REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN THE ISOLATED PERFUSED HAMSTER HEART

by

© Grant Michael Hatch

A thesis submitted to the Faculty of Graduate Studies

The University of Manitoba

In partial fulfillment of the requirements for the degree

Doctor of Philosophy

Department of Biochemistry, Faculty of Medicine

1988

ISBN 0-315-51604-6

REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN THE ISOLATED PERFUSED HAMSTER HEART

BY

GRANT MICHAEL HATCH

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Patrick C. Choy for both his guidance, patience, encouragement and above all his enthusiasm that he instilled in me. Pat, you are more than a friend.

I would also like to thank my co-workers and colleagues in the department of Biochemistry, University of Manitoba for their discussions and suggestions. Gilbert, Monika, Cheryl, Leona, Lore, and "the guys" Paul and Chris. A special thanks to Dr. Karmin O and Willem Stevens for the collaboration and Dr. Y-Z Cao for helpful discussions.

A special thanks to my supervisory committee, Dr. F.C. Stevens, Dr. J.M. Bowness, Dr. E.T. Pritchard and above all Dr. S.L. Pelech.

A most sincere thankyou to my two good friends Teresa and Tracy for encouragement and help with the statistics.

Finally, I would like to thank my mom and family for their love and faith, and Shellie for her love and encouragement.

To mom

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF ABBREVIATIONS

ACS	Aqueous counting scintillant
ADP	Adenosine-5'-diphosphate
ANSA	1-Amino-2-napthol-4-sulphonic acid
ATP	Adenosine-5'-triphosphate
C	Carbon
^{o}C	Degrees Celsius
Ca^{+2}	Calcium
cAMP	Adenosine-3',5'-monophosphate
$CaCl_2$	Calcium chloride
CDP	Cytidine-5'-diphosphate
$CHCl_3$	Chloroform
CH_3COOH	Acetic acid
CH_3OH	Methanol
$(C_2H_5)_2O$	Diethylether
Ci	Curie
CMP	Cytidine-5'-monophosphate
Co.	Company
CoA	Coenzyme A
Corp.	Corporation
CTP	Cytidine-5'-triphosphate
$CuSO_4$	Copper sulfate
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g	Gravitational force
GC	Gas chromatography

HCl	Hydrochloric acid
H_2O	Water
H_3PO_4	Phosphoric acid
H_2SO_4	Sulphuric acid
HPLC	High performance liquid chromatography
hr	Hour
K_2HPO_4	Potassium phosphate dibasic
KCl	Potassium chloride
KHB	Krebs-Henseleit buffer
KH_2PO_4	Potassium phosphate monobasic
Km	Michaelis-Menten constant
l	Litre
M	Molar
min	Minute
mCi	Millicurie
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
ml	Millilitre
mm	Millimetre
mM	Millimolar
N	Normal
N_2	Nitrogen
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4 $7H_2O$	Sodium phosphate dibasic heptahydrate

NaH_2PO_4	Sodium phosphate monobasic
NaOH	Sodium hydroxide
NCS	Ammonium base counting scintillant
NH_4HCO_3	Ammonium bicarbonate
NH_4OH	Ammonium hydroxide
nm	Nanometre
nmol	Nanomole
O_2	Oxygen
P	Statistical probability
Pi	Phosphate
PPi	Inorganic phosphate
R_f	Relative mobility
s	Second
TCA	Trichloroacetic acid
tlc	Thin-layer chromatography
Tris	Trizma base
µ	Micron
µCi	Microcurie
µl	Microlitre
µM	Micromolar
µmol	Micromole
unit	Amount of enzyme which catalyzes the conversion of 1 µmole substrate/min
U.V.	Ultraviolet
v	Volume
w	Weight

ABSTRACT

Phosphatidylcholine is the principle phospholipid in the mammalian heart and the majority of this phospholipid is synthesized via the CDP-choline pathway. There are at least three tiers of control of phosphatidylcholine biosynthesis via the CDP-choline pathway. However, detailed studies on these control mechanisms have not been conducted. The objective of this study was to investigate these three tiers of control of phosphatidylcholine biosynthesis using the isolated perfused hamster heart model.

Choline uptake by the isolated hamster heart has been shown to be inhibited by exogenous ethanolamine. In this study, the effect of amino acids on choline uptake was investigated. Hamster hearts were perfused with labeled choline, and in the presence of glycine, L-alanine, L-serine, L-phenylalanine ($\geq$0.1 mM), choline uptake was enhanced 20-38%. L-Arginine, L-lysine, L-glutamate and L-aspartate did not influence choline uptake. The rate of phosphatidylcholine biosynthesis was unaffected by all amino acids tested. Enhancement of choline uptake by neutral amino acids was not additive or dose dependent but required a concentration threshold. The enhancement of choline uptake by neutral amino acids was not influenced by prior perfusion with the same amino acid. Exogenous choline had no effect on the uptake of amino acids. We postulate that the neutral amino acids are not cotransported and modulation of choline uptake is facilitated by direct interaction of the neutral amino acids with the choline transport system.

In the hamster heart, the hydrolysis of phosphocholine to choline by a non-specific phosphatase has been suggested. We demonstrated that this phosphatase was located in the microsomal fraction. Unlike alkaline phosphatase, this phosphatase was uninhibited by amino acids. The pH optimum and heat sensitivity of the phosphocholine phosphatase was found to differ from alkaline phosphatase. Phosphocholine did not inhibit the hydrolysis of p-nitrophenylphosphate, but a "mixed-type" inhibition of the hydrolysis of phosphocholine was observed in the presence of p-nitrophenylphosphate. Our results clearly indicate that these two activities originate from separate and distinct enzymes.

Since ATP and CTP are required cofactors for the biosynthesis of phosphatidylcholine via the CDP-choline pathway, the effect of a lowered ATP and CTP levels on phosphatidylcholine biosynthesis in the hamster heart was investigated. Reduction of ATP and CTP levels was facilitated by perfusion of the hamster heart under hypoxic conditions. The heart was pulse-labeled with radioactive choline, then perfused for 30,45 and 60 min under hypoxic or normoxic conditions. ATP and CTP levels were significantly reduced at 60 min of hypoxic perfusion and the incorporation of radioactivity into phosphatidylcholine was also decreased. Analysis of the choline-containing metabolites of the CDP-choline pathway revealed that radioactivity had

accumulated in the phosphocholine fraction with a reduction in the CDP-choline fraction. However, the pool sizes of these choline-containing metabolites remained the same between the control and hypoxic hearts, and there was no difference in the phospholipid contents between these two groups. From the specific radioactivity of CDP-choline and the radioactivity incorporated into phosphatidylcholine, the rate of phosphatidylcholine synthesized was estimated to be 41.5 nmol/min/g for the control heart and 42.6 nmol/min/g for the hypoxic heart. The reduced incorporation of radioactivity into phosphatidylcholine in the hypoxic hearts was a direct result of the lowered specific radioactivity of CDP-choline. To further investigate the compensatory changes for maintaining phosphatidylcholine biosynthesis during hypoxia, the activities of the enzymes for the CDP-choline pathway were determined. Hypoxia caused an increase in the microsomal cytidylyltransferase activity with a corresponding decrease in the cytosolic enzyme activity. Interestingly, an accumulation of fatty acids in the cytosol was detected. It appears that the lowering of the CTP level during hypoxia resulted in a reduction in the labeling of CDP-choline and phosphatidylcholine. At the same time, the accumulation of fatty acids in the cytosol caused the redistribution of cytidylyltransferase from the cytosolic to the microsomal form. The translocation of the cytidylyltransferase to the microsomal form might be one of the compensatory mechanisms for the heart to sustain the

synthesis of a functional level of CDP-choline and thus maintain the rate of phosphatidylcholine biosynthesis.

INTRODUCTION

I. THE BIOLOGICAL MEMBRANE

Biological membranes contain a large and diverse variety of lipids. In the Singer and Nicholson fluid mosaic model, membrane lipids provide a fluid bilayer which allows rapid lateral diffusion of lipid and protein in the plane of the membrane (Figure 1) (1,2). The ability of membrane lipids (phospholipids) to assume basic bilayer organization is due to their amphipathic nature, which is characterized by the presence of both a polar (hydrophilic) head group region and a nonpolar (hydrophobic) tail region. The polar regions orient toward the aqueous phase while the nonpolar regions are oriented away from such phase.

In eukaryotic membranes the glycerol-based phospholipids are predominant, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin (2). Sphingosine lipids, including sphingolipids and the glycosphingolipids are also present as major fractions. Glycolipids, present in smaller quantities, are carbohydrate-containing glycerol-based lipids. Cholesterol, a major component of plasma membranes, may exist in equimolar amounts with phospholipids.

The inner and outer leaflets of membrane bilayers may exhibit different lipid compositions (2). A general feature of plasma membrane asymmetry is that the majority of

