of washed microsomes. 2 µg of pure cytidylyltransferase (initially "phosphorylated" with cAMP dependent protein kinase) was incubated with alkaline phosphatase (0-40 units) bound to agarose at 4 °C. After 1 h, alkaline phosphatase was removed by centrifugation at 2000 x g for 5 min. The "dephosphorylated" cytidylyltransferase was incubated at 37 °C with 1 mg washed microsomes in Tris-HCl buffer. After 30 min, the incubation mixture was fractionated into the pellet (◆), and the supernatant (□) by centrifugation at 130,000 x g for 1 h. The supernatant was assayed in the presence of exogenous lipid. The results are the average of three separate experiments ± S.D.
fold (0.56±0.08 to 1.15±0.06 nmol/min/ml) in the presence of 40 units of alkaline phosphatase. If alkaline phosphatase were incubated with buffer, without cytidylyltransferase, and subsequently removed by centrifugation and the buffer incubated with washed microsomes, this did not result in an increase in cytidylyltransferase activity in the microsomes (data not shown). This indicated that the alkaline phosphatase was not "leaking" off the agarose support into the buffer and activating the cytidylyltransferase by dephosphorylation of a putative microsomal regulator protein.

We determined whether the decrease in the cytidylyltransferase activity in the supernatant with increasing alkaline phosphatase concentrations seen above correlated with a decrease in the phosphate content of the cytidylyltransferase. Fig. 57, shows that the amount of $^{32}P$ incorporated into the cytidylyltransferase in the supernatant decreased with increasing alkaline phosphatase concentrations. This indicates that the decrease in the cytidylyltransferase activity in the supernatant with increasing alkaline phosphatase concentrations corresponded directly to a decrease in $^{32}P$ incorporation into the cytidylyltransferase.

Table 6 shows the effect of phosphorylating/dephosphorylating conditions on the binding of cytidylyltransferase to washed microsomes. The cytidylyltransferase activity, at the start of the incubation in the supernatant fraction, was very much greater than the activity in the pellet. Incubation of the cytidylyltransferase with 150 units of cAMP-dependent protein kinase, resulted in a 2 fold decrease (3.2±0.12 to 1.8±0.01 nmol/min) in the
Figure 57. Incubation of $^{32}$P-labeled cytidylyltransferase under dephosphorylating conditions in the presence of washed microsomes. Cytidylyltransferase, which had been "phosphorylated" with [$\gamma$-$^{32}$P]ATP using cAMP dependent protein kinase for 30 min at 30 °C, was passed through a Q-Sepharose column to remove the cAMP dependent protein kinase. The cytidylyltransferase was eluted from the column and then incubated, at 4 °C with continuous shaking, with 0-40 units of alkaline phosphatase bound to agarose. After 1h, the alkaline phosphatase was removed by centrifugation at 2000 x g for 5 min. The supernatant was then incubated at 37 °C with 1 mg of microsomal protein. After 30 min, the incubation mixture was fractionated into the supernatant and the pellet by centrifugation at 130,000 x g for 1h. The cytidylyltransferase in the supernatant was precipitated with 20% trichloroacetic acid and then analyzed on SDS-PAGE and the gel exposed to autoradiography.
Table 6. Effect of phosphorylating and dephosphorylating conditions on pure cytidylyltransferase in the presence of washed microsomes. The cytidylyltransferase was incubated with 200 μM oleate, 150 units cAMP-dependent protein kinase (cAMP PK), or with 50 units alkaline phosphatase bound to agarose (AP). In another instance, the cytidylyltransferase was first incubated with 50 units alkaline phosphatase and then with 150 units cAMP-dependent protein kinase (AP + cAMP PK) or with 150 units cAMP-dependent protein kinase and then with 200 μM oleate (cAMP PK + oleate). The cytidylyltransferase was then incubated with washed microsomes at 37 °C. After 30 min, the incubation mixture was fractionated into the supernatant and the pellet (microsomes) by centrifugation at 130,000 x g for 1 h. The supernatant was assayed in the presence of exogenous lipid. The results are the average ± standard deviations, of three separate preparations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Supernatant (nmol/min)</th>
<th>Microsome (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.8 ± 0.7</td>
<td>3.2 ± 0.12</td>
</tr>
<tr>
<td>cAMP-PK</td>
<td>59.9 ± 0.1</td>
<td>1.8 ± 0.01</td>
</tr>
<tr>
<td>AP</td>
<td>50.9 ± 0.3</td>
<td>10.5 ± 0.03</td>
</tr>
<tr>
<td>AP + cAMP-PK</td>
<td>58.0 ± 0.9</td>
<td>4.7 ± 0.05</td>
</tr>
<tr>
<td>Oleate</td>
<td>43.7 ± 1.1</td>
<td>18.9 ± 0.07</td>
</tr>
<tr>
<td>cAMP-PK + oleate</td>
<td>44.1 ± 0.9</td>
<td>18.8 ± 0.04</td>
</tr>
</tbody>
</table>
cytidylyltransferase activity compared to control in the pellet fraction. Incubation of the cytidylyltransferase with microsomes, after treatment with alkaline phosphatase (40 units), resulted in a 3 fold increase (3.2±0.12 to 10.5±0.03 nmol/min) in the pellet fraction activity. If however the cytidylyltransferase was first incubated with alkaline phosphatase (40 units) and then with the cAMP dependent protein kinase in the presence of microsomes, the enzyme activity in the pellet fraction dropped by 56% (10.53±0.03 to 4.67±0.05 nmol/min). If the incubation with cAMP-dependent protein kinase was carried out in the presence of 200 µM oleate, the effects of phosphorylation were abolished. This confirms our earlier finding that the effects of reversible phosphorylation are only seen in the absence of oleate.

**Pure Cytidylyltransferase And Phosphatidylcholine Vesicles**

*Incubation of pure cytidylyltransferase with phosphatidylcholine vesicles under phosphorylating conditions (5.3.1.1.).*

We wished to distinguish whether phosphorylation was only directly affecting the cytidylyltransferase or was also affecting some other protein component on the microsomal membrane which might modify the cytidylyltransferase activity. We prepared PC vesicles in the absence of oleate. Pure cytidylyltransferase was incubated under phosphorylating conditions in the presence of PC vesicles. Fig. 58, shows the effect of incubation of the cytidylyltransferase with pure catalytic subunit of cAMP-dependent protein kinase. Incubation with
Figure 58: Effect of cAMP-dependent protein kinase on alkaline phosphatase-treated cytidylyltransferase in the presence of phosphatidylcholine vesicles. 1 μg cytidylyltransferase (which had been pre-incubated with alkaline phosphatase) in Tris-HCl, pH 7.4 buffer, was incubated with 4 mM MgCl₂, 0.1 mM ATP, and cAMP dependent protein kinase (0-110 units) at 37 °C in the presence of phosphatidylcholine vesicles. After 20 min, 20 μl was removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. The results are the average of three separate experiments ± S.D.
0-110 units of cAMP-dependent protein kinase, resulted in a 30% decrease (3.99±0.11 to 2.82±0.07 nmol/min/ml) in the cytidylyltransferase activity. In this case the cytidylyltransferase was initially incubated with alkaline phosphatase prior to cAMP-dependent protein kinase. Incubation of cytidylyltransferase with cAMP-dependent protein kinase in the absence of ATP had no effect on the enzyme activity. Incubation of the cytidylyltransferase with 110 units of cAMP-dependent protein kinase in the presence of pure protein kinase inhibitor (283) prevented the decrease in the cytidylyltransferase activity seen above (ie, the cytidylyltransferase activity was 3.95 ± 0.08 nmol/min/ml).

We examined whether the decrease in cytidylyltransferase activity with increasing cAMP-dependent protein kinase seen above, corresponded to an increase in $^{32}$P incorporation into the cytidylyltransferase. The experiment in Fig. 58 was repeated with [$\gamma$-$^{32}$P]ATP instead of unlabeled ATP. Fig. 59 shows that the amount of $^{32}$P incorporated into the cytidylyltransferase increased with increasing cAMP-dependent protein kinase concentrations. This indicates that the decrease in the cytidylyltransferase activity with increasing cAMP-dependent protein kinase concentrations is directly related to an increase in the phosphate content of the cytidylyltransferase. In the presence of purified protein kinase inhibitor (283), the incorporation of $^{32}$P into the cytidylyltransferase by cAMP-dependent protein kinase was blocked.

**Incubation of pure cytidylyltransferase with phosphatidylcholine vesicles under dephosphorylating conditions (5. 3. 2. 1.).**
Figure 59. Incorporation of $^{32}$P into cytidylyltransferase under phosphorylating conditions in the presence of phosphatidylcholine vesicles. 1 µg cytidylyltransferase ("dephosphorylated") was incubated with 4 mM MgCl$_2$ and [γ-$^{32}$P]ATP in the presence of 0 unit (lane 1), 14 units (lane 2), 28 units (lane 3), 56 units (lane 4), 83 units (lane 5), and 110 units (lane 6) of pure cAMP dependent protein kinase and 0.2 mM phosphatidylcholine vesicles at 37 °C. Lane 7 and 8 contains 110 units of cAMP dependent protein kinase + 250 nM or 500 nM pure protein kinase inhibitor respectively. After 20 min, the incubation mixture was passed through a Q-Sepharose column. The cytidylyltransferase was eluted, precipitated with 20% trichloroacetic acid and then analyzed on SDS-PAGE and the gel exposed to autoradiography.
We wished to distinguish whether dephosphorylation was only directly affecting the cytidylyltransferase or was also affecting some other protein component on the microsomal membrane which might modify the cytidylyltransferase activity. Fig. 60, shows the effects of incubation of the cytidylyltransferase with PC vesicles in the presence of 0-20 units of alkaline phosphatase bound to agarose. A 60% increase \((2.83\pm0.05 \text{ to } 4.53\pm0.04 \text{ nmol/min/ml})\) in the cytidylyltransferase activity was obtained. We examined whether this increase in cytidylyltransferase activity was due to a decrease in the phosphate content of the cytidylyltransferase. Fig. 61 shows that the amount of \(^{32}\text{P}\) incorporated into the cytidylyltransferase decreased with increasing alkaline phosphatase concentrations. This indicates that the increase in the cytidylyltransferase activity with increasing alkaline phosphatase concentrations corresponded directly to a decrease in \(^{32}\text{P}\) incorporation into the cytidylyltransferase.

The above results demonstrated that the effects on cytidylyltransferase activity were due to effects directly on the enzyme and not on a putative modifier protein.

**Time course of incorporation of \(^{32}\text{P}\) into the cytidylyltransferase**

(5. 3. 1. 1.).

Purified cytidylyltransferase, which had or had not been "dephosphorylated" with alkaline phosphatase, was incubated with \([\gamma-^{32}\text{P}]\text{ATP}\) and cAMP-dependent protein kinase (150 units) for different time intervals (Fig. 62). It is seen that if the cytidylyltransferase was "dephosphorylated" prior to incubation with \([\gamma-^{32}\text{P}]\text{ATP}\), then 2 fold more \(^{32}\text{P}\) \((0.2 \text{ mol P/mol subunit})\) was
Figure 60. Effect of alkaline phosphatase on pure cytidylyltransferase activity in the presence of phosphatidylcholine vesicles. 1 μg cytidylyltransferase (initially "phosphorylated" with cAMP dependent protein kinase) in 20 mM Tris-HCl, pH 7.4 buffer was incubated with alkaline phosphatase (0-20 units) bound to agarose at 4 °C. After 1h, alkaline phosphatase was removed by centrifugation at 2000 x g for 5 min. The "dephosphorylated" cytidylyltransferase was then incubated at 37 °C with phosphatidylcholine vesicles. After 20 min, 20 μl was removed and assayed for cytidylyltransferase activity in the absence of exogenous lipid. The results are the average of four separate experiments.
Figure 61. Incubation of $^{32}$P-labeled cytidylyltransferase under dephosphorylating conditions in the presence of phosphatidylcholine vesicles. 1 μg cytidylyltransferase, which had been initially "phosphorylated" with [γ-$^{32}$P]ATP using pure cAMP dependent protein kinase, was incubated with 0-20 units alkaline phosphatase at 4 °C. After 1 h, the alkaline phosphatase was removed by centrifugation and the "dephosphorylated" cytidylyltransferase incubated with phosphatidylcholine vesicles at 37 °C. After 20 min, the cytidylyltransferase was precipitated with 20% trichloroacetic acid and then analyzed on SDS-PAGE and the gel exposed to autoradiography.
Figure 62. Time course of amount of $^{32}\text{P}$ incorporated into normal and alkaline phosphatase-treated cytidylyltransferase. 1 μg of cytidylyltransferase, which had (♦) or had not (□) been incubated with 40 units alkaline phosphatase, was incubated with 4 mM MgCl$_2$, 150 units cAMP dependent protein kinase and [$\gamma$-$^{32}\text{P}$]ATP for different time intervals. The reaction mixture was passed through a Q-Sepharose column to separate the cAMP dependent protein kinase. The cytidylyltransferase was eluted, precipitated and subsequently analyzed on SDS-PAGE. Bands corresponding to cytidylyltransferase were excised from the gel and counted (panel A). Panel B represents a typical autoradiogram for such a time course. A; normal cytidylyltransferase, B; cytidylyltransferase which had been "dephosphorylated" prior to rephosphorylation.
incorporated into the enzyme after 120 min (or 0.12 mol P/mol subunit after 30 min). With non "dephosphorylated" cytidylyltransferase, only 0.086 mol P/mol subunit was incorporated after 120 min (or 0.05 mol P/mol subunit after 30 min). The incorporation of $^{32}\text{P}$ into the cytidylyltransferase reached a plateau after 120 min.

Analysis of the amino acid phosphorylated in the cytidylyltransferase (5.4.1.1).

Serine is the amino acid phosphorylated on the cytidylyltransferase by cAMP-dependent protein kinase as shown in Fig. 63.

Incorporation of $^{32}\text{P}$ into pure cytidylyltransferase in vitro and analysis by 2D-PAGE (5.5.1.1).

Cytidylyltransferase was phosphorylated in vitro using cAMP-dependent protein kinase and analyzed by 2D-PAGE. The enzyme was transferred onto a nitrocellulose membrane using the Western-blot technique, detected with antibody, and the membrane exposed to autoradiography (Fig. 64). A number of spots of varying intensity corresponding to cytidylyltransferase were detected.

Incorporation of $^{32}\text{P}$ into cytidylyltransferase in vivo and analysis by 2D-PAGE (5.5.2.1.).

Hepatocytes were incubated with $[^{32}\text{P}]$inorganic phosphate to label the cytidylyltransferase in vivo. The cells were homogenized and the cytosolic content isolated. The cytidylyltransferase was
Figure 63. Analysis of amino acid phosphorylated on cytidylyltransferase. 1 μg of cytidylyltransferase was incubated under phosphorylating conditions with cAMP-dependent protein kinase (150 units) and 0.1 mM [γ-32P]ATP for 60 min at 37 °C. The protein was precipitated with trichloroacetic acid and then the precipitate hydrolyzed with 6N HCl for 2 h at 110 °C under N₂, and analyzed by thin layer electrophoresis. Phosphoamino acids were separated by electrophoresis toward the anode for 3 h at 500 V. Amino acids were visualized with 0.2% ninhydrin and the plate exposed to autoradiography. Lane A, 32P labelled cytidylyltransferase; B, cAMP dependent protein kinase; C, "cold" phosphoamino standards; D, 32P labelled histones.
Figure 64. Incorporation of $^{32}$P into cytidylyltransferase in vitro and analysis by 2D-PAGE. 2 μg of cytidylyltransferase was incubated under phosphorylating conditions with cAMP-dependent protein kinase (150 units) and 0.1 mM [$\gamma$-$^{32}$P]ATP for 60 min at 37 °C. The protein was precipitated with trichloroacetic acid and analyzed on SDS-PAGE. The protein was transferred to a nitrocellulose membrane by the Western-blot technique and the cytidylyltransferase detected with antibody. The nitrocellulose was subsequently exposed to autoradiography. The prestained standards (phosphorylase, 130000; BSA, 75000; ovalbumin, 50000; carbonic anhydrase 39000; soyabean trypsin inhibitor, 27000; lysozyme, 17000) are shown on the left hand side. cAMP-dependent protein kinase was not separated from cytidylyltransferase in this experiment.
partially purified before analyzing by 2D-PAGE. The proteins were transfered onto a nitrocellulose membrane and the cytidylyltransferase detected with antibody. The membrane was subsequently exposed to autoradiography. Although a number of spots aligned with the position of the cytidylyltransferase on the film, there were also many other proteins which were heavily phosphorylated and migrated close to the cytidylyltransferase, thus not allowing clear interpretation of whether cytidylyltransferase is phosphorylated *in vivo* (data not shown).
REGULATION OF CYTIDYLYLTRANSFERASE BY Ca\textsuperscript{2+}

\textit{In Vitro}

Effect of Ca\textsuperscript{2+} and EGTA on post mitochondrial supernatant (6.1.1.1.).

Incubation of post mitochondrial supernatant with increasing concentrations of Ca\textsuperscript{2+} resulted in a rapid decrease in cytidylyltransferase activity (Fig. 65). In the presence of 5 mM Ca\textsuperscript{2+}, the cytidylyltransferase activity decreased by 50% (from 0.8 ± 0.01 to 0.4 ± 0.02 nmol/min/mg).

Incubation of post mitochondrial supernatant with increasing concentrations of EGTA resulted in a slight increase in cytidylyltransferase activity (Fig. 66). In the presence of 12.5 mM EGTA, the cytidylyltransferase activity increased by 13% (from 0.77 ± 0.01 to 0.90 ± 0.03 nmol/min/mg). Since EGTA is a specific chelator of Ca\textsuperscript{2+}, this suggested that endogenous Ca\textsuperscript{2+} may have been chelated which resulted in an increase in cytidylyltransferase activity.

A time course of the effects of EGTA and Ca\textsuperscript{2+} on post mitochondrial supernatant was carried out (Fig. 67). Although the effects of EGTA and Ca\textsuperscript{2+} are noticeable within 10 min of incubation with post mitochondrial supernatant, the effects are more pronounced after 30 min. Incubation of post mitochondrial supernatant with 5 mM Ca\textsuperscript{2+} resulted in a decrease in cytidylyltransferase activity compared to control while incubation with 12.5 mM EGTA resulted in an increase in cytidylyltransferase activity compared to control.
Figure 65. Treatment of post mitochondrial supernatant with Ca$^{2+}$. Post mitochondrial supernatant was incubated, in a total volume of 600 μl, with various concentrations of CaCl$_2$ at 37 °C. After 60 min, 20 μl of sample was removed and assayed for cytidylyltransferase activity in the absence of lipid. The results are the average of three separate experiments ± S.D.

Figure 66. Treatment of post mitochondrial supernatant with EGTA. Post mitochondrial supernatant was incubated, in a total volume of 600 μl, with various concentrations of EGTA at 37 °C. After 60 min, 20 μl of sample was removed and assayed for cytidylyltransferase activity in the absence of lipid. The results are the average of three separate experiments ± S.D.
Fractionation of post mitochondrial supernatant after treatment with Ca\textsuperscript{2+} and EGTA (6.1.1.2.).

We tried to establish whether the decrease or increase in cytidylyltransferase activity on incubation of post mitochondrial supernatant with Ca\textsuperscript{2+} or EGTA, is due to the translocation of the enzyme between the microsomal (active) and the cytosolic (inactive) compartment. Post mitochondrial supernatant was incubated with Ca\textsuperscript{2+} or EGTA and then fractionated into the cytosolic and microsomal fraction (Fig. 68). In the control incubation, most of the cytidylyltransferase activity resided in the microsomal fraction, due to the 37 °C incubation, with little activity in the cytosolic fraction. Incubation with EGTA resulted in a slight increase in the microsomal fraction compared to the control and a corresponding slight decrease in the cytosolic fraction. Incubation with Ca\textsuperscript{2+} resulted in a decrease in the microsomal fraction and a corresponding increase in the cytosolic fraction compared to the control. Thus the decrease in cytidylyltransferase activity on incubation of post mitochondrial supernatant with Ca\textsuperscript{2+} is due to translocation of the enzyme from the microsomal (active) to the cytosolic (inactive) fraction.