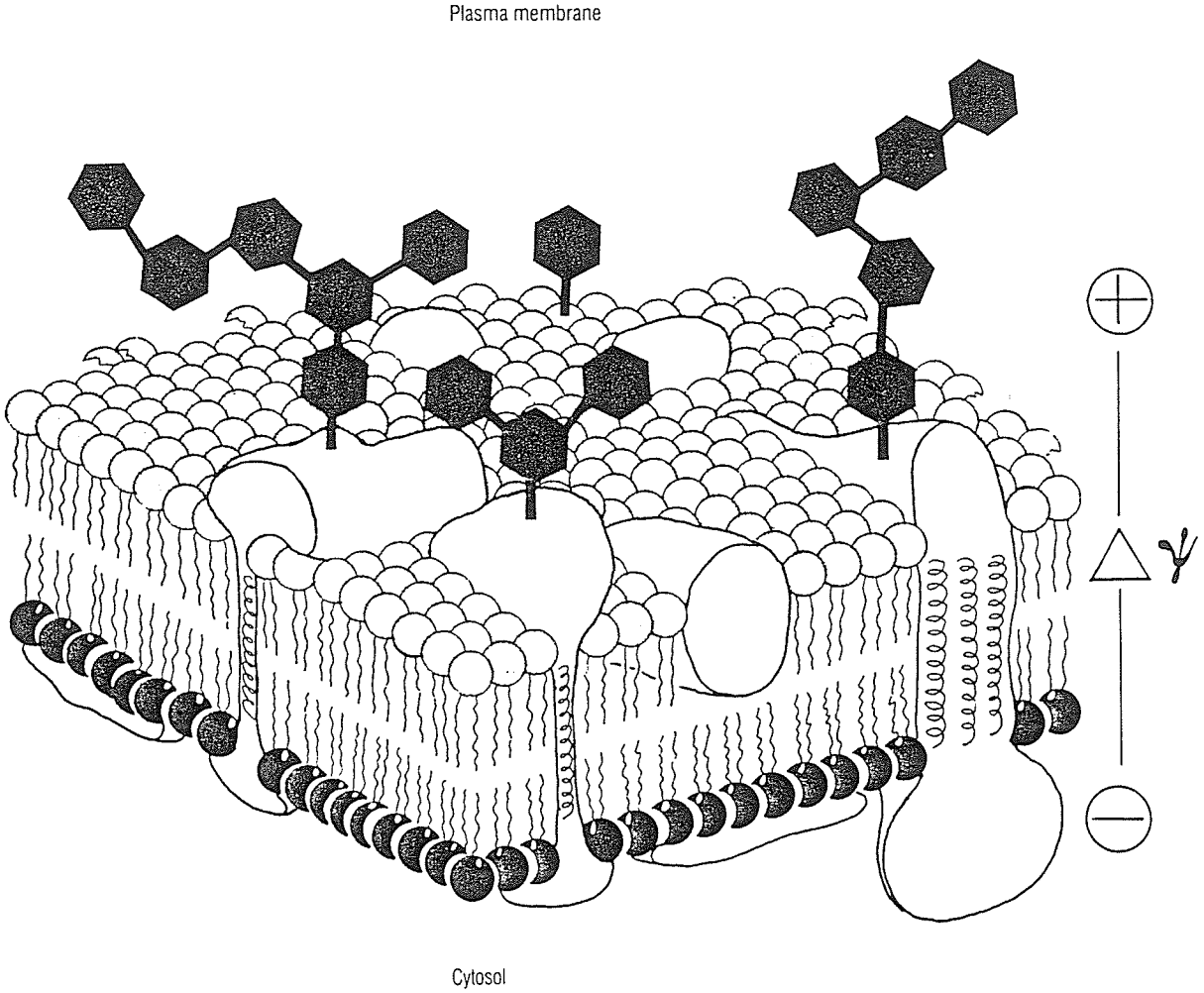

Figure 1 The plasma membrane

phospholipids that exhibit a net negative charge (phosphatidylserine and phosphatidylinositol) are found on the cytosolic half of the bilayer. Proteins appear to be involved in maintaining this asymmetry. The ability of membrane lipids to self-assemble into fluid bilayer structures is consistent with two major roles in membranes: establishing a permeability barrier and providing a matrix with which membrane proteins are associated (2).

II. PHYSICAL AND FUNCTIONAL ROLES OF PHOSPHATIDYLCHOLINE IN MEMBRANES

Three distinct types of choline containing glycerophosphatides have been identified (3). They are (i) diacyl-glycerophosphocholine, commonly known as phosphatidylcholine, (ii) alkenylacyl-glycerophosphocholine, and (iii) alkylacyl-glycerophosphocholine. Alkenylacyl-glycerophosphocholine, also known as plasmenylcholine or choline plasmalogen, exists in significant quantities only in some mammalian hearts and other electrically active organs. For example, 30-40% of total choline containing glycerophosphatides in human, guinea pig and rabbit hearts are composed of alkenylacyl-glycerophosphatide, but it is present in very low quantities in rat and hamster heart (4,5). Alkylacyl-glycerophosphocholine is found as a minor phospholipid component in all mammalian tissues.

Phosphatidylcholine is the major phospholipid in the

heart and most mammalian tissues, and it acts as an important structural component (6). Phosphatidylcholine is composed of a polar headgroup region and a glycerol backbone to which fatty acids are esterified (Figure 2) (7). The choline headgroup distinguishes phosphatidylcholines from other phospholipid molecules. The C-1 position of the glycerol backbone is usually esterified with a saturated fatty acid, while unsaturated fatty acids are esterified primarily at the C-2 position.

In addition to its role as a structural component of cellular membranes, phosphatidylcholine plays important functional roles (8). Phosphatidylcholine (i) serves as a major component of pulmonary surfactant (9) which prevents atelectasis of the lung (10), (ii) is a major source of arachidonic acid for prostaglandin biosynthesis (11), (iii) aids in bile solubilization (12), and (iv) is required for D-beta-hydroxybutyrate dehydrogenase activity (13).

III. PHOSPHATIDYLCHOLINE BIOSYNTHESIS

In mammalian tissues, several known pathways are responsible for the formation of phosphatidylcholine (14). The majority of phosphatidylcholine is formed via the CDP-choline pathway (Figure 3) (15). Alternatively, phosphatidylethanolamine is progressively methylated to phosphatidylcholine by the transfer of methyl groups from S-adenosylmethionine (16). This pathway contributes significantly to phosphatidylcholine formation in the liver

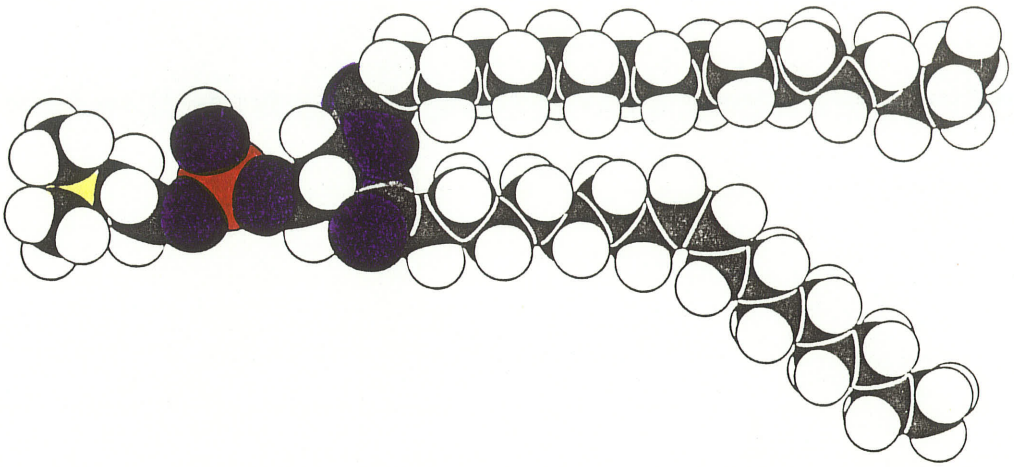

Figure 2 Space filling model of a phosphatidylcholine molecule

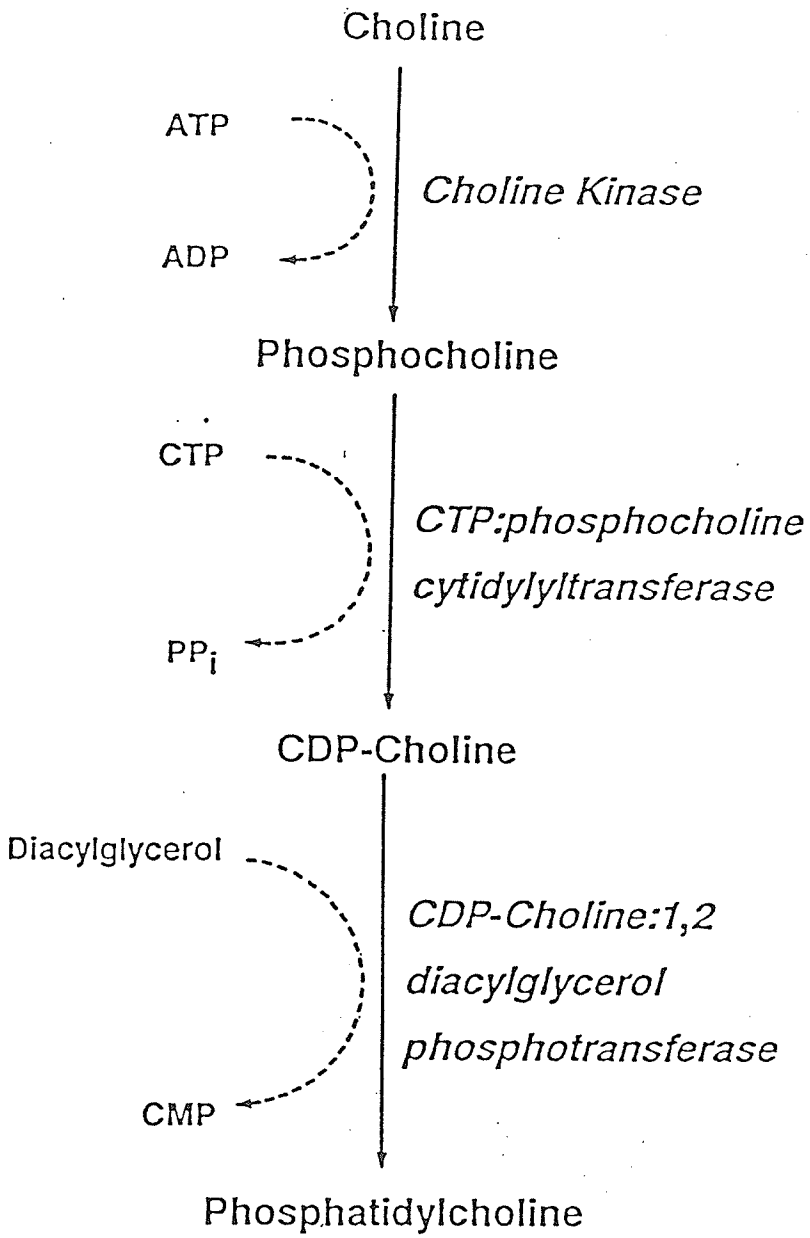

Figure 3 The CDP-choline pathway

(16), but it is completely absent in rat intestine (17). Another possible pathway is the Ca^{+2} mediated base exchange of choline for other phospholipid head groups (18). Once phosphatidylcholine is made, its acyl groups can be modified by a deacylation-reacylation process (19). This process may be important for the introduction of polyunsaturated fatty acids into phosphatidylcholine. Alternatively, the product of deacylation, lysophosphatidylcholine, can be condensed with another lysophosphatidylcholine molecule to form phosphatidylcholine and glycerophosphocholine (20).

a) The CDP-choline pathway

The CDP-choline pathway was first elucidated by Kennedy and his co-workers in the 1950's (Figure 3) (15). The first step of the pathway involves the phosphorylation of choline to phosphocholine catalyzed by the enzyme choline kinase. This reaction involves the utilization of the high energy compound ATP. Choline kinase is found exclusively in the cytosolic fraction of rat liver (21). In the second step of the pathway, phosphocholine is converted to CDP-choline at the expense of the high energy compound CTP. The enzyme responsible for the catalysis is CTP:phosphocholine cytidylyltransferase. Cytidylyltransferase is an ambiquitous enzyme (22) found in both the cytosolic and microsomal fractions of rat liver homogenate (21). Both cytosolic and microsomal forms are immunologically identical (23), and the cytosolic form has recently been purified (24). In the final step of

the pathway, 1,2-diacylglycerol:CDP-choline cholinephosphotransferase catalyzes the condensation of CDP-choline with 1,2-diacylglycerol to form phosphatidylcholine. This enzyme is located exclusively on the cytoplasmic side of the microsomal fraction (25). This reaction is readily reversible in rat liver (26). The *in vivo* formation of CDP-choline from phosphatidylcholine is slow compared to the formation of phosphatidylcholine from CDP-choline (27).

b) The methylation pathway

The progressive methylation of phosphatidylethanolamine, with S-adenosylmethionine as methyl group donor, was first demonstrated by Bremer and Greenberg (16). The reaction is catalyzed by a single microsomal enzyme phosphatidylethanolamine N-methyltransferase, recently purified to homogeneity (28). The pathway accounts for as much as 40% of the total phosphatidylcholine synthesized in rat liver (29).