Post mitochondrial supernatant was fractionated after incubation with various concentrations of Ca\textsuperscript{2+} (Fig. 69). As the concentration of Ca\textsuperscript{2+} increased, the cytidylyltransferase activity associated with the cytosolic fraction decreased while the activity in the microsomal fraction increased. With 5 mM Ca\textsuperscript{2+}, the cytosolic activity decreased from 4.0 to 2.5 nmol/min/mg while the microsomal activity increased from 0.4 to 1.2 nmol/min/mg.
Figure 67. Time course of treatment of post mitochondrial supernatant with Ca\(^{2+}\) and EGTA. Post mitochondrial supernatant was incubated, in a total volume of 600 \(\mu\)l, with buffer, 5 mM CaCl\(_2\), or 12.5 mM EGTA at 37 °C. After various time intervals, 20 \(\mu\)l of sample was removed and assayed for cytidylyltransferase activity in the absence of lipid. The results are the average of three separate experiments ± S.D.

Figure 68. Fractionation of post mitochondrial supernatant after treatment with Ca\(^{2+}\) and EGTA. Post mitochondrial supernatant was incubated, in a total volume of 4 ml, with buffer, 5 mM CaCl\(_2\), or 12.5 mM EGTA at 37 °C. After 60 min, the sample was fractionated into the cytosolic and the microsomal fraction by centrifugation at 150,000 x \(g\) for 1 h. The microsomes were resuspended in 2 ml of Tris-HCl buffer with a tight-fitting dounce homogenizer. The cytidylyltransferase in the cytosolic fraction was assayed in the presence of 200 \(\mu\)M PC-oleate vesicles. The results are the average of three separate experiments ± S.D.
Treatment of post mitochondrial supernatant with various cations (6. 1. 1. 3.).

We examined whether the effect of Ca$^{2+}$ on post mitochondrial supernatant could be mimicked by other cations (Fig. 70). Incubation with Mg$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$ resulted in no alteration in cytidylyltransferase activity in the cytosol and the microsomes compared to the control. However, incubation with Ca$^{2+}$ resulted in an increase in cytidylyltransferase activity in the cytosol and a decrease in activity in the microsomes compared to the control. Thus, the effect of Ca$^{2+}$ is quite specific and cannot be mimicked by other cations.

Treatment of post mitochondrial supernatant with Ca$^{2+}$ in the presence and absence of calmodulin (6. 1. 1. 4.).

We examined whether the effects of Ca$^{2+}$ on post mitochondrial supernatant are mediated through the actions of calmodulin, which is known to bind Ca$^{2+}$ (Fig. 71). Incubation of post mitochondrial supernatant with various concentrations of Ca$^{2+}$ resulted in an increase in cytidylyltransferase activity in the cytosol and a decrease in the microsomes compared to the control. If the effects of Ca$^{2+}$ are mediated through calmodulin, then the presence of calmodulin should further enhance the effects of Ca$^{2+}$. However, the presence of calmodulin reversed the effects of Ca$^{2+}$ and the enzyme profile was similar to that in the control. This indicated that calmodulin was not mediating the effects of Ca$^{2+}$ on the inhibition of cytidylyltransferase
Figure 69. Fractionation of post mitochondrial supernatant after treatment with various concentrations of Ca$^{2+}$. Post mitochondrial supernatant was incubated, in a total volume of 4 ml, with various concentrations of CaCl$_2$ at 37 °C. After 60 min, the sample was fractionated into the cytosolic and the microsomal fraction by centrifugation at 150,000 x g for 1 h. The microsomes were resuspended in 2 ml of Tris-HCl buffer with a tight-fitting dounce homogenizer. The cytidylyltransferase in the cytosolic fraction was assayed in the presence of 200 μM PC-oleate vesicles.

Figure 70. Treatment of post mitochondrial supernatant with various cations. Post mitochondrial supernatant was incubated, in a total volume of 4 ml, with buffer, 5 mM CaCl$_2$, 5 mM MgCl$_2$, 5 mM MnCl$_2$, or 5 mM ZnCl$_2$ at 37 °C. After 60 min, the sample was fractionated into the cytosolic and the microsomal fraction by centrifugation at 150,000 x g for 1 h. The microsomes were resuspended in 2 ml of Tris-HCl buffer with a tight-fitting dounce homogenizer. The cytidylyltransferase in the cytosolic fraction was assayed in the presence of 200 μM PC-oleate vesicles.
activity. It further suggested that only the presence of free Ca\(^{2+}\) could lead to the inhibition of cytidylyltransferase activity.

**Effect Of Ca\(^{2+}\) In Vivo**

Digitonin-mediated release of cytidylyltransferase from hepatocytes by Ca\(^{2+}\) (6. 1. 2. 1.).

Hepatocytes were treated with various concentrations of calcium chloride for 5 or 10 min and the release of cytidylyltransferase examined by digitonin treatment. Figure 72A shows the effect on cytidylyltransferase release after 5 min incubation of Ca\(^{2+}\) with hepatocytes. As the concentration of Ca\(^{2+}\) increased, there was a substantial decrease in the amount of cytidylyltransferase released by digitonin treatment compared to control. With 7 mM Ca\(^{2+}\) in the medium, only 1.7 ± 0.1 nmol/min/mg cytidylyltransferase is released compared to 5.1 ± 0.2 nmol/min/mg in the control after 8 min. Incubation with Ca\(^{2+}\) in the medium for 10 min resulted in a slightly larger decrease in digitonin mediated cytidylyltransferase release (Fig. 72B). For 10 min, only 1.0 ± 0.1 nmol/min/mg cytidylyltransferase was released compared to 5.0 ± 0.3 nmol/min/mg in the control after 8 min. Therefore Ca\(^{2+}\) caused a rapid decrease in the amount of cytidylyltransferase released by digitonin treatment. Digitonin permeabilized cells have been shown to release rapidly cytosolic enzymes into the medium but not membrane bound enzymes (274). This is illustrated by the rapid release of lactate dehydrogenase, which is soluble, but not cholinephosphotransferase which is membrane bound. Therefore the above results indicated that Ca\(^{2+}\) was causing a decrease in the
Figure 7j. Treatment of post mitochondrial supernatant with Ca\(^{2+}\) in the presence or absence of calmodulin. Post mitochondrial supernatant was incubated, in a total volume of 4 ml, with buffer (A), 2.5 mM CaCl\(_2\) (B), 5 mM CaCl\(_2\) (C), 5 mM CaCl\(_2\) + 5000 units calmodulin (D), or 5 mM CaCl\(_2\) + 10000 units calmodulin (E) at 37 °C. After 60 min, the sample was fractionated into the cytosolic and the microsomal fraction by centrifugation at 150,000 x g for 1 h. The microsomes were resuspended in 2 ml of Tris-HCl buffer with a tight-fitting dounce homogenizer. The cytidylyltransferase in the cytosolic fraction was assayed in the presence of 200 μM PC-oleate vesicles.
amount of cytidylyltransferase in the cytosol resulting in less enzyme being released by digitonin.

**Digitonin-mediated release of cytidylyltransferase from hepatocytes by Ca\(^{2+}\) in the presence of verapamil (6.1.2.2).**

To examine if Ca\(^{2+}\) was mediating its effect on cytidylyltransferase release through an intracellular or extracellular component, cytidylyltransferase release by Ca\(^{2+}\) was examined in the presence of a Ca\(^{2+}\) channel blocker, verapamil. Figure 73A shows the effect of incubating hepatocytes for 5 min in the presence of different concentrations of verapamil and 7 mM Ca\(^{2+}\). It was observed that verapamil can partially reverse the decrease in cytidylyltransferase release caused by 7 mM Ca\(^{2+}\). The presence of 7 mM Ca\(^{2+}\) in the medium caused 1.7 ± 0.1 nmol/min/mg of cytidylyltransferase to be released compared to 5.1 ± 0.2 nmol/min/mg in the control. Incubation with 150 μM verapamil in the presence of 7 mM Ca\(^{2+}\) resulted in 3.7 ± 0.1 nmol/min/mg of cytidylyltransferase being released. Incubation of hepatocytes with verapamil for 10 min did not result in further increase in cytidylyltransferase release (Fig. 73B). Thus, it would appear that Ca\(^{2+}\) entering the cell via the Ca\(^{2+}\) channels was partly decreasing the release of cytidylyltransferase by digitonin treatment.

**Digitonin-mediated release of cytidylyltransferase from hepatocytes by Ionophore A23187 (6.1.2.3).**

We wanted to examine if the Ca\(^{2+}\) ionophore A23187 could mimic the effects of high concentrations of Ca\(^{2+}\) in the medium on
Figure 72. Effect of Ca$^{2+}$ on digitonin mediated release of cytidylyltransferase. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing 0 mM (□), 2 mM (■), 4 mM (▲), or 7 mM (◆) calcium chloride for 5 min (A) or 10 min (B). The medium was subsequently removed, the cells washed with 2 ml/dish of phosphate buffered saline and then 1 ml of digitonin solution added to each dish. After 2, 4, or 8 min, the digitonin solution was removed and assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
cytidylyltransferase release. Hepatocytes were incubated in the presence of 2 mM Ca$^{2+}$ (the normal concentration of Ca$^{2+}$ in Eagle's medium) and various concentrations of Ca$^{2+}$ ionophore A23187 for 5 or 10 min and the release of cytidylyltransferase by digitonin examined. Figure 74A shows the effect of various concentrations of ionophore on cytidylyltransferase release after 5 min incubation with hepatocytes. Higher concentrations of ionophore could partially mimic the effect of Ca$^{2+}$ on cytidylyltransferase release. With 10 μM ionophore, 2.4 ± 0.1 nmol/min/mg of cytidylyltransferase is released after 8 min of digitonin treatment compared to 3.4 ± 0.1 nmol/min/mg in the control (p<0.01). The maximum release of cytidylyltransferase in the absence of ionophore plus 2 mM Ca$^{2+}$ was similar to the experiments described in Fig. 72 and 73. The results after 10 min incubation of hepatocytes with ionophore were similar (Fig. 74B). Thus, it appears that the Ca$^{2+}$ ionophore A23187 can partially mimic the effects of Ca$^{2+}$ on cytidylyltransferase release.