It had been proposed that phospholipid methylation played an important functional role in modulating the microviscosity of cell membranes and some physiological responses (30). However, the enzyme activity in extrahepatic tissues is extremely low, and only minute amounts of phosphatidylethanolamine are methylated in membranes in which marked changes in microviscosity are observed (31). Hence, the functional role of phospholipid methylation remains an open question.

c) The base exchange pathway

Dils and Hubscher demonstrated a Ca^{+2}-mediated exchange reaction in which choline was exchanged with another phospholipid headgroup in rat liver microsomes (18). This pathway is the primary route for the biosynthesis of phosphatidylserine in the liver (32). Choline, ethanolamine and serine exchange with phosphatidylserine and phosphatidylcholine while serine and ethanolamine exchange with phosphatidylethanolamine (32). The exchange reactions are presumably catalyzed by different enzymes (33,34).

d) Resynthesis pathway

Phosphatidylcholine can be remodelled by a deacylation-reacylation cycle first proposed by Lands (19). One of the acyl group linkages in phosphatidylcholine may be hydrolyzed by phospholipase A. The resulting lysophospholipid is reacylated back to the parent molecule upon transfer of an acyl group from acyl-CoA to the lysophosphatidylcholine. The transfer of an acyl group from acyl-CoA:1-acylglycerophosphocholine is catalyzed by acyl:1-acylglycerophosphocholine acyltransferase, while transfer of acyl groups from acyl-CoA to 2-acyl-CoA:2-acylglycerophosphocholine is catalyzed by acyl-CoA:2-acyl-glycerophosphocholine acyltransferase. These two microsomal enzymes appear to be distinct from each other (35). Deacylation-reacylation is thought to be a facile mechanism

to obtain "tailor-made" phosphatidylcholine with the appropriate acyl groups (19,36,37). This process appears to be important in many tissues but is extremely essential in lung where dipalmitoylphosphatidylcholine accounts for greater than 70% of the phospholipid species (38).

e) Transacylation pathway

Marinetti (20) first demonstrated the formation of phosphatidylcholine by transacylation of 2 molecules of lysophosphatidylcholine. The reaction is catalyzed by lysophosphatidylcholine:lysophosphatidylcholine transacylase in the cytosolic fraction (39). Although such a reaction has been shown to occur, its importance in mammalian tissues apart from lung has yet to be shown (40).

IV. BIOSYNTHESIS OF NEW PHOSPHATIDYLCHOLINE IN THE HAMSTER HEART

Choline is the common precursor for phosphatidylcholine formation via the CDP-choline pathway and the base exchange pathway. The contribution of these two pathways have been evaluated in the isolated hamster heart (41). The heart was perfused with labeled choline, and total uptake of choline by the hearts was found to be linear from 5-60 min of perfusion and with 0.1-50 uM choline. Analysis of the lipid extract after perfusion clearly showed that the majority of radioactivity was located in the phosphatidylcholine fraction. A distinct lag in the incorporation of label into

phosphatidylcholine was observed during the first 15 min of perfusion. Since the specific radioactivity of labeled choline in the heart was constant throughout the perfusion, the lag period suggested that the majority of the labeled phosphatidylcholine was not formed by the base exchange reaction. Analysis of the choline-containing metabolites of the aqueous fraction revealed that greater than 70% of accumulated labeled choline was associated with the phosphocholine fraction, with the remainder in the choline and CDP-choline fractions. Very little radioactivity was detected in betaine, indicating that choline oxidation is only a minor pathway for choline metabolism and the majority of choline taken up is committed to phosphatidylcholine biosynthesis via the CDP-choline pathway. From the average specific radioactivity of CDP-choline at 30 and 60 min of perfusion, the rate of new phosphatidylcholine formation via the CDP-choline pathway in the hamster heart was estimated to be 39 nmol/min/g wet weight (41).

The contribution of phosphatidylcholine formation by the methylation of phosphatidylethanolamine was studied by perfusing the isolated hamster heart with labeled ethanolamine (41). The radioactivities incorporated into phosphatidylethanolamine and phosphatidylcholine at different periods of perfusion were determined. From the average specific radioactivity of phosphatidylethanolamine at 30-60 min of perfusion, the rate of phosphatidylcholine formed via progressive methylation was estimated to be 1.0

nmol/min/g wet weight. Thus, the methylation pathway appears to have only a minor contribution to new phosphatidylcholine biosynthesis in the hamster heart (41).

V. CONTROL OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS VIA THE CDP-CHOLINE PATHWAY

At least three tiers of control of phosphatidylcholine biosynthesis via the CDP-choline pathway have been identified in hamster heart: (a) at the level of choline uptake, (b) the energy status of the organ and (c) modulation of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase.

a) Choline uptake

Choline was discovered in 1862 by Strecker (42) and its structure elucidated in 1866 by Baeyer (43). However, it was not until 1932 that Best and Huntsman (44) demonstrated choline to be an essential ingredient in the diet. It was thought that choline was an essential vitamin until Wilson *et al* in 1960 (45) described the enzymatic synthesis of choline from ethanolamine in rat liver. However, enzymatic synthesis of choline is probably of little significance *in vivo*, since choline deficiency can be induced in a number of animal species (46). By far the majority of choline is supplied from the diet (44). The liver is the chief organ for regulating the plasma concentration of choline. The liver attempts to maintain a constant supply of choline in

the plasma (10-20 μM) (47,48) and only fails to do so under conditions of extreme starvation (49).

A distinct transport system for choline was first described in rat kidney by Sung and Johnstone (50). Choline is taken up by the isolated perfused rat liver by both saturable and non-saturable components (47). The apparent Km for choline uptake for the saturable component was calculated to be 170 μM. The non-saturable component was attributed to simple diffusion of choline across the plasma membrane. The demonstration of choline uptake by mammalian heart is well documented (5,41,51-53). In the hamster heart, choline is taken up by a saturable mechanism with a Km of 0.1 mM (41).

Dietary studies have shown that phosphatidylcholine biosynthesis is affected by the supply of choline (54,55). Similar results have been obtained from Ehrlich-Lettre-Ascites tumor cells (56) and Novikoff Hepatoma cells (57). In these cell lines, when the choline concentration in the medium was limiting, there was a lowered conversion of choline to phosphocholine and subsequently into phosphatidylcholine . However, at concentrations of choline greater than the calculated Km's for choline uptake, the rate of phosphatidylcholine biosynthesis was independent of the choline concentration in the medium (56,57). In these two cell lines, choline uptake appeared to be rate-limiting for phosphatidylcholine biosynthesis. Inhibition of the specific

component of choline uptake has been demonstrated in chlorophenylthio-cAMP treated rat hepatocytes (58). In these cells, choline uptake and phosphatidylcholine biosynthesis were reduced when cells were treated with the cAMP analogue for 1.5-15 hr. The inhibition of choline uptake by the cAMP analogue was independent of choline concentration in the medium.

Although choline uptake has been shown to be inhibited by by a number of compounds including hemicholinium-3 (59) and chlorocholine (56), very limited information is available regarding the in vivo regulation of such uptake by metabolic inhibitors. Ethanolamine is structurally similar to choline, and it is present in hamster plasma in significant quantities (0.9 mM) (60). The effect of ethanolamine on choline uptake and phosphatidylcholine biosynthesis in hamster heart has been investigated (61). When the isolated hamster heart was perfused with labeled choline in the presence of 0.1-0.5 mM ethanolamine, incorporation of label into phosphatidylcholine was decreased 26-63%. Ethanolamine was found to inhibit the uptake of choline in a competitive manner, but it did not affect the conversion of choline to phosphocholine or the biosynthesis of phosphatidylcholine. Inhibition of choline uptake by ethanolamine for up to 60 min in the isolated heart did not cause any significant change in the intracellular choline pool size. It appeared that the intracellular choline concentration was conserved

by recycling, and the maintenance of the choline pool would enable the heart to sustain the rate of phosphatidylcholine biosynthesis, at least for short periods. However, it is not known whether the intracellular choline level can be sustained throughout an extended period of low choline uptake. The fact that a small amount of choline was released by the isolated heart during perfusion (41) suggests that the intracellular choline pool may eventually be depleted over a prolonged period of low choline uptake. The source of this released choline might originate from phosphocholine that was hydrolyzed by an alkaline (phosphocholine) phosphatase present in the heart (41,62). These findings imply that phosphatidylcholine biosynthesis in the heart may be affected if choline uptake is limited for prolonged periods of time.

b) The energy status of the heart

Both ATP and CTP are required co-factors in the CDP-choline pathway, and the availability of these high energy compounds may affect the rate of phosphatidylcholine biosynthesis (14). An increase in cytoplasmic CTP in polio-infected Hela cells was shown to cause an enhancement of phosphatidylcholine biosynthesis in these cells, but the increase in ATP level did not affect the phosphorylation of choline (63). In 3-deaza-adenosine-treated rats, enhancement of phosphatidylcholine formation was accompanied by an increase in the hepatic concentration of CTP (64). A

pathological model was employed to examine the effects of ATP and CTP levels on phosphatidylcholine biosynthesis in the hamster heart (65). Through autosomal recessive inheritance, cardiomyopathy develops spontaneously in a strain of inbred Syrian hamsters (BIO 14.6 strain) in which the myocardium exhibits degenerative lesions with 100% incidence (66). With the 150-200 day-old myopathic animals, a 34% decrease of the ATP and CTP concentrations in the hearts was observed (65). When the myopathic hearts were perfused with labeled choline for 60 min, a 22% increase in the incorporation of radioactivity into phosphatidylcholine was observed when compared to controls. Inspite of the increase in the labeling of phosphatidylcholine in the myopathic heart, the total cardiac phosphatidylcholine level remained unchanged throughout the development of the disease.

To elucidate the cause of the increase in labeling of phosphatidylcholine during cardiomyopathy, the activities of the enzymes involved in phosphatidylcholine formation in myopathic hamster hearts were assayed and compared with normal hearts (65). There were no detectable changes in the methylation of phosphatidylethanolamine or base exchange reaction, but the CTP:phosphocholine cytidylyltransferase activity was elevated. Analysis of the metabolites in the CDP-choline pathway revealed that the labeling and pool size of choline and phosphocholine were unchanged, but the labeling and pool size of CDP-choline in the myopathic

hearts were decreased by 28% and 40%, respectively. This uneven distribution caused a 20% increase in the specific radioactivity of CDP-choline. Since CDP-choline is the immediate precursor for phosphatidylcholine formation, it was concluded that the increase in labeling of phosphatidylcholine in the myopathic heart was a direct reflection of the higher specific radioactivity of CDP-choline. Based on the specific radioactivity and the labeling of phosphatidylcholine, the net amount of phosphatidylcholine synthesized in the myopathic hamster heart was estimated. The rate of phosphatidylcholine synthesis in the myopathic heart was not different from the normal heart, and the reduction in CDP-choline formation in the myopathic heart was postulated to be caused by a decrease in CTP concentration during the development of cardiomyopathy. The precise biochemical mechanism for the activation of the cytidylyltransferase in this study was unknown. However, the activation of cytidylyltransferase was postulated to be one of the compensatory mechanisms for the myopathic heart to maintain a minimum CDP-choline level to prevent any reduction of net phosphatidylcholine biosynthesis. This study suggested that the intracellular CTP concentration is an important factor for the maintenance of phosphatidylcholine biosynthesis via the CDP-choline pathway in hamster heart (65).

c) Modulation of CTP:Phosphocholine cytidylyltransferase

The rate-limiting role of cytidylyltransferase in the CDP-choline pathway has been identified in the liver, lung, brain and other mammalian tissues (67). In rat liver, this is supported by the fact that the pool size of choline is substantially lower than phosphocholine (27,68). Such pool sizes imply a rapid conversion of choline to phosphocholine. In addition, the phosphocholine pool is substantially higher than the CDP-choline pool (27). However, more definitive proof was obtained from pulse-chase studies (41,68). Isolated hamster hearts were perfused with labeled choline for 5 min and subsequently perfused with non-labeled choline for 1-60 min (41). The radioactivities in choline, phosphocholine, CDP-choline and phosphatidylcholine at different periods of the chase were determined. The labelings of choline and CDP-choline were low and remained unchanged at all time points of the chase. The labeling of phosphocholine was much higher and maximum labeling was reached immediately after the pulse. As the radioactivity in phosphocholine decreased during the chase, an increase in phosphatidylcholine labeling was observed. The results indicated that the rate-limiting step occurs at the conversion of phosphocholine to CDP-choline, catalyzed by CTP:phosphocholine cytidylyltransferase.

As previously mentioned, CTP:phosphocholine cytidylyltransferase is located in both cytosolic and microsomal

fractions in mammalian tissues (21). The microsomal cytidylyltransferase is regarded as the active form of the enzyme and the cytosolic cytidylyltransferase a storage form (64,69-71). The translocation of the enzyme between these two subcellular compartments represents a plausible mechanism for the regulation of its activity and thus phosphatidylcholine biosynthesis (72). Evidence for the translocation of cytidylyltransferase has been obtained in rat liver (73), rat hepatocytes (74), HeLa cells (75,76), rat lung (77), chick embryonic myoblasts (78-80), and in the lung (81) and liver (71) of fetal rats.

Two mechanisms have been identified for the promotion of translocation of cytidylyltransferase. In the presence of exogenous long chain fatty acids, phosphatidylcholine biosynthesis was stimulated with a concomitant increase in the conversion of phosphocholine to CDP-choline (74). Cytidylyltransferase activity in the microsomal fraction was increased with a corresponding decrease of enzyme activity in the cytosolic fraction. It was postulated that fatty acids promoted the binding of the cytosolic enzyme to the microsomal membrane where the enzyme could be activated by membrane phospholipids (82,83). Another mechanism for enzyme translocation is the reversible phosphorylation-dephosphorylation mechanism (84), where the phosphorylated enzyme is the less active form. In rat hepatocytes, the addition of glucagon, phosphodiesterase inhibitors and cAMP analogues inhibited phosphatidylcholine biosynthesis (85).

Rat liver cytosolic enzyme activity was elevated 5-fold when treated with cAMP-dependent protein kinase inhibitors. The phosphorylation-dephosphorylation of cytidylyltransferase as a mechanism for the translocation of the enzyme is an attractive hypothesis. However, there is only limited evidence that the dephosphorylated form (more active form) of the enzyme is actually associated with the microsomal fraction. The phosphorylated sites on the enzyme have not yet been identified.

There is no direct evidence that the heart cytidylyltransferase is regulated by translocation. Nevertheless, the importance of the microsomal form of the enzyme in the regulation of phosphatidylcholine biosynthesis has been demonstrated. When isolated hamster hearts were perfused with exogenous stearic acid, phosphatidylcholine biosynthesis was stimulated, and such stimulation was attributed to a corresponding increase in the activity of the microsomal cytidylyltransferase (86). However, no translocation of cytidylyltransferase from the cytosolic to the microsomal fraction was detected. In addition, there are some major differences in the regulation of phosphatidylcholine biosynthesis by fatty acids between the liver and heart. Only stearic acid was found to be effective in hamster hearts (86), whereas in rat hepatocytes (74), stimulatory effects were produced by all fatty acids examined. The stimulation of phosphatidylcholine

biosynthesis by stearic acid in the hamster heart appeared to be dependent on the intracellular accumulation of the fatty acid to a critical level to produce effective modulation of cytidylyltransferase activity (86). In rat hepatocytes, activation of cytidylyltransferase can be duplicated *in vitro* by the addition of free fatty acids into the assay mixture (74). Such direct activation of enzyme activity was not observed with the cytidylyltransferase from the hamster heart (86). The inability of stearic acid to activate hamster heart cytidylyltransferase *in vitro* suggests that modulation of enzyme activity by fatty acid is not the same between the heart and the liver.

VI. RESEARCH AIMS

Phosphatidylcholine is the major phospholipid in the mammalian heart (6). However, the regulation of its biosynthesis via the CDP-choline pathway is largely unknown. The overall objective of this study was to identify regulatory mechanisms involved in phosphatidylcholine biosynthesis via the CDP-choline pathway in hamster heart. Two separate experimental approaches were used to elucidate these mechanisms.

The regulation of choline uptake may be a plausible mechanism for the control of phosphatidylcholine biosynthesis in the heart. Choline uptake in the isolated perfused hamster heart was shown to be inhibited in a competitive fashion by ethanolamine (61). Initially the effect of glycine on choline uptake and phosphatidylcholine biosynthesis in the isolated perfused hamster heart was investigated. Glycine was chosen since it is structurally similar to ethanolamine and is found in significant quantities in hamster plasma (87). Because of its structural analogy to ethanolamine, glycine was postulated to inhibit choline uptake in a similar manner. Subsequently, the effect of other neutral, basic and acidic amino acids on choline uptake and phosphatidylcholine biosynthesis were examined.

The requirement of ATP and CTP in the CDP-choline pathway implied that the availability of these high energy compounds may affect the rate of phosphatidylcholine biosynthesis

(14). A decrease in ATP and CTP levels were observed in the hearts of 150-200 day old myopathic hamsters (65). The reduction in the level of ATP did not affect the phosphorylation of choline, but the diminished level of CTP caused a decrease in the conversion of labeled phosphocholine to CDP-choline. To prevent reduction of net phosphatidylcholine biosynthesis, CTP:phosphocholine cytidylyltransferase activity was elevated 2-fold as a compensatory mechanism for maintaining a funtional pool of CDP-choline. At present the mechanism for triggering the increase in cytidylyltransferase is undefined. Since the decrease in CTP level in the myopathic heart was instilled over a prolonged period, it was not clear whether the increase in cytidylyltransferase activity could be triggered by a rapid reduction of ATP and CTP levels. The isolated heart perfused under hypoxic conditions is an established model for decreasing the overall oxygen delivery to below the critical level required to support the metabolic ATP demands of the tissue (88,89). Since ATP is a required precursor for CTP biosynthesis, a decrease in cardiac ATP may also cause a reduction in cardiac CTP level. Thus, the effect of hypoxia on phosphatidylcholine biosynthesis in the isolated perfused hamster heart was investigated.

Examination of phosphatidylcholine biosynthesis under conditions which influence choline uptake and the energy status of the heart will lead to an understanding of how the

biosynthesis of this important structural and functional component of the myocardial membrane is regulated.

EXPERIMENTAL PROCEDURES

MATERIALS

I Experimental Animals

Syrian Golden hamsters (85-115 g, body weight) were used throughout the study. Hamsters were maintained on Purina Hamster Chow and tap water, ad libitium, in a temperature- and light-controlled room.

II Chemicals, Enzymes and Radioisotopes

Aqueous counting scintillant was obtained from Amersham Corp. Dowex AG1-X8 exchange resin (chloride form) was obtained from Bio-Rad Laboratories. Silica G25 thin-layer chromatography plates were obtained from Brinkman. Sucrose, ATP, CTP, choline chloride, phosphocholine, CDP-choline, bovine serum albumin, butylated hydroxy toluene, tetraphenylboron, Trizma base (reagent grade) and activated charcoal were obtained from Sigma Chemical Co. Phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, cardiolipin and sphingomyelin were obtained from Serdary Research Laboratories. 3-Heptanone and 2',7'-dichlorofluorescein were obtained from Eastman Kodak Co. Celite 545 (AW) and BF-3 Methanol kit were obtained from Supelco Inc. Fatty acid test kit was obtained from Boehringer-Mannheim. Activated charcoal was obtained from Sigma Chemical Co. All other chemicals were of reagent grade and obtained from either

Sigma Chemical Co. or Fisher Scientific Co. All solutions were prepared with distilled water and adjusted to the desired pH.

Alkaline phosphatase (Type IIIs), phosphodiesterase (Type II) and choline kinase were obtained from Sigma Chemical Co.

[Methyl-^{3}H]choline, CDP-[Methyl-^{14}C]choline, [γ-^{32}P]ATP, [5-^{3}H]Cytidine-5'-triphosphate, phospho[Methyl-^{14}C]choline and [5-^{3}H]cytidine-5'-triphosphate were obtained from New England Nuclear. Phospho[Methyl-^{3}H]choline was synthesized enzymatically from [Methyl-^{3}H]choline by the procedure of Paddon and Vance (90).