**Digitonin-mediated release of cytidylyltransferase from hepatocytes by vasopressin (6. 1. 2. 4.).**

Since it had been observed that short term incubation with Ca$^{2+}$ or Ca$^{2+}$ ionophore could decrease cytidylyltransferase release by digitonin treatment, we wanted to examine if compounds which raised cytosolic Ca$^{2+}$ levels could have similar effects on cytidylyltransferase release. Vasopressin, which has been shown to raise cytosolic Ca$^{2+}$ levels (285), was incubated with hepatocytes for 5 or 10 min in the presence of 2 mM Ca$^{2+}$ and the release of cytidylyltransferase by digitonin examined. After 5 min incubation
Figure 73. **Effect of verapamil on digitonin mediated release of cytidylyltransferase.** Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing 0 mM Ca\(^{2+}\) (□), or 7 mM Ca\(^{2+}\) + 0 μM (□), 50 μM (■), 100 μM (▲), or 150 μM (■) verapamil for 5 min (A) or 10 min (B). The medium was subsequently removed, the cells washed with 2 ml/dish of phosphate buffered saline and then 1 ml of digitonin solution added to each dish. After 2, 4, or 8 min, the digitonin solution was removed and assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
Figure 74. Effect of ionophore A23187 on digitonin mediated release of cytidylyltransferase. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing 2 mM Ca\(^{2+}\) and 0 µM (□), 5 µM (■), or 10 µM (▲) ionophore A23187 for 5 min (A) or 10 min (B). The medium was subsequently removed, the cells washed with 2 ml/dish of phosphate buffered saline and then 1 ml of digitonin solution added to each dish. After 2, 4, or 8 min, the digitonin solution was removed and assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results. (* p<0.01)
with various concentrations of vasopressin, there was only a slight decrease in the amount of cytidylyltransferase released by digitonin compared to control (Fig. 75A). However, the effect was more pronounced after 10 min incubation (Fig. 75B). With 200 nM vasopressin in the medium, 2.5 ± 0.2 nmol/min/mg of cytidylyltransferase is released after 8 min of digitonin treatment compared to 3.4 ± 0.1 nmol/min/mg in the control (p< 0.01). Therefore it would appear that vasopressin can partially mimic the effect of Ca\(^{2+}\) on cytidylyltransferase release, presumably by raising the cytosolic Ca\(^{2+}\) levels.

Subcellular fractionation after treatment with Ca\(^{2+}\) and verapamil (6.1.3.1.).

Since Ca\(^{2+}\) and Ca\(^{2+}\) mobilizing agents were decreasing release of cytidylyltransferase by digitonin treatment, we wanted to examine whether or not this decrease was due to translocation of the cytosolic form to the microsomes or whether the decrease was due to inhibition of the cytosolic activity by some other mechanisms.

Hepatocytes treated with various concentrations of Ca\(^{2+}\), were homogenized and fractionated into the cytosolic and microsomal components. Figure 76 shows that with increasing Ca\(^{2+}\) concentrations, there was a decrease in the amount of cytidylyltransferase in the cytosol and an increase in the microsomes. With 7 mM Ca\(^{2+}\) in the medium, the cytosolic cytidylyltransferase level decreased to 1.2 nmol/min/mg compared to 4.0 nmol/min/mg in the control. Correspondingly, the microsomal level rose to 2.4 nmol/min/mg from 0.6 nmol/min/mg in the control.
Figure 75. Effect of vasopressin on digitonin mediated release of cytidylyltransferase. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing 2 mM Ca\(^2+\) and 0 nM (□), 50 nM (■), 100 nM (▲), or 200 nM (▲) vasopressin for 5 min (A) or 10 min (B). The medium was subsequently removed, the cells washed with 2 ml/dish of phosphate buffered saline and then 1 ml of digitonin solution added to each dish. After 2, 4, or 8 min, the digitonin solution was removed and assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results. (* p< 0.01)
Figure 76. Effect of Ca$^{2+}$ on cytidylyltransferase translocation. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing various concentrations of Ca$^{2+}$ (▲ ■), or Ca$^{2+}$ + 150 μM verapamil (△ □) for 10 min. The medium was subsequently removed, and the cells washed with 2 ml/dish of phosphate buffered saline. The cells were scraped and homogenized with 40 strokes of a tight fitting dounce homogenizer in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM PMSF, and 2 mM DTT. The extract was centrifuged at 10,000 x g for 10 min at 4 °C. The resulting supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The cytosolic (△ ▲) and the microsomal (□ ■) fractions were assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
Verapamil blocked the translocation of cytidylyltransferase to the microsomes caused by low concentrations of Ca\(^{2+}\), but higher concentrations of Ca\(^{2+}\) largely overcame the effects of verapamil. Thus, translocation of the cytidylyltransferase from the cytosolic compartment to the microsomal compartment could account for two thirds of the decrease of cytidylyltransferase being released by digitonin treatment.

Subcellular fractionation after treatment with vasopressin and Ionophore A23187 (6.1.3.2.).

The effects of various concentrations of Ca\(^{2+}\) ionophore A23187 and vasopressin on cytidylyltransferase translocation were examined. The cytidylyltransferase decreased in the cytosolic compartment (from 4.0 nmol/min/mg to 3.0 nmol/min/mg) while it increased in the microsomal compartment (from 0.7 nmol/min/mg to 1.2 nmol/min/mg) upon treatment with 400 nM vasopressin (Fig. 77). Similarly, cytidylyltransferase decreased in the cytosolic compartment (from 4.1 nmol/min/mg to 2.9 nmol/min/mg) while it increased in the microsomal compartment (from 0.6 nmol/min/mg to 1.5 nmol/min/mg) upon treatment with 10 μM ionophore A23187 (Fig. 78). Therefore Ca\(^{2+}\) and Ca\(^{2+}\) mobilizing agents were found to promote translocation of cytidylyltransferase from the cytosol to the microsomes, which resulted in less cytidylyltransferase being released by digitonin treatment.

Effect of Ca\(^{2+}\) on PC synthesis (6.1.4.1.).
Figure 77. Effect of vasopressin on cytidyltransferase translocation. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing various concentrations of vasopressin for 10 min. The medium was subsequently removed, and the cells washed with 2 ml/dish of phosphate buffered saline. The rest of the procedure is as in Figure 77. The data is the mean of duplicate determinations. The experiment was done three times with similar results.

Figure 78. Effect of ionophore A23187 on cytidyltransferase translocation. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing various concentrations of ionophore A23187 for 10 min. The medium was subsequently removed, and the cells washed with 2 ml/dish of phosphate buffered saline. The rest of the procedure is as in Figure 77. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
The next step was to analyse the effect of Ca\(^{2+}\) on PC synthesis. Since the above results showed that Ca\(^{2+}\) could modulate cytidylyltransferase activity, then Ca\(^{2+}\) should also modulate PC synthesis since the rate of the cytidylyltransferase catalyzed step governs the rate of PC synthesis.

Initially hepatocytes were pulsed with \([\text{methyl-}^{3}\text{H}]\text{choline chloride}\) in the presence of different concentrations of Ca\(^{2+}\). After 10 min, the lipid from the cells was extracted and the radioactivity in the PC fraction analysed (Fig. 79). It was observed that Ca\(^{2+}\) caused a significant \((p< 0.01)\) increase in the rate of formation of PC. With 7 mM Ca\(^{2+}\) in the medium, the radioactivity associated with the PC fraction increased to \(9.0 \pm 0.2 \times 10^5\) dpm/dish from \(6.0 \pm 0.3 \times 10^5\) dpm/dish in the control.

A time course of the effect of Ca\(^{2+}\) on PC synthesis during the pulse was carried out (Fig. 80). The presence of Ca\(^{2+}\) caused an increase in label incorporation into PC as early as after 5 min. However, longer incubation time intervals did not lead to further increase in label incorporation into PC as compared to incorporation after 5 min.

It could be argued that the increase in label in PC could be the result of differential uptake of choline label by the cells in the presence of Ca\(^{2+}\). To overcome this possibility, the cells were pulsed with \([\text{methyl-}^{3}\text{H}]\text{choline chloride}\) in the absence of Ca\(^{2+}\) for 30 min, and then chased without label for 10 min in the presence of various concentrations of Ca\(^{2+}\). Figure 81A and 81B show the radioactivity associated with the choline and phosphocholine fractions respectively. As the Ca\(^{2+}\) concentrations were increased, the label
Figure 79. Effect of Ca\(^{2+}\) during pulse on PC synthesis. Hepatocytes were pulsed with 15 \(\mu\)Ci/dish of [methyl-\(^3\)H]choline chloride for 10 min in choline- and methionine-free modified Eagle's medium in the presence of various concentrations of Ca\(^{2+}\). The medium was subsequently removed and the cellular lipids extracted and analysed. The results are the average of three separate determinations \(\pm\) S.D. (* \(p< 0.01\)).