METHODS

A. Amino Acid Perfusion Experiments and Characterization Of Alkaline Phosphatase And Phosphocholine Phosphatase

I. Perfusion Of The Isolated Hamster Heart

Hamsters were sacrificed by decapitation, and the heart excised, cleaned of extraneous tissue and washed with Krebs-Henseleit buffer (91). Buffer was prepared by combining 100 ml solution A, 10 ml solution B, 5 ml solution C and distilled H_2O to a volume of 1 litre. Solution A contained: 70.1 g/l NaCl, 21 g/l $NaHCO_3$ and 9.91 g/l dextrose. Solution B contained 3.55 g/100 ml KCl 2.94 g/100 ml $MgSO_4$ and 1.63 g/100 ml NaH_2PO_4. Solution C contained 3.73 g/100 ml $CaCl_2$. All solutions were stored separately at 4^oC. The heart was cannulated via the aorta and perfused in the Langendorff mode (92). All perfusions were performed at 37^oC with a flow rate of 3.0 ml/min. The heart was perfused with KHB for 10 min to allow the heart to stabilize and restore regular rhythm. The heart was then perfused with radioactive or non-radioactive compounds for various intervals. Electrocardiac recordings were obtained by placing one electrode on the aortic cannula and the other in the solution bathing the heart. This allowed for the assessment of both ventricular and atrial activities simultaneously. The viability of the heart under these conditions could be maintained for at least 4 hr of perfusion (93). Subsequent to perfusion, 10 ml of KHB was forced through the heart to remove the residual

radioactive or non-radioactive compounds in the vessels. The heart was cut open, blotted dry and wet weight determined.

II. Subcellular Fractionation Of The Heart

Hamster hearts were perfused for 60 min with KHB. Subsequent to perfusion, a 20% heart homogenate was prepared in 0.145 M NaCl-5 mM Tris-HCl (pH 7.4) buffer. Homogenization for 15 s was performed twice using a Polytron homogenizer (Brinkmann PT10/35). The homogenate was centrifuged at 10,000 x g for 15 min (Sorvall RC-5 Superspeed Refrigerated Centrifuge with SS34 rotor). The pellet was discarded and the post-mitochondrial supernatant was centrifuged at 105,000 x g for 60 min (Beckmann Ultracentrifuge with Ti 60 rotor). The resulting supernatant was designated the cytosolic fraction. The pellet was resuspended in 5 ml buffer and homogenized in a Dounce homogenizer (Type B). The homogenate was centrifuged at 105,000 x g for 60 min. The supernatant was discarded and the pellet was resuspended in 1 ml buffer and homogenized in a Dounce homogenizer (Type A). The resulting suspension was designated the microsomal fraction.

For the studies in which alkaline phosphatase and phosphocholine phosphatase were characterized, a 10% heart homogenate was prepared in 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4). The homogenate was centrifuged 1,000 x g for 10 min, and the resulting supernatant was centrifuged at

12,500 x g for 15 min. The pellet was resuspended in 5 ml buffer B (0.15 M Tris-HCl, pH 8.0) and homogenized in a Dounce homogenizer (Type B). The suspension was recentrifuged at 12,500 x g for 15 min. The pellet was resuspended in 3 ml buffer A (0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and homogenized in a Dounce homogenizer (Type A). The homogenate was designated the mitochondrial fraction. The supernatant from the first centrifugation (designated the post-mitochondrial supernatant) at 12,500 x g was centrifuged at 105,000 x g for 60 min. The supernatant was discarded and the pellet resuspended in 5 ml buffer B and homogenized in a Dounce homogenizer (Type B). The homogenate was centrifuged at 105,000 x g for 60 min and the resulting pellet resuspended in 1 ml buffer A and homogenized in a Dounce homogenizer (Type A). The resulting suspension was designated the microsomal fraction.

III. Isolation Of Radioactive Choline-containing Metabolites

Hamster hearts were perfused for 15,30,45 and 60 min with 10 ml KHB containing 0.01 mM [Methyl-^{3}H]choline (5 µCi/ml) in the absence and presence of amino acid. Subsequent to perfusion, hearts were homogenized in 20 ml $CHCl_3$:CH_3OH (1:1 ; v/v), and 20 ul was taken for determination of total uptake of labeled choline. The homogenate was placed in a round bottom flask to which was added $CHCl_3$ and 0.05 M KCl until a final ratio $CHCl_3$:CH_3OH:0.05 M KCl of 4:2:3 (v/v/v)

was obtained. This allowed for separation into organic and aqueous phases. The solvent in the aqueous phase was evaporated under reduced pressure at 37°C and resuspended in 1 ml water. A 25 µl aliquot was applied onto a tlc plate and Choline, phosphocholine, CDP-choline fractions were separated in a solvent system containing CH_3OH:0.6% NaCl:NH_4OH (50:50:5 ; v/v/v). CDP-choline was visualized under ultraviolet light and choline and phosphocholine were visualized by exposure to iodine vapor. Gel containing choline, phosphocholine and CDP-choline was removed from the plate and placed in a scintillation vial to which was added 100 µl acetic acid and 10 ml of ACS. The radioactivity was determined using channels' ratio calibration method in a scintillation counter (LKB Model 1211). The solvent in the organic phase was evaporated under reduced pressure and resuspended in 1 ml $CHCl_3$:CH_3OH (2:1 ; v/v). An aliquot (50 µl) was placed on a tlc plate and phosphatidylcholine was separated from other phospholipids in a solvent system containing $CHCl_3$:CH_3OH:H_2O:NH_4OH (70:30:4:1 ; v/v/v/v). Phosphatidylcholine migration was determined using a standard and was visualized with iodine vapor. Gel containing phosphatidylcholine was removed from the plate and the radioactivity determined.

IV. Enzyme Assays

All test tubes used in all assays were treated with dimethyldichlorosilane (2% in 1,1,1-trichloroethane)

followed by heating in an oven at 110°C. The treatment prevents reagents and solutions from adhering to the tubes.

Alkaline phosphatase activity in the microsomal fraction was determined by the procedure of Cox and Griffin (94). The assay mixture contained 0.4 M Tris-HCl, (pH 10), 1 mM $MgCl_2$, 10 mM p-nitrophenylphosphate and 0.02 mg of microsomal protein in a total volume of 0.5 ml. The reaction mixture was incubated at 37° C for 30 min, and the reaction was terminated by the addition of 1.0 ml 0.25 N NaOH. The amount of p-nitrophenol formed was determined by absorbance at 410 nm, with the aid of a p-nitrophenol standard. The reaction mixture without the addition of microsomal fraction was used as control. The reaction was linear for at least 40 min.

Phosphocholine phosphatase activity was determined by the formation of [Methyl-^{3}H]choline from phospho[Methyl-^{3}H]choline (90). The reaction mixture contained 0.1 M Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 10 mM phospho[Methyl-^{3}H]choline and 0.06 mg microsomal protein in a total volume of 0.1 ml. The reaction mixture was incubated at 37°C for 30 min, and the reaction terminated by boiling for 2 min. The reaction mixture without the addition of microsomal fraction was used as control. After termination of the reaction, 1 ml of H_2O was added to the assay mixture, and the entire reaction mixture was applied to a AG1-X8 anion exchange column (1.0 ml bed volume). [Methyl-^{3}H]choline in the reaction mixture was eluted from the column by the addition of 10 ml H_2O and

the radioactivity in a 2 ml aliquot of the eluant was determined. The reaction was linear for at least 40 min.

Choline kinase in the cytosolic fraction (21) was assayed with [Methyl-^{3}H]choline as described by Weinhold and Rethy (95). The reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, 0.5 mM [Methyl-^{3}H]choline, cytosol and H_2O in a final volume of 200 µl. The reaction mixture was incubated at 37^{o}C for 20 min and terminated by boiling the tubes for 3 min. The reaction mixture was then centrifuged at 2,000 rpm for 10 min in a bench top centrifuge (Beckman Model TJ6). An aliquot (100 µl) of the resulting supernatant was placed on a tlc plate and phosphocholine was separated from choline in a solvent system containing CH_3OH:0.6 % NaCl:NH_4OH (50:50:5 ; v/v/v). The phosphocholine fraction was visualized with iodine vapor, removed and the radioactivity determined. The reaction was linear for at least 30 min.

Cholinephosphotransferase in the microsomal fraction (96) was assayed by the method of Arthur and Choy (93). The reaction mixture contained 0.1 M Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 5 mM EDTA, 0.2 uM diacylglyceride (prepared in 0.015% TWEEN-20, sonicated for 30 s), 0.4 mM CDP[Methyl-^{14}C]choline, microsomal fraction and H_2O in a final volume of 1 ml. The reaction mixture was incubated at 37^{o}C for 15 min and terminated by the addition of 2 ml of $CHCl_3$:CH_3OH (1:1 ; v/v). Phase separation was caused by the addition of

1 ml $CHCl_3$ and 0.5 ml H_2O. The reaction mixture was then centrifuged at 2,000 rpm for 10 min. The aqueous phase was removed and the organic phase washed twice with 2 ml 40% CH_3OH in H_2O. The resulting organic phase was placed in a scintillation vial and the solvent evaporated under a stream of nitrogen for radioactivity determination. The reaction was linear for at least 20 min.

CTP:phosphocholine cytidylyltransferase in both the microsomal and cytosolic fractions (21) was determined by the method of Choy et al. (96). Assays were performed exactly 4 hr after tissue homogenization, since cytosolic cytidylyltransferase aggregates after prolonged storage (96). The reaction mixture contained 0.012 M magnesium acetate, 0.1 M Tris-succinate (pH 8.0), 2.5 mM CTP, 1.0 mM phospho[Methyl-^{3}H]choline, heart cytosolic or microsomal fraction and H_20 in a final volume of 100 µl. The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by boiling the tubes for 3 min. The reaction mixture was centrifuged at 2,000 rpm for 10 min and 50 µl of the resulting supernatant applied to a tlc plate. CDP-choline was separated from phosphocholine by a solvent system containing CH_3OH:0.6% NaCl:NH_4OH (50:50:5 ; v/v/v). The CDP-choline fraction was visualized under ultraviolet light and the gel containing CDP-choline removed and the radioactivity in CDP-choline determined.