Figure 80. Time course of Ca\(^{2+}\) effect during pulse on PC synthesis. Hepatocytes were pulsed with 15 \(\mu\)Ci/dish of [methyl-\(^3\)H]choline chloride for various time intervals in choline- and methionine-free modified Eagle's medium in the absence (■) or presence (□) of 7 mM Ca\(^{2+}\). The medium was subsequently removed and the cellular lipids extracted and analysed.
associated with both the choline and phosphocholine fraction decreased. This was expected if Ca\(^{2+}\) were activating the cytidylyltransferase since both choline and phosphocholine, which occur before the cytidylyltransferase catalyzed step, would decrease. Figure 81C shows the effect of various concentrations of Ca\(^{2+}\) on incorporation of label into PC. It is observed that with 7 mM Ca\(^{2+}\) in the medium, the label associated with PC increases to 3.8 ± 0.1 x 10\(^5\) dpm/dish from 2.7 ± 0.1 x 10\(^5\) dpm/dish in the control (p< 0.01). Apparently Ca\(^{2+}\) can stimulate PC synthesis by activating the cytidylyltransferase.

**Effect of Ca\(^{2+}\) on PC degradation (6. 1. 4. 2.).**

The next step in the investigation was to determine if PC degradation were increased since Ca\(^{2+}\) can cause activation of phospholipase A activity.

Hepatocytes, from a choline deficient rat, were pulsed with \([\text{methyl-}^3\text{H}]\text{choline chloride for 2 h. In contrast to cells from normal rats, the use of cells from choline deficient rats allowed most of the label to be incorporated into PC. The cells were chased without label for different time intervals in the presence or absence of 7 mM Ca}^{2+}. Figure 82A shows the effect of Ca\(^{2+}\) on cellular LPC formation. Ca\(^{2+}\) caused an increase in LPC formation with time. The incorporation of label increased from 1.2 ± 0.1 x 10\(^5\) dpm/dish to 2.0 ± 0.1 x 10\(^5\) dpm/dish (p< 0.01) with Ca\(^{2+}\) in the medium after 30 min, whereas in the control, the label increased to 1.5 ± 0.1 x 10\(^5\) dpm/dish after the same time interval.
Figure 81. Effect of Ca\(^{2+}\) during pulse-chase on PC synthesis. Hepatocytes were pulsed with 15 μCi/dish of [methyl-\(^{3}\)H]choline chloride for 30 min in choline- and methionine-free modified Eagle's medium. After this interval, the medium was removed, the cells washed with 2 ml/dish of unlabelled medium prior to incubation with 3 ml/dish of unlabelled medium containing 28 μM choline chloride and various concentrations of Ca\(^{2+}\) for 10 min. The medium and the cells were removed and the lipid and choline containing water soluble compounds extracted. The total radioactivity (cell+medium) in choline (A), phosphocholine (B), and PC (C) was determined. The results are the average of three separate determinations ± S.D. (*p< 0.01).
Since hepatocytes also secrete lipids into the medium (286), the effect of \( \text{Ca}^{2+} \) on LPC secretion into the medium was investigated (Fig. 82B). \( \text{Ca}^{2+} \) caused an increase in LPC secretion to \( 3.1 \pm 0.1 \times 10^5 \) dpm/dish compared to \( 2.1 \pm 0.2 \times 10^5 \) dpm/dish in the control after 30 min (\( p < 0.01 \)). Therefore, \( \text{Ca}^{2+} \) stimulated degradation of PC by a phospholipase reaction to generate LPC.

Since LPC can be degraded further to form GPC by lysophospholipase activity, the effect of \( \text{Ca}^{2+} \) on GPC formation was examined. There was only a slight effect of \( \text{Ca}^{2+} \) on GPC formation. Within the cell (Fig. 83A), the GPC increased from \( 1.0 \pm 0.02 \times 10^5 \) dpm/dish to \( 1.4 \pm 0.03 \times 10^5 \) dpm/dish with \( \text{Ca}^{2+} \) in the medium after 30 min, whereas in the control, the label increased to \( 1.3 \pm 0.02 \times 10^5 \) dpm/dish (\( p < 0.1 \)). In the medium (Fig. 83B), the label in GPC increased to \( 5.3 \pm 0.13 \times 10^4 \) dpm/dish in \( \text{Ca}^{2+} \) treated, whereas in the control, it increased to \( 4.9 \pm 0.22 \times 10^4 \) dpm/dish after 30 min (\( p < 0.1 \)). Therefore \( \text{Ca}^{2+} \) did not cause a significant increase in GPC formation either in the cell or in the medium.

**Digitonin-mediated release of cytidylyltransferase by phospholipase C and A2 (6. 2. 1. 1.).**

Hepatocytes were treated with various concentrations of phospholipase C for 10 min and the release of cytidylyltransferase examined by digitonin treatment (Figure 84). As the phospholipase C concentration increased, this resulted in a decrease in the amount of cytidylyltransferase released. With 0.9 units/dish of phospholipase C in the medium, there was only \( 2.7 \pm 0.2 \) nmol/min/mg of
Figure 82. Effect of $Ca^{2+}$ on LPC formation. Hepatocytes, from choline deficient rats, were pulsed for 2 h with 15 $\mu$Ci/dish of $[methyl-^3H]$choline chloride in choline- and methionine-free modified Eagle's medium. Subsequently, the medium was removed and the cells washed with 2 ml/dish of unlabelled medium. The cells were incubated for 0-30 min in unlabelled medium containing 28 $\mu$M choline chloride, and 0 mM (□) or 7 mM $Ca^{2+}$ (♦). The cellular and medium lipids were extracted and analysed. The radioactivity associated with the LPC fraction in the cell (A) and the medium (B) was determined. The results are the averages of three separate determinations ± S.D. (* p< 0.01; + p< 0.01).
Figure 83. Effect of Ca\(^{2+}\) on GPC formation. Hepatocytes, from choline deficient rats, were pulsed for 2 h with 15 μCi/dish of \([\text{methyl-}^{3}\text{H}]\text{choline chloride}\) in choline- and methionine-free modified Eagle’s medium. Subsequently, the medium was removed and the cells washed with 2 ml/dish of unlabelled medium. The cells were incubated for 0-30 min in unlabelled medium containing 28 μM choline chloride, and 0 mM (□) or 7 mM Ca\(^{2+}\) (◆). The cellular and medium lipids were extracted and analysed. The radioactivity associated with the GPC fraction in the cell (A) and the medium (B) was determined. The results are the averages of three separate determinations ± S.D. (* p< 0.1; + p< 0.1).
cytidylyltransferase released compared to $3.8 \pm 0.1$ nmol/min/mg released after 8 min of digitonin treatment.

Similarly, hepatocytes were treated with various concentrations of phospholipase A$_2$ for 5 or 10 min and the release of cytidylyltransferase examined by digitonin treatment. After 5 min incubation (Fig. 85A), the amount of cytidylyltransferase released from phospholipase A$_2$ treated cells was less than the release from control cells. However, the effect of phospholipase A$_2$ treatment was more pronounced after 10 min incubation (Fig. 85B). Incubation with 0.9 units/dish of phospholipase A$_2$ for 10 min resulted in $2.4 \pm 0.1$ nmol/min/mg of cytidylyltransferase released compared to $3.6 \pm 0.3$ nmol/min/mg in the control after 8 min of digitonin treatment. Thus, both phospholipase A$_2$ and C can decrease the amount of cytidylyltransferase released by digitonin treatment compared to control.

**Subcellular fractionation after treatment with phospholipase A$_2$ (6.2.2.1.).**

Since phospholipase A$_2$ was decreasing the release of cytidylyltransferase by digitonin treatment, we wanted to examine whether or not this decrease was due to translocation of the cytosolic form to the microsomes or whether the decrease was due to inhibition of the cytosolic activity by some other mechanisms.

Hepatocytes treated with phospholipase A$_2$, were homogenized and fractionated into the cytosolic and microsomal components. Figure 86A shows that with increasing phospholipase A$_2$, there was a decrease in the amount of cytidylyltransferase in the cytosol and an
increase in the microsomal fraction. With 0.9 units/dish of phospholipase A$_2$, the cytidylyltransferase decreased in the cytosolic fraction from 4.0 to 2.8 nmol/min/mg while it increased in the microsomal fraction from 0.8 to 1.8 nmol/min/mg. Thus, translocation of the cytidylyltransferase from the cytosol to the microsomes can account for over 75% of the decrease in cytidylyltransferase activity released by digitonin treatment.

Hepatocytes were incubated with 0.6 units/dish of phospholipase A$_2$ for various time intervals and then homogenized and fractionated into the cytosolic and microsomal fraction (Fig. 86B). As the time of incubation of cells with phospholipase A$_2$ increased, this resulted in a decrease in cytidylyltransferase activity in the cytosolic fraction and an increase in the microsomal fraction. After 20 min incubation, the cytidylyltransferase activity in the cytosol decreased from 3.9 to 2.7 nmol/min/mg while it increased in the microsomal fraction from 0.8 to 1.8 nmol/min/mg.

Effect of phospholipase A$_2$ on PC synthesis (6. 2. 3. 1.).

Initially hepatocytes were pulsed with [methyl-$^3$H]choline chloride for 30 min and then chased with unlabelled medium containing various concentrations of phospholipase A$_2$ for 10 min. When we extracted the cellular lipids and analyzed incorporation of the label into PC, we found a significant amount of label incorporated into the LPC fraction with very little incorporation into PC (data not shown). This suggested that the label was initially being incorporated into PC but this was then quickly degraded by the phospholipase A$_2$ to form LPC. To overcome this problem, we incubated hepatocytes
Figure 84. Effect of phospholipase C on digitonin mediated release of cytidylyltransferase. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing 0 units (∎), 0.15 units (∆), 0.3 units (■), 0.6 units (▲), or 0.9 units (■) phospholipase C (from Clostridium perfringens) for 10 min. The medium was subsequently removed, the cells washed with 2 ml/dish of phosphate buffered saline and then 1 ml of digitonin solution added to each dish. After 2, 4, or 8 min, the digitonin solution was removed and assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
Figure 85. **Effect of phospholipase A\textsubscript{2} on digitonin mediated release of cytidylyltransferase.** Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing 0 units (□), 0.15 units (▲), 0.3 units (▲), 0.6 units (●), or 0.9 units (■) phospholipase A\textsubscript{2} (from *Naja mocambique*) for 5 min (A) or 10 min (B). The medium was subsequently removed, the cells washed with 2 ml/dish of phosphate buffered saline and then 1 ml of digitonin solution added to each dish. After 2, 4, or 8 min, the digitonin solution was removed and assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
Figure 86. Effect of phospholipase A$_2$ on cytidylyltransferase translocation. Hepatocytes were incubated with 3 ml/dish of modified Eagle's medium containing various concentrations of phospholipase A$_2$ for 10 min (A) or 0.6 units phospholipase A$_2$ for various time intervals (B). The medium was subsequently removed, and the cells washed with 2 ml/dish of phosphate buffered saline. The cells were scraped and homogenized with 40 strokes of a tight fitting dounce homogenizer in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM PMSF, and 2 mM DTT. The extract was centrifuged at 10,000 x g for 10 min at 4 °C. The resulting supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The cytosolic (square) and the microsomal (■) fractions were assayed for cytidylyltransferase activity. The data is the mean of duplicate determinations. The experiment was done three times with similar results.
with phospholipase $A_2$ for various time intervals prior to pulsing the cells. The medium was removed and the cells pulsed with $[methyl-\textsuperscript{3}H]$choline chloride for 30 min and chased with unlabelled medium. Figure 87A shows that as the concentration of phospholipase $A_2$ were increased, this resulted in an increase in label associated with PC. Incubation with 0.9 units/dish of phospholipase $A_2$ resulted in an increase in label into PC from $2.4 \pm 0.02 \times 10^5$ dpm/dish to $3.1 \pm 0.1 \times 10^5$ dpm/dish ($p< 0.01$).

A time course of the effects of 0.6 units/dish of phospholipase $A_2$ on PC synthesis was carried out (Fig. 87B). With increasing time intervals, the label into PC increased. After 20 min incubation, the label into PC increased from $2.6 \pm 0.03 \times 10^5$ dpm/dish to $3.4 \pm 0.05 \times 10^5$ dpm/dish. Thus, presence of phospholipase $A_2$ can increase incorporation of label into PC.
Figure 87. Effect of phospholipase A$_2$ on PC synthesis. Hepatocytes were incubated with various concentrations of phospholipase A$_2$ for 10 min (A) or 0.6 units phospholipase A$_2$ for various time intervals (B). The medium was removed and the cells pulsed with 15 $\mu$Ci/dish of [methyl-$^3$H]choline chloride for 30 min in choline- and methionine-free modified Eagle's medium. After this interval, the medium was removed, the cells washed with 2 ml/dish of unlabelled medium prior to incubation with 3 ml/dish of unlabelled medium containing 28 $\mu$M choline chloride for 20 min. The medium and the cells were removed and the total radioactivity in PC determined. The results are the average of three separate determinations ± S.D. (* $p<0.01$).
DISCUSSION

Purification of CTP:phosphocholine cytidylyltransferase (7.1.1.1.).

Initial purification of the cytidylyltransferase proved to be very tedious and time consuming with little success. The main problem encountered was increasing loss of enzyme activity as the purification increased. The instability of the enzyme could be overcome by using lipids but this led to aggregation of the enzyme which in turn meant dissociation of the enzyme with detergents before further purification. Since dissociation of the aggregated enzyme was variable (for some unknown reasons), we tended not to use lipids for stabilization. The enzyme was found to be stabilized to some extent by the inclusion of the detergent Triton-X100 in the purification buffers.

Choy et al (69) had reported purification of the cytidylyltransferase by aggregation/deaggregation. Despite extensive efforts, we were unable to reproduce the results. The main problem was that aggregation and dissociation was variable and contaminating proteins co-purified with the cytidylyltransferase. Therefore, we avoided using the aggregated enzyme and instead used non-aggregated cytosolic enzyme as the source of starting material. Despite combining conventional chromatography with high performance chromatography using FPLC, we were still unable to achieve complete purification of the cytidylyltransferase, although we did manage an almost 1000-fold purification. We tried other chromatographic techniques in combination with FPLC but this did not lead to an increase in purification. One of the factors which
hindered our progress of purification was the rapid loss of enzyme activity of the highly purified protein.

Weinhold et al (72) reported purification of the cytidylyltransferase by acid precipitation of the aggregated enzyme and then dissociation and purification by conventional chromatography. However, when we tried to reproduce the procedure, two problems were encountered. The first was the enzyme, at the end of the purification, was still contaminated with a large number of proteins which became visible when the sample was analyzed on SDS-PAGE and silver stained. The second problem was that we could not detect in our preparations the presence of both the 39 and the 48 Kd subunits of cytidylyltransferase reported by Weinhold et al (72). This suggested that our enzyme preparations were not homogenous. Therefore, we modified Weinhold et al's procedure by employing linear gradients instead of step gradients and by using FPLC to enable us to purify the enzyme to homogeneity. Finally, we were able to achieve purification of the cytidylyltransferase to homogeneity. The purified enzyme migrated as a single band of molecular weight ~42 Kd when analyzed on SDS-PAGE and silver stained. The cytidylyltransferase was purified over 34,000-fold by our procedure with a recovery of 8.4%. Later work by Feldman and Weinhold (287) confirmed our finding that the purified cytidylyltransferase was a single subunit when analyzed by SDS-PAGE of molecular weight ~45 Kd.

Characterization of the purified cytidylyltransferase

Analysis by PAGE (7. 2. 1. 1.)
When the purified cytidylyltransferase was analyzed by native-PAGE, the protein migrated as a single band of molecular weight ~90 Kd. This suggested that the native form of the enzyme may be a dimer. This contradicts the earlier findings of Choy et al (69) and Weinhold et al (72) who both suggested the enzyme to be a tetramer. It may be that during electrophoresis, the tetrameric form dissociated into a dimer. However, when the purified enzyme was analyzed on a gel filtration column, the enzyme eluted at a $K_{av}$ value which corresponded to a molecular weight of ~129,000 after subtracting the molecular weight of a Triton X-100 micelle. This value seems closer to the enzyme being a dimer than a tetramer. It must be cautioned that the molecular weight of Triton X-100 micelle was estimated from its theoretical structure, and this may have been altered during chromatography on a gel filtration column.

Analysis of the purified cytidylyltransferase by IEF-PAGE showed the enzyme to migrate as a single band with a pI of ~5.8. Thus the enzyme had a net negative charge in the buffer systems used during the purification. Analysis of the purified enzyme by 2D-PAGE showed the enzyme to exist of at least two isoforms. These isoforms seem to differ in their pI while the molecular weight seems to be similar.

**Lipid activation of purified cytidylyltransferase (7. 2. 1. 2.).**

We analyzed the lipid requirement of the purified enzyme for activation. Vesicles made from commercial PC-oleate, PS and PI had been shown to stimulate CT activity (22, 53, 65). Similarly, we found vesicles made from PC-oleate, PS, PI, and TRLP (extracted from rat
liver microsomes) gave maximal stimulation of the enzyme activity. Since PC-oleate was less expensive than PS and PI, this was subsequently used for enzyme activation. The stimulation by PS and PI may be of physiological importance since the activation of the cytidylyltransferase by these lipids is to the same extent as with TRLP. Since PS and PI account for only a small portion (~5%) of the total lipid present in the microsomes, then a small change in the PS and PI content of the membranes may lead to a dramatic effect on cytidylyltransferase activation and translocation to the microsomal membrane. It was also evident that charged lipids give a better activation of the cytidylyltransferase than neutral lipids. Lysolipids, such as LPC and LPE, were not very potent activators of the enzyme. One surprising finding was that oleate, in the absence of exogenous lipids, also partially activated the cytidylyltransferase. This appears to contradict Choy et al.’s (284) finding that neutral lipids, including fatty acids, do not activate the non-aggregated enzyme. Weinhold et al. (72) found a slight activation of the cytidylyltransferase by oleate alone. It may be that there is still a small amount of lipid tightly associated with cytidylyltransferase which, in the presence of oleate, can partially activate the enzyme. Another possibility is that oleate can partition into the Triton X-100 micelle to provide a hydrophobic environment, similar to that provided PC-oleate vesicles, which can partially activate the enzyme.

**Generation of polyclonal antibodies (7. 2. 1. 3.).**

Although we followed standard procedures, attempts to generate polyclonal antibodies in rabbits were largely unsuccessful,
with only a weak antibody titre generated. Whether this was due to
the cytidylyltransferase acting as a poor antigen or whether the
method of sample preparation and injection were inadequate is
unclear. Dr. Harris Jamil, using a procedure similar to our's, was
successful in generating weak antibodies in the chicken. This
antibody detected cytidylyltransferase by the Western-blot
technique and inhibited cytidylyltransferase activity, but was unable
to immunoprecipitate the enzyme in solution using protein-A
Sépharose. Therefore, attempts to study the cytidylyltransferase
under \textit{in vivo} situations were hindered due to this aspect.

When purified cytidylyltransferase was analyzed by 2D-PAGE
and detected by immuno-blot, several isoforms of the enzyme were
detected. These isoforms may differ in the degree of glycosylation of
the protein, since cytidylyltransferase was found to bind to Con-A
Sépharose and was eluted with high concentrations of mannose. If
the cytidylyltransferase is glycosylated, treatment with
neuraminidase should cause a shift in the isoform profile on 2D-
PAGE.

\textbf{Regulation of cytidylyltransferase by reversible phosphorylation \textit{in}
\textit{vitro}}

\textit{In post mitochondrial supernatant (7. 3. 1. 1.).}

It was previously demonstrated, \textit{in vitro}, that the
cytidylyltransferase may be regulated by
phosphorylation/dephosphorylation (74). This conclusion was
derived from experiments involving incubations of rat liver cytosol
in the presence of Mg.ATP (substrate for protein kinase) or NaF
(protein phosphatase inhibitor) which led to inhibition of the cytidylyltransferase activity. Incubation with protein kinase inhibitor reversed the inhibition of the cytidylyltransferase activity by Mg.ATP. Similarly, we found incubation of post mitochondrial supernatant under phosphorylating conditions (using cAMP-dependent protein kinase, or Mg-ATP) led to an inhibition, while incubation under dephosphorylating conditions (using alkaline phosphatase) led to a stimulation of the cytidylyltransferase activity. The inhibition or stimulation was due to reciprocal changes of the cytidylyltransferase between the cytosolic and the microsomal fractions.