V. Radioactive Amino Acid Incorporation Into Hearts

Hamster hearts were perfused for 60 min with 10 ml KHB containing 0.01 mM [2-^{3}H]glycine or 1.0 mM L-[3-^{3}H]alanine in the absence and presence of 0.01 mM choline. The total uptake of radioactive amino acid and incorporation into protein was determined as described by Inui and Ishioku (97). Subsequent to perfusion, the hearts were homogenized in 2 ml distilled H_2O and a 100 µl aliquot taken for determination of total uptake of radioactivity. NCS tissue solubilizer was added to facilitate digestion of the homogenate. Protein in the homogenate was precipitated by the addition of 3 ml of 14% TCA. The precipitate was sedimented by centrifugation at 2,000 rpm for 10 min. The acid-soluble fraction was removed, and the pellet was washed twice with 3 ml of 7% TCA and twice with C_2H_5OH:$[C_2H_5]_2O$:$CHCl_3$ (2:2:1 ; v/v/v) and then dissolved in 0.05 M NaOH. A 0.5 ml aliquot was taken for determination of radioactivity and a 5 µl aliquot taken for protein determination.

VI. Enzymatic Synthesis Of Phosphocholine

One unit of choline kinase was dissolved in 5.0 ml H_2O (pH 8.0) and placed in a conical Amicon filter (CF-25). The solution was centrifuged at 2,000 rpm for 40 min in a Sorvall Model RC-5 high speed centrifuge. The material in the cone was reconstituted in 5.0 ml 0.02 M $Na_2HPO_4.7H_2O$, pH 8.0 and centrifuged again under the same conditions. The yield was approximately 300 µl of enzyme preparation.

Phospho[Methyl-^{3}H]choline was synthesized enzymatically from [Methyl-^{3}H]choline. The reaction mixture contained 0.5 mCi [Methyl-^{3}H]choline, 9.75 ul of 10 mM choline iodide, 50 µl of 100 mM $MgCl_2$, 50 µl of 10 mM ATP, 25 µl of 1.0 M Tris-HCl pH 8.0, 115.25 µl H_2O pH 8.0 and 0.8 units of dialyzed choline kinase. The reaction mixture was incubated for 60 min at 37°C and the reaction was terminated by boiling in a water bath for 3 min. The reaction mixture was centrifuged at 1,500 rpm for 15 min in a bench top centrifuge. The supernatant was applied to a G25 silica gel plate (100 µl/ 4 cm band). Standard phosphocholine (50 µl of 100 mM) was placed on an adjacent lane. The plate was developed in a solvent system containing 105 ml CH_3OH:0.6% NaCl:NH_4OH (50:50:5 ; v/v/v). The part of the tlc plate containing the phosphocholine standard was exposed to iodine vapor to estimate the R_f of the phosphocholine fraction. The labeled phosphocholine was eluted from the gel by washing with 5 ml H_2O (4 times). Recovery of radioactivity was >70%.

VII. Estimation Of Protein

Protein was determined by a modified method of Lowry et al. (98). Aliquots of subcellular fractions were incubated in 1.5 ml of 0.66 N NaOH at room temperature overnight. To each sample 1.5 ml of solution A (100 ml of 13% Na_2CO_3; 3 ml of 4% sodium potassium tartarate; and 3 ml of 4% $CuSO_4$) was added, and the mixture was allowed to stand at room temperature for 10 min. Phenol reagent (0.5 ml) was added to

each sample. The mixture was vortexed and allowed to stand at room temperature for one half hour. Absorbance was measured at 625 nm against a blank. Fatty acid free albumin (1 mg/ml) was used as a standard. Absorbance was linear to 0.1 mg of protein.

VIII. Estimation Of Lipid Phosphorus

The phospholipid content in hamster heart was determined by the method of Bartlett (99). The tubes used for phosphorus analysis were soaked in chromic acid overnight followed by extensive washing with distilled water to remove residual phosphorus on the tubes. Gel containing phospholipid was removed from tlc plates and placed into acid washed tubes. Perchloric acid (1.1 ml of 70%) was added to the gel and the mixture was incubated at 160^{o}C overnight. Subsequent to cooling at room temperature, 8 ml of H_2O and 0.8 ml of 5% ammonium molybdate were added to the mixture followed by the addition of 0.2 ml ANSA. The mixture was placed in a boiling H_2O bath for at least 10 min. The mixture was cooled to room temperature and centrifuged at 2,000 rpm for 10 min in a bench top centrifuge. The absorbance of the resulting supernatant was read at 830 nm. The phosphorus content was determined from a standard curve prepared using KH_2PO_4 (10 µg/ml) as standard. Blanks containing silica gel were employed to take into account any non-specific absorbance.

B. Hypoxic Perfusion Experiments

I. Perfusion Of Hamster Hearts

Hamster hearts were perfused in the Langendorff mode (92) with Krebs-Henseleit buffer (91) as described in A.I. Specifically, the hearts were pulse-labeled for 30 min with 10 ml of 95% O_2/5% CO_2-saturated buffer containing 0.01 mM [Methyl-^{3}H]choline (5 µCi/ml). Subsequently, hearts were perfused (chased) with KHB containing 0.01 mM choline for 30, 45 and 60 min. The chase buffer was saturated with either 95% O_2/5% CO_2 (control) or 95% N_2/5% CO_2 (hypoxic). The PO_2 of the control buffer was 522±15 mm Hg and the PO_2 of the hypoxic buffer was 22±5 mm Hg and remained at this level throughout the chase.

II. Determination Of Radioactivity Incorporated Into Choline Containing Metabolites

Subsequent to perfusion, the hearts were weighed and homogenized in 20 ml $CHCl_3$:CH_3OH (1:1 ; v/v). A 20 µl aliquot of the homogenate was taken for the determination of total radioactivity. The homogenate was allowed to separate into two phases by the addition of $CHCl_3$ and 0.05 M KCl until a 4:2:3 ratio of $CHCL_3$:CH_3OH:0.05 M KCl (v/v/v) was obtained. The solvents in the organic and aqueous phases were evaporated under reduced pressure at 37°C. The residue from the organic phase was resuspended in 1 ml $CHCl_3$:CH_3OH (2:1 ; v/v). The aqueous phase residue was resuspended in 1

ml H_2O. The radioactivity incorporated into choline-containing metabolites was determined as described in A.III.

III. Enzyme Assays

Subsequent to perfusion, the hearts were weighed, and a 20% homogenate (w/v) in 0.145 M NaCl/5 mM Tris-HCl (pH 7.4) was prepared. The postmitochondrial fraction was obtained by centrifugation at 10,000 x g for 15 min. The postmitochondrial fraction was then centrifuged at 105,000 x g for 60 min to yield the cytosolic and crude microsomal fractions. The crude microsomal fraction was washed once with the same buffer and the resultant pellet resuspended in 1 ml buffer and designated the microsomal fraction. Glucose-6-phosphatase, employed as a microsomal marker enzyme, in the homogenate and microsomal fraction was determined by a modification of the method of Moore (100). The reaction mixture contained 0.45 ml substrate, heart microsomal fraction, 0.012% deoxycholate, and water in a total volume of 0.6 ml. The substrate contained 0.1 M sodium-glucose-6-phosphate pH 6.5, 35 mM histidine pH 6.5, sodium-EDTA pH 7.0 and water in a ratio of 2:5:1:1, respectively. The reaction mixture was incubated at 37°C for 30 min and 2.5 ml 8% TCA was added to stop the reaction. The reaction was linear for at least 40 min. The tubes were kept on ice for at least 30 min and centrifuged at 2,000 rpm for 5 min in a bench top centrifuge. The supernatant (2 ml) was removed for the estimation of phosphorus released. Water

(2.3 ml) was added to the supernatant followed by the addition of 0.5 ml 2.5% (w/v) ammonium molybdate in 5 N H_2SO_4. After addition of 0.2 ml of 2.5% ANSA the mixture was vortexed and allowed to stand at room temperature for 20 min. The absorbance at 660 nm against a H_2O blank was determined. The amount of phosphorus in the sample was estimated from a standard phosphorus curve. The activity of glucose-6-phosphatase in the microsomes was between 18-19% of the enzyme activity in the homogenate. Choline kinase, CTP:phosphocholine cytidylyltransferase, CDP-choline:1,2-diacylglycerol cholinephosphotransferase and phosphocholine phosphatase were assayed as described in section A.IV. In some experiments total and specific cytidylyltransferase activities in the cytosol were assayed in the presence of total hamster liver lipid extract (1 mg phospholipid/ml reaction mixture) to obtain maximal stimulation of the cytosolic enzyme (82,83). Total microsomal cytidylyltransferase activity was calculated based upon yield of microsomes. The total cytosolic cytidylyltransferase activity was calculated based on the complete activation of the enzyme in the presence of lipids.

IV. Extraction Of Total Hamster Liver Lipids

Hamster total liver lipid was extracted as previously described (101,102). Hamster liver (5 g) was homogenized in 15 ml $CHCl_3$:CH_3OH (1:2 ; v/v). The homogenate was centrifuged at 2,000 rpm in a bench top centrifuge. The

pellet was extracted twice with 10 ml of $CHCl_3$:CH_3OH:H_2O (1/2/0.8 ; v/v/v). The extracts were pooled, placed in a separatory funnel and a biphasic mixture was obtained with the addition of 10 ml $CHCl_3$ and 10 ml H_2O. The organic phase was removed and the aqueous phase washed with 20 ml of $CHCl_3$:CH_3OH:H_2O (86:14:1 ; v/v/v). The organic phases were pooled and evaporated under reduced pressure. The dried lipid extract was resuspended in $CHCl_3$:CH_3OH (2:1 ; v/v), and lipid phosphorus was determined by the method of Bartlett (99). The phospholipid solution was adjusted to a final concentration of 1 mg/ml and stored at -20°C.

V. Metabolic Fate Of CDP-choline

The metabolic fate of CDP-choline was estimated as described by Choy (65). Hearts were perfused under control or hypoxic conditions for 60 min. Subsequent to perfusion, the hearts were homogenized in 3.5 ml 0.25 M sucrose. An aliquot was removed for the determination of protein. Aliquots of the homogenate containing 1.5 mg of protein were incubated with 1 µmol CDP-[^{14}C]choline (1 µCi/µmol) in 0.05 M K_2HPO_4 buffer (pH 7.4), in a total volume of 0.5 ml, for 15 min at 37°C. Two ml of $CHCl_3$:CH_3OH (2:1 ; v/v) were added to stop the reaction. To obtain phase separation 1 ml of H_2O was added to the mixture. The metabolites in each phase were analyzed by thin-layer chromatography as described in A.III.