Using purified cytidylyltransferase and washed microsomes (7.3.1.2.).

However, the above and previous studies (74) could not eliminate the possibility that the cytidylyltransferase activity might be regulated by a modulator protein which in turn was subject to control by phosphorylation/dephosphorylation. Conclusive evidence for the regulation of the cytidylyltransferase activity by phosphorylation/dephosphorylation awaited the purification of the enzyme to homogeneity.

Incubation of the pure cytidylyltransferase ("dephosphorylated") with washed microsomes in the presence of cAMP dependent protein kinase led to an increase in the cytidylyltransferase activity in the supernatant and a corresponding decrease in the microsomal fraction. Incubation of the pure cytidylyltransferase, after "dephosphorylation" with alkaline
phosphatase, with washed microsomes led to a decrease in the cytidylyltransferase activity in the supernatant and a corresponding increase in the microsomal fraction.

**Incorporation of $^{32}$P into cytidylyltransferase (7.3.1.3.).**

Cytidylyltransferase was found to be a substrate for cAMP-dependent protein kinase *in vitro*. Phosphate was incorporated into serine residues on the cytidylyltransferase. We wanted to correlate the increase or decrease in cytidylyltransferase activity in the supernatant fraction under reversible phosphorylating conditions seen above with alteration in $^{32}$P incorporation into the cytidylyltransferase. The increase in the cytidylyltransferase activity in the supernatant on incubation with cAMP-dependent protein kinase was found to be directly related to an increase in $^{32}$P incorporation into the cytidylyltransferase. However, the changes in the enzyme activity were substantially greater than the stiochiometry of $^{32}$P incorporation. A number of reasons could have accounted for the low incorporation of $^{32}$P into the cytidylyltransferase. One possibility is that the enzyme was passed through a column to separate the kinase and since the recovery from the column is not 100% (more likely ~60-70%), then the amount of enzyme recovered was less than that at the start. Since the enzyme in the assay at the start was used for calculation of the amount of enzyme present, then the value for $^{32}$P incorporation may be underestimated. Another possibility for the low incorporation is that the enzyme was eluted in a large volume from the Q-Sepharose column, then not all the enzyme may have been precipitated by TCA. Thus,
not all the "phosphorylated-enzyme" may have been applied onto SDS-PAGE, since it is very difficult to precipitate small amounts (1 μg) of protein with TCA. Another possibility is that we may have over-estimated the amount of enzyme present in the assay at the start, since the Bio-Rad protein assay is not very sensitive in the 1 μg range. All these possibilities could have accounted for the low stiochiometric incorporation of $^{32}$P into the cytidylyltransferase.

The decrease in the cytidylyltransferase activity in the supernatant on incubation with alkaline phosphatase correlated with a decrease in $^{32}$P incorporation into the cytidylyltransferase.

Using purified cytidylyltransferase with phosphatidylcholine vesicles (7. 3. 1. 4.).

Incubation of the pure cytidylyltransferase with phosphatidylcholine vesicles in the presence of pure cAMP-dependent protein kinase led to a decrease in the cytidylyltransferase activity. The decrease in the cytidylyltransferase activity was directly related to an increase in $^{32}$P incorporation into the cytidylyltransferase. Incubation of the cytidylyltransferase, after "dephosphorylation" with alkaline phosphatase, with phosphatidylcholine vesicles led to an increase in cytidylyltransferase activity. The increase in cytidylyltransferase activity was directly related to a decrease in $^{32}$P incorporation into the cytidylyltransferase.

Thus, cytidylyltransferase activity is subject to regulation by phosphorylation/dephosphorylation in vitro.
It was observed that the effects of phosphorylation/dephosphorylation on the cytidylyltransferase activity were not seen in the absence of lipid or microsomal membranes. Since the cytidylyltransferase requires membrane lipids for activation, it appears that phosphorylation/dephosphorylation was affecting the affinity of the cytidylyltransferase for binding to the lipid or microsomal membranes.

The regulation of cytidylyltransferase binding to lipid and microsomal membranes by phosphorylation/dephosphorylation was not observed in the presence of 0.2 mM oleate. Therefore, all experiments were done in the absence of exogenous oleate. The results indicate that the cytidylyltransferase contains a site(s) which can be phosphorylated. When phosphate was incorporated into this site(s), this decreased the affinity of the cytidylyltransferase for membranes resulting in more enzyme being recovered in the supernatant. We postulate that a conformational change in the enzyme could account for the enzyme translocation. If phosphate were removed from this site(s), the cytidylyltransferase might revert back to its original conformation and the affinity for membranes would be increased resulting in less enzyme being recovered in the supernatant. Interaction of the cytidylyltransferase with oleate may keep the cytidylyltransferase in a conformation which still has a high affinity for membranes and may explain why oleate can overcome the effects of phosphorylation/dephosphorylation on the cytidylyltransferase activity.

Incorporation of $^{32}$P into the cytidylyltransferase demonstrated that if the cytidylyltransferase were incubated with alkaline
phosphatase prior to incubation with cAMP dependent protein kinase, 2 fold more phosphate was incorporated. This is consistent with our hypothesis that the cytosolic form of the cytidylyltransferase may already be phosphorylated. Removal of this endogenous phosphate with alkaline phosphatase would allow more $^{32}$P to be incorporated into the cytidylyltransferase. Examination of the amount of phosphate incorporated per cytidylyltransferase subunit shows a small but significant amount incorporated. This may be explained in that we are not totally removing all the endogenous phosphate from the cytidylyltransferase. The intestinal alkaline phosphatase is probably not the actual protein phosphatase in vivo responsible for the dephosphorylation of the cytidylyltransferase. Thus in vitro, we are only partially dephosphorylating the cytidylyltransferase, resulting in a lower incorporation of $^{32}$P.

In general, cAMP-mediated protein phosphorylations have been associated with a stimulation of degradative pathways and an inhibition of biosynthetic pathways. The effects of cAMP on lipid synthesis is consistent with this observation. Elevated intracellular levels of cAMP have led to inhibition of fatty acid and cholesterol synthesis (287). In these cases, phosphorylation reduced the activity of the regulatory enzymes acetyl-CoA carboxylase (288) and HMG-CoA reductase (289) respectively. Previous work with cultured hepatocytes showed that incubation with cAMP analogues led to inhibition of phosphatidylcholine synthesis (75). Therefore, it is not surprising to find that phosphatidylcholine synthesis may also be regulated via phosphorylation/dephosphorylation of the regulatory enzyme cytidylyltransferase, in a cAMP dependent manner.
Phosphorylation of cytidylyltransferase in vivo (7.3.1.5.).

The next step was to prove that cytidylyltransferase is phosphorylated/dephosphorylated in vivo. The essential reagent for these studies is an antibody to cytidylyltransferase that can be precipitated. Despite considerable efforts, we have been unable to prepare an antibody which can be used to precipitate the cytidylyltransferase in solution. However, we have used the chicken antibody, which can detect the cytidylyltransferase on immuno-blot, to detect the cytidylyltransferase which had been isolated from hepatocytes incubated with $^{32}$P. Although we detected spots on the film in the region where the cytidylyltransferase migrated, it was difficult to estimate whether the cytidylyltransferase was indeed phosphorylated in vivo since a large number of contaminating proteins, which were also heavily phosphorylated, migrated close to the cytidylyltransferase. Therefore, "phosphorylated" cytidylyltransferase needs to be purified further before definitive results on whether the enzyme is phosphorylated in vivo can be obtained.

Regulation of cytidylyltransferase activity by Ca$^{2+}$

In post mitochondrial supernatant (7.4.1.1.).

Ca$^{2+}$ was found to inhibit, while EGTA stimulated cytidylyltransferase activity in the post mitochondrial supernatant in a time and concentration dependent manner (it must be noted that the concentrations of Ca$^{2+}$ used were not physiological, which are in the μM range rather than mM). The inhibition and the stimulation
were caused by reciprocal changes in the cytidylyltransferase activity between the microsomal and the cytosolic compartment. The effect with Ca\(^{2+}\) was specific since other cations did not mimic the effect. The effects of Ca\(^{2+}\) on cytidylyltransferase are not mediated through calmodulin, since the presence of calmodulin reversed the effects of Ca\(^{2+}\). Thus it seems that only free Ca\(^{2+}\) can inhibit cytidylyltransferase activity, while chelation with EGTA and calmodulin reverses the inhibition. The inhibition of cytidylyltransferase activity by Ca\(^{2+}\) may be the result of Ca\(^{2+}\) forming "bridges" with phospholipids on the disrupted microsomal membrane leading to aggregation of the membrane. This may prevent the phospholipids from activating the cytidylyltransferase leading to a decrease in the cytidylyltransferase associated with the microsomes.

Digitonin-mediated release of cytidylyltransferase from hepatocytes

(7. 4. 2. 1.).

High concentrations of Ca\(^{2+}\) (7 mM) in the medium caused a marked decrease, within 10 min, in the amount of cytidylyltransferase released by digitonin treatment. This effect of Ca\(^{2+}\) can be partially overcome by the use of a Ca\(^{2+}\) channel blocker, verapamil. This indicated that some of the Ca\(^{2+}\) may be entering the cell via voltage-dependent Ca\(^{2+}\) channels in the plasma membrane and affecting cytidylyltransferase release. This observation is further supported since the Ca\(^{2+}\) ionophore A23187, which is a mobile ion carrier allowing Ca\(^{2+}\) to move into the cytosol down its electrochemical gradient, can mimic partially the effects of Ca\(^{2+}\) on
cytidylyltransferase release. Vasopressin, which is known to cause an increase in cytosolic Ca\(^{2+}\) concentrations as a result of inositol trisphosphate formation (285), also decreased the release of cytidylyltransferase by digitonin treatment. These effects of Ca\(^{2+}\) and Ca\(^{2+}\)-mobilizing agents in hepatocytes are opposite to those observed in vitro using post mitochondrial supernatant.