VI. Measurement Of Choline, Phosphocholine And CDP-choline Pool Sizes

a) Isolation Of Choline, Phosphocholine And CDP-choline

Choline, phosphocholine and CDP-choline pool sizes were determined by a modification of the procedure of Vance et al. (68). Three groups of hearts were used for the study: a) non-perfused hearts b) hearts perfused for 60 min with 95% O_2-saturated buffer and c) hearts perfused with 95% N_2-saturated buffer. Immediately after perfusion, the hearts were frozen in liquid N_2. Subsequently, the hearts were weighed and homogenized in 20 ml of $CHCl_3$:CH_3OH (1:1 v/v). To each homogenate was added chromatographically pure [Methyl-^{3}H]choline, phospho[Methyl-^{14}C]choline and CDP-[Methyl-^{14}C]choline, in order to estimate recoveries. To obtain phase separation 10 ml of H_2O were added to the homogenate. The aqueous phase was removed and the solvent evaporated under reduced pressure at 37°C. The organic phase was washed twice with 10 ml of CH_3OH:H_2O (2:1 ; v/v) and then discarded. The aqueous phases from the two washes were combined and evaporated under reduced pressure at 37°C and reconstituted in 5 ml water. This was applied to a Dowex AG1-X8 (OH^- form) column (1 x 30 cm) that had been previously equilibrated with CH_3OH:H_2O (1:1 ; v/v). Choline was eluted into a round bottom flask with 100 ml of CH_3OH:H_2O (1:1 ; v/v) followed by 10 ml of H_2O. Phosphocholine and CDP-choline were eluted with 200 ml of 0.4 M NH_4HCO_3 into a round bottom flask. The eluates were evaporated under reduced pressure at 37°C. Phosphocholine

and CDP-choline were redissolved in 5 ml water and applied to a Norite A-cellite (1:1 v/v) column (1 x 5 cm). Phosphocholine was eluted into round bottom flasks with 20 ml of H_2O followed by the addition of 15 ml of 2% C_2H_5OH to the column. CDP-choline was then eluted into round bottom flasks with 30 ml of 40% C_2H_5OH containing 1% NH_4OH. The eluates were evaporated under reduced pressure at 37°C and reconstituted in 2 ml of H_2O. The phosphocholine fraction was digested with 10 units of E. Coli alkaline phosphatase (type II) for 2 hr at 37°C. The CDP-choline fraction was digested with 10 units of alkaline phosphatase and 10 units of Crotalus adementeus phosphodiesterase (type III) for 3 hr at 37°C. The reaction mixtures were lyophilized and the residue extracted twice with 70% C_2H_5OH. The C_2H_5OH was evaporated under a stream of N_2 gas and the choline, phosphocholine and CDP-choline fractions dissolved separately in 4 ml of H_2O. The choline in each fraction was extracted twice with 3 ml of 10 mg/ml tetraphenylboron in 3-heptanone. The choline in each fraction was back extracted from the 3-Heptanone tetraphenylboron with 0.5 ml 0.4 N HCl followed by a second extraction with 3 ml 1.0 N HCl. The HCl extracts from each sample were combined, lyophilized and redissolved in 1 ml of H_2O. An aliquot of this suspension was taken for the determination of radioactivity. The yield was calculated based on the % of radioactivity recovered in each sample.

b) Quantitation Of Choline Pools

The amount of choline in each fraction was determined by quantitative conversion of choline to [γ-^{32}P]phosphocholine. The conversion was catalyzed by choline kinase in the presence of [γ-^{32}P]ATP by a modification of the method of McCamon and Stetzler (103). Choline kinase was prepared as decribed in section A.VI. The reaction mixture contained 40 µl of cocktail, 50 µl of sample, standard or H_2O and 2 units of choline kinase in a final volume of 110 µl. The cocktail was a mixture of 125 µl of 0.1 M $MgCl_2$, 250 µl of 30 mM [γ-^{32}P]ATP (10 µCi/µmol), 835 µl of 0.2 M NaH_2PO_4 buffer pH 8.0 and H_2O in a final volume of 1.25 ml. The reaction mixture was incubated at 37°C for 1 hr, and placed on ice to stop the reaction. Ice cold 0.2 M barium acetate (100 µl) was added, and after 15 min, the reaction mixture was centrifuged at 4,000 rpm for 10 min at 0°C (Sorvall RC-5 Superspeed Refrigerated Centrifuge with SS-34 rotor). Ice cold barium acetate (25 µl) and 6 µl of 30 mM ATP were added to 150 ul of the resulting supernatant. The mixture was centrifuged as before. Of the reulting supernatant 100 µl was applied to a charcoal/celite (1:1 v/v) column (0.5 x 3 cm). [γ-^{32}P]phosphocholine was eluted into glass vials with 2 ml of H_2O and the radioactivity determined by scintillation counting. A standard curve was established and the choline concentration from each original choline, phosphocholine and CDP-choline fraction estimated from the curve. Samples containing an internal standard of 5 nmol of

choline were used to determine the degree of conversion of choline to phosphocholine.

VII. Determination Of Nucleotide Levels

Subsequent to perfusion, hearts were immediately frozen in liquid N_2 and stored at $-70^{o}C$. The hearts were weighed and homogenized in 6 ml of $CHCl_3$:CH_3OH (2:1 v/v). For recovery determination, 2 µl of [5-^{3}H]cytidine-5'-triphosphate was added to the homogenates. To cause phase separation 3 ml of H_2O was added to the homogenate. The aqueous phase was removed and the organic phase washed twice with 3 ml H_2O. The aqueous phase extracts were pooled and concentrated by evaporation under reduced pressure. The extract was resuspended in 200 µl H_2O. The nucleotide content in each sample was analyzed by high performance liquid chromatography using a Beckman Model 110 HPLC. The sample was diluted 20 times, and 20 ul was applied to a 5 µ Ultrasphere-ODS reversed-phase column. Nucleotides were eluted from the column with 0.03 M KH_2PO_4 containing 0.02 M tetrabutyl ammonium phosphate in 19% acetonitrile, pH 2.65 adjusted with H_3PO_4. The analytical conditions for the HPLC were; sensitivity: 0.005, flow rate: 1.2 ml/min, wavelength: 254 nm, temperature: ambient. Under these conditions ATP had a typical retention time of 10.0 min and CTP that of 7.7 min.

VIII. Analysis of Phospholipids

Subsequent to perfusion, the hearts were homogenized in 20 ml $CHCl_3$:CH_3OH (2:1 ; v/v). To the homogenate 0.05 M KCl was added until a final ratio of $CHCl_3$:CH_3OH:0.05 M KCl of 4:2:3 (v/v/v) was obtained. After phase separation, the organic phase was extracted twice with 10 ml $CHCl_3$:0.05 M KCl (1:1 ; v/v) and the extracts were pooled. The volume of the pooled extracts was reduced under N_2 and the phospholipid fractions in each extract were separated by thin-layer chromatography as described in A.III. The phospholipid content was determined as described by Bartlett (99).

IX. Measurement Of Fatty Acid Levels

Subsequent to perfusion, hearts were weighed and homogenized in 5 ml 0.145 N NaCl/5 mM Tris-HCl (pH 7.4). The homogenates from each group were pooled and an aliquot of the homogenate was taken for the determination of fatty acid content. Cytosolic fraction was prepared as described in B.III. The fatty acid content in the homogenate and cytosol were determined by the method of Shimizu et al. (104) using the Boehringer-Mannheim fatty acid determination kit. Fatty acids from cytosol were analyzed as described by Metcalfe and Schnitz (105). Briefly, to 16 ml of supernatant was added 20 ml $CHCl_3$ and 40 ml CH_3OH (butylated hydroxytoluene was added to all solutions). To this mixture 44 ml of 1.0 M KCl was added. The phases were allowed to separate and the aqueous phase was removed. The organic phase was evaporated

under reduced pressure at 37°C and the extract resuspended in 2 ml $CHCl_3$:CH_3OH (2:1 ; v/v). Fatty acids were separated by thin-layer chromatography with a solvent system containing heptane:isopropylether:CH_3COOH (60:40:4 ; v/v/v). The plate was air dried and sprayed with 0.25% dichloro-fluorescein in C_2H_5OH. Fatty acids were visualized under ultraviolet light. Silica gel containing fatty acids were removed from the plate and the fatty acids were extracted from the gel by washing twice with 6 ml of $CHCL_3$:CH_3OH:H_2O:CH_3COOH (50:39:10:1 ; v/v/v/v). The extracts were combined and 4 ml of 4 M NH_4OH was added to remove the dichlorofluorescein. The aqueous phase was removed and the organic phase was washed with 6 ml of CH_3OH:H_2O (1:1 ; v/v). The organic phase was filtered through phase separation paper (Whatman 1PS) into a screwcap tube and the solvent was removed under a stream of N_2.

Fatty acid species in cytosol were determined by gas liquid chromatography as previously described (86). To each sample 0.5 ml of BF-3 Methanol reagent was added (Supleco 3020) and the solution was vortexed and sonicated for 15 s. The mixture was placed in a H_2O bath at 96°C for 15 min. Subsequently, 0.5 ml of H_2O was added. The solution was washed twice with 1.5 ml of petroleum ether. The upper phases were removed, combined and evaporated under a stream of N_2. The residue was dissolved in 100 µl hexane and an aliquot injected into a Shimadzu GC Mini 2 equiped with

temperature programmer. Fatty acid methyl ester peaks were identified by comparison with standards. The areas under the peaks were measured using a Beckman 450 Data Controller Integrator and the percentage distribution of the methylesters determined.

X. Statistical Analysis

All results are depicted as mean$\pm$standard deviation (number of experiments) unless otherwise indicated. A two-tailed Students' t-test and Dunnets' t-test (when appropriate) were used for the determination of significance. The level of significance was defined as $P<0.05$ for both t-tests.

RESULTS

A. The Effect Of Amino Acids On Choline Uptake And Phosphatidylcholine Biosyntheis

I. The effect of amino acids on labeled-choline uptake and incorporation of labeled material into choline containing metabolites.

The effect of glycine on choline uptake was examined by a time-course study. Isolated hamster hearts were perfused for 15-60 min with 10 ml Krebs-Henseleit buffer containing 0.01 mM [Methyl-^{3}H]choline (5 μCi/ml) in the absence and presence of 1.0 mM glycine. Subsequent to perfusion, the heart was homogenized in 20 ml $CHCl_3$:CH_3OH (1:1 v/v) and an aliquot taken for determination of radioactivity. Total incorporation of radioactivity was linear from 15-60 min of perfusion in control and glycine perfused hearts (Figure 4). Significant enhancement of choline uptake by 1.0 mM glycine was observed at 45 and 60 min (Figure 4). The difference was most significant at 60 min of perfusion.