The decrease in cytidylyltransferase release by Ca\(^{2+}\) and Ca\(^{2+}\) mobilizing agents upon digitonin treatment is largely (about two-thirds) due to translocation of the enzyme from the cytosolic compartment to the microsomal compartment. Verapamil can partially block the translocation indicating that intracellular Ca\(^{2+}\) was involved in translocation. Since the microsomal cytidylyltransferase is the active form of the enzyme, the above results indicate that Ca\(^{2+}\) and Ca\(^{2+}\) mobilizing agents can activate the cytidylyltransferase. This observation is opposite to that observed in vitro where Ca\(^{2+}\) caused an inhibition of cytidylyltransferase activity. The reason why we do not observe an inhibition by Ca\(^{2+}\) of cytidylyltransferase activity in hepatocytes is not known, possibly the microsomal membrane is altered in vitro but intact in hepatocytes and so the phospholipid sites may not be exposed to form bridges with Ca\(^{2+}\) and thus lead to inhibition of cytidylyltransferase.

**Ca\(^{2+}\)** stimulates PC synthesis (7. 4. 2. 1.).

Ca\(^{2+}\) caused an increase in PC synthesis within 10 min of treatment of hepatocytes. The increased labelling of PC was not due to differential uptake of choline label, but due to acceleration of the
cytidylyltransferase catalysed reaction as evidenced by the decrease in the label in both choline and phosphocholine.

Previous work of Graham et al (189) had shown inhibition of PC synthesis by verapamil (150 μM) and EGTA (2 mM). Verapamil, which blocked the Ca$^{2+}$ channels, produced a 49% decrease in [methyl-$^3$H]choline incorporation into PC, while EGTA, which chelates Ca$^{2+}$, produced a 32% decrease in incorporation into PC compared to controls. Our work supports their findings in that Ca$^{2+}$ can stimulate PC synthesis by activating the cytidylyltransferase catalysed reaction.

Our results showing activation of the cytidylyltransferase by Ca$^{2+}$ and Ca$^{2+}$ mobilizing agents, which in turn would mean stimulation of PC synthesis, are contrary to the finding of Tijburg et al (188) who showed a slight inhibition of PC synthesis by vasopressin. Since their procedure involved incubation of cells for longer time periods with the hormone compared to ours, the prolonged exposure may have resulted in a decrease in cytidylyltransferase activity leading to an inhibition in PC synthesis. However, our studies are in agreement with Pollard and Brindley (187) who showed a stimulation of oleate incorporation into total phospholipids. The reason why we examined the effects of Ca$^{2+}$ on cytidylyltransferase for short time periods only was because Ca$^{2+}$ is rapidly sequestered by living cells and so the effects are only transient.

Alemany et al (185) also showed an inhibition of PC synthesis by vasopressin. They reported that treatment of microsomes with low concentrations of Ca$^{2+}$ can decrease the $V_{\text{max}}$ of CDP-choline:1, 2-
diacylglycerol cholinephosphotransferase without affecting the $K_m$. However they did not examine the effect of Ca$^{2+}$ on the cytidylyltransferase. The stimulation of cytidylyltransferase in our experiments by vasopressin and ionophore, A23187, which would appear to be opposite to the findings of Alemany et al, may be the result of inclusion of 2 mM Ca$^{2+}$ in the medium during the incubations.

**Ca$^{2+}$ stimulates PC degradation (7. 4. 2. 2.).**

Ca$^{2+}$ caused an increase in the degradation of PC resulting in increased formation of LPC both in the cell and the medium. This indicated that Ca$^{2+}$ was stimulating a phospholipase A reaction. There was no significant increase in GPC formation indicating that lysophospholipase activity was not stimulated.

**Possible mechanism of Ca$^{2+}$-mediated stimulation of PC synthesis**

Since Ca$^{2+}$ stimulates the production of LPC, indicating activation of a phospholipase A activity, the other product of this reaction, fatty acid, has been shown to activate the cytidylyltransferase (63, 276). This may be the mechanism of how Ca$^{2+}$ is activating the cytidylyltransferase and PC synthesis in our cells. It may be postulated that first Ca$^{2+}$ activates phospholipase A activity leading to the production of LPC and fatty acid. The latter then activates the cytidylyltransferase by translocation to the microsomes which in turn results in stimulation of PC synthesis. At the present time there is no evidence to support or refute this hypothesis.
Another important effect of Ca$^{2+}$ includes activation of protein kinase C (190). This enzyme has also been shown to exist as a soluble and a membrane bound form. Phorbol esters, which have been shown to stimulate PC synthesis (208), can rapidly activate the protein kinase C by substituting for the endogenous activator, diglyceride (203). Whether protein kinase C plays a direct role in stimulation of the cytidylyltransferase and thus of PC synthesis, is not clear. Cook and Vance (222) reported that partially purified protein kinase C did not affect the cytidylyltransferase activity in vitro.

Ca$^{2+}$ is also responsible for the activation of Ca$^{2+}$-calmodulin dependent protein kinases (290). However, it has been reported previously that phosphorylating conditions involving cAMP activated protein kinases in vivo (75), and in vitro (74) led to an inhibition of the cytidylyltransferase and PC synthesis. It is possible that one set of kinases may inhibit cytidylyltransferase while another set of kinases may activate the cytidylyltransferase.

The stimulation of cytidylyltransferase and PC synthesis by Ca$^{2+}$ in our cells may be partially the result of Ca$^{2+}$ interacting with the plasma membrane and activating a regulatory protein which in turn leads to activation of cytidylyltransferase. This might explain why ionophore, A23187, and vasopressin, which both elevate Ca$^{2+}$ concentrations in the cell, cannot stimulate the cytidylyltransferase to the same extent as incubation with Ca$^{2+}$ alone. In addition, verapamil can only block partially the stimulation caused by Ca$^{2+}$ indicating that Ca$^{2+}$ may affect both intracellular and extracellular components which in turn can activate cytidylyltransferase.
Phospholipase $A_2$ decreases digitonin-mediated release of cytidylyltransferase from hepatocytes (7. 5. 1. 1.).

Incubation of hepatocytes in the presence of phospholipase $A_2$ led to a decrease in the amount of cytidylyltransferase released by digitonin treatment. Fractionation of the cells showed that treatment of cells with phospholipase $A_2$ led to a translocation of the enzyme from the cytosolic to the microsomal compartment. This effect was concentration and time dependent. Since one of the products of phospholipase $A_2$ reaction is free fatty acid, then production of this might stimulate cytidylyltransferase activity leading to translocation of the enzyme from the cytosol to the microsomes. Since it was also demonstrated in vitro that addition of free fatty acids can lead to translocation of the enzyme to the microsomes, treatment of cells with phospholipase $A_2$ confirms the idea that free fatty acids might regulate cytidylyltransferase activity in vivo. However, there is no evidence in support of this proposal.

Phospholipase $A_2$ stimulates PC synthesis (7. 5. 2. 1.)

Pulsing hepatocytes with [methyl-$^3$H]choline, after the cells had been pre-treated with phospholipase $A_2$, led to an increase in the label incorporated into PC. If however, the cells were pulsed and then chased in the presence of phospholipase $A_2$, the incorporation of the label into PC was insignificant compared to the label incorporated into LPC. This indicated that presence of phospholipase $A_2$ in the chase medium may be degrading the labelled PC to generate LPC.
The stimulation of cytidylyltransferase and PC synthesis by phospholipase A$_2$ compares well with the reported activation of cytidylyltransferase and PC synthesis by phospholipase C (64, 66, 67). This suggests, in agreement the idea postulated by Sleight and Kent (64, 66, 67), that degradation of PC by phospholipases may represent the signal to increase cytidylyltransferase activation and in turn PC synthesis. Thus, as the degradation of PC is increased, this is counterbalanced by an increase in PC synthesis which helps to maintain the PC pool at a steady level. Therefore, regulation of phospholipase activity \textit{in vivo} not only controls the degradation of PC but appears to control indirectly the rate of PC synthesis.
Although the cytidylyltransferase has been purified to homogeneity and characterized in vitro, a number of questions still remain unanswered. For instance, we still do not know whether the regulation of cytidylyltransferase activity by reversible phosphorylation, seen in vitro, is of physiological importance in vivo. To answer this question, a precipitating antibody to cytidylyltransferase is required, which we were unable to obtain. Therefore extensive efforts, using various techniques, should be made to obtain a precipitating antibody. Not only can this antibody be useful to determine whether the cytidylyltransferase is phosphorylated in vivo, but can also be used for purification of the enzyme. If cytidylyltransferase were phosphorylated in vivo, what type(s) of protein kinase catalyses the phosphorylation and what phosphoprotein phosphatase catalyses the dephosphorylation? The amino acid sequence of the cytidylyltransferase needs to be determined in order to identify which amino acid(s) is phosphorylated in vivo. Also it needs to be determined whether cytidylyltransferase phosphorylated at multiple sites and the presence of which hormone is responsible for phosphorylation at a particular site?

The native subunit structure of cytidylyltransferase needs to be determined in detail. Is the enzyme dimeric or tetrameric? Do all the enzyme subunits have catalytic activity or do some subunits have a regulatory role? Is phosphate incorporated into all the subunits?
The mechanism of the effects of Ca\(^{2+}\) needs to be identified. Is the regulation of PC synthesis by Ca\(^{2+}\) through stimulation of protein kinase C or phospholipase A\(_2\) activity? Are similar kinds of mechanisms utilized by Ca\(^{2+}\) mobilizing agents such as vasopressin and angiotensin II? The nature of the effects of free fatty acids on cytidylyltransferase activation needs also to be evaluated. Do free fatty acids alter configuration of the cytidylyltransferase or do they alter lipid arrangement which leads to stimulation of cytidylyltransferase activity?

Answers to these questions should enable the precise mechanism of regulation of cytidylyltransferase, and in turn of PC synthesis, to be identified.
REFERENCES