To determine whether the enhancement of choline uptake was specific to glycine or could be caused by other amino acids, isolated hearts were perfused for 15-60 min with 10 ml Kreb-Henseleit buffer containing 0.01 mM [Methyl-^{3}H]choline (5 μCi/ml) in the absence and presence of 1.0 mM L-alanine. Total incorporation of radioactivity was linear from 15-60 min of perfusion in control and L-alanine perfused hearts (Figure 5). Significant enhancement of choline uptake by 1.0 mM L-alanine was observed at 30, 45

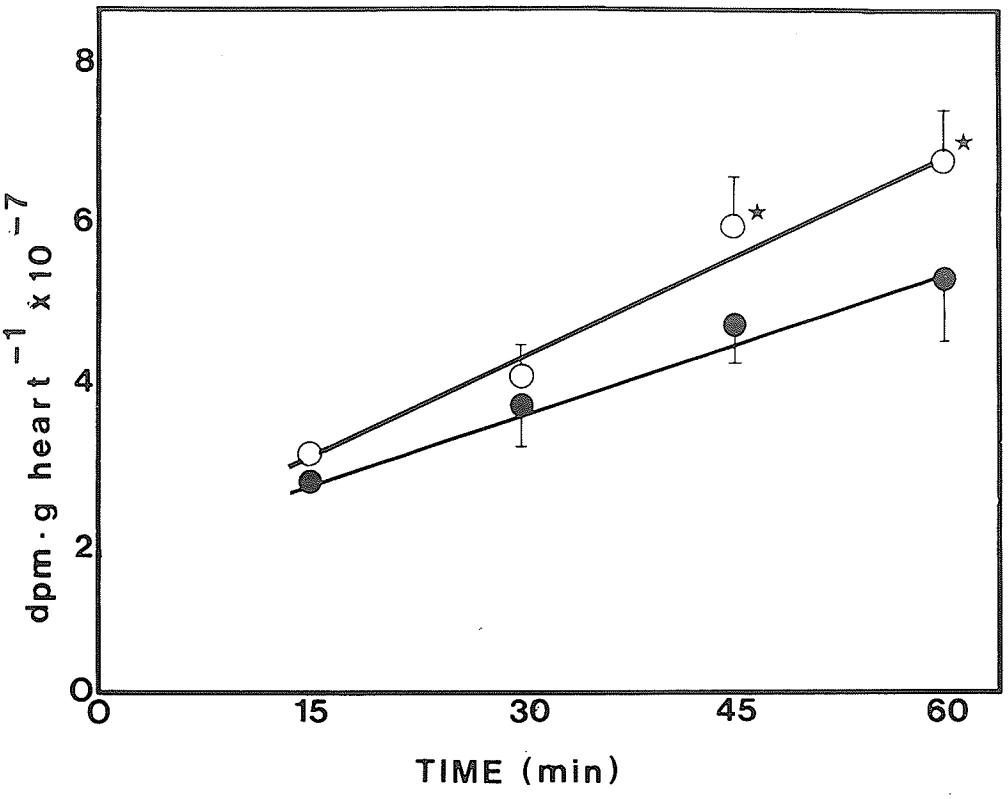

Figure 4. Total [Methyl-^{3}H]choline uptake in hearts perfused with glycine.

Isolated hearts were perfused for 15,30,45 and 60 min in the absence (●) and presence (O) of 1.0 mM glycine as described in Experimental Procedures. Subsequent to perfusion, hearts were homogenized in $CHCl_3$:CH_3OH (1:1 v/v) and an aliquot taken for determination of radioactivity. Points with vertical bars represent the mean of at least three separate experiments. Points without vertical bars represent the mean of two experiments. The vertical bars are standard deviations. $^*P<0.05$.

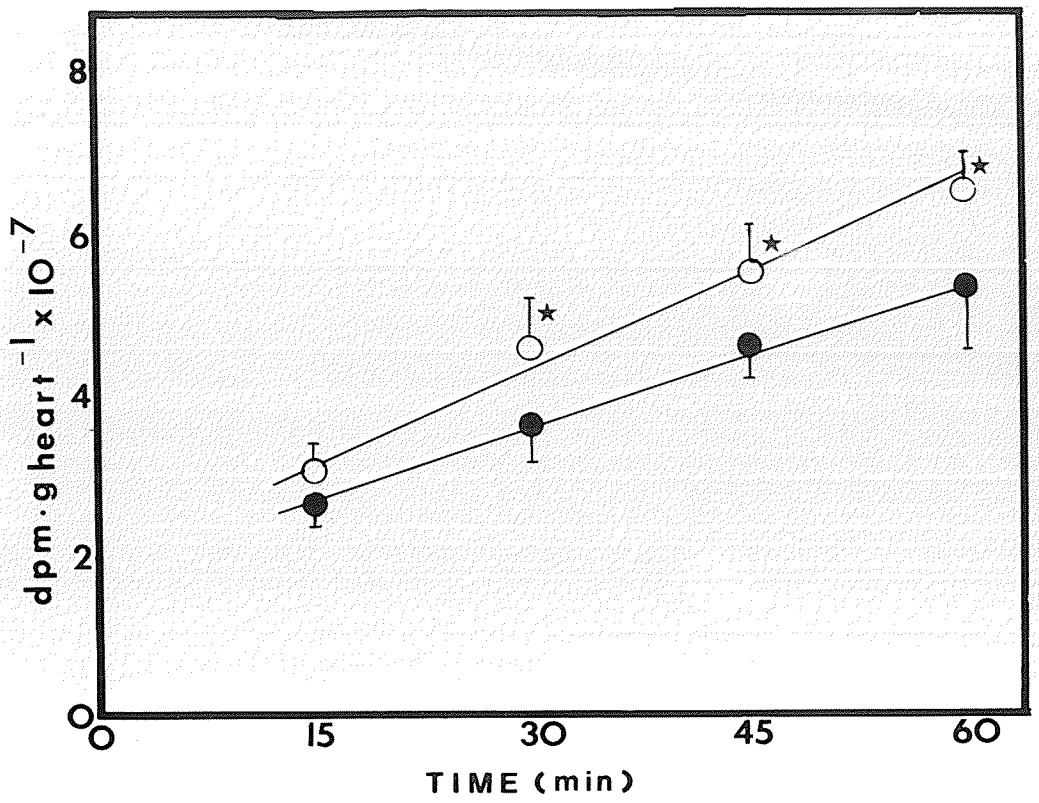

Figure 5. Total [Methyl-^{3}]choline uptake in hearts perfused with L-alanine.

Isolated hearts were perfused for 15,30,45 and 60 min in the absence (●) and presence (O) of 1.0 mM L-alanine as described in Experimental Procedures. Subsequent to perfusion, hearts were homogenized in $CHCl_3$:CH_3OH (1:1 v/v) and an aliquot taken for determination of radioactivity. Points with vertical bars represent the mean of at least three separate experiments. The vertical bars are standard deviations. $^*P<0.05$.

and 60 min of perfusion (Figure 5). As was the case with glycine, the enhancement of choline uptake was most significant at 60 min of perfusion.

The effect of glycine and L-alanine on phosphatidylcholine biosynthesis in the isolated hamster heart was examined. The tissue homogenate was separated into aqueous and organic phases by the addition of $CHCl_3$ and 0.05 M KCl. More than 90% of the radioactivity in the organic phase was associated with the phosphatidylcholine fraction in all cases. The presence of 1.0 mM glycine or 1.0 mM L-alanine had no effect on the labeling of phosphatidylcholine (Figures 6,7).

The choline-containing metabolites in the aqueous phase were examined. In all cases, the sum of the radioactivities recovered from choline, phosphocholine and CDP-choline accounted for 99% of the radioactivity in the aqueous phase. In the presence of 1.0 mM glycine or 1.0 mM L-alanine, the labeling of choline and CDP-choline remained the same as in the controls (Figures 8,9). Increases in the labeling of the phosphocholine fractions were detected at 45 and 60 min of perfusion with glycine (Figure 8) and 30, 45 and 60 min of perfusion with L-alanine (Figure 9), quantitatively accounting for the difference in labeled-choline uptake (Figure 4,5).

To determine whether the enhancement of choline uptake by

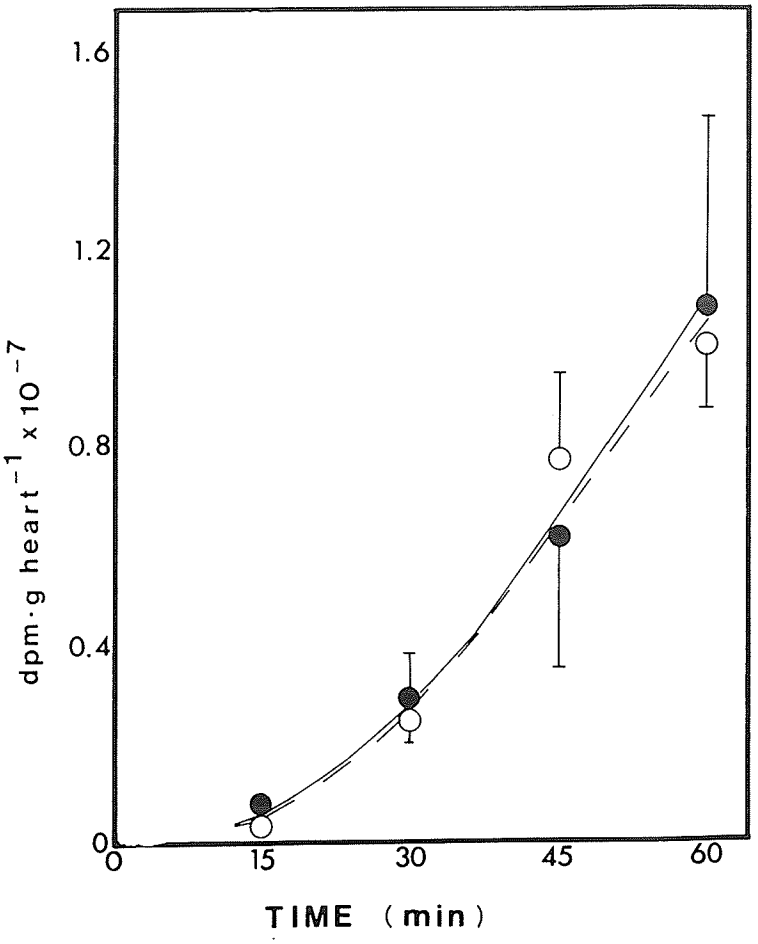

Figure 6. The incoporation of [Methyl-^{3}H]choline into phosphatidylcholine in glycine perfused hearts. Isolated hearts were perfused for 15,30,45 and 60 min in the absence (●) and presence (O) of 1.0 mM glycine and the radioactivity in phosphatidylcholine determined as described in Experimental Procedures. Points with vertical bars represent the mean of at least three separate expeiments. Points without vertical bars represent the mean of two separate experiments. The vertical bars are standard deviations.

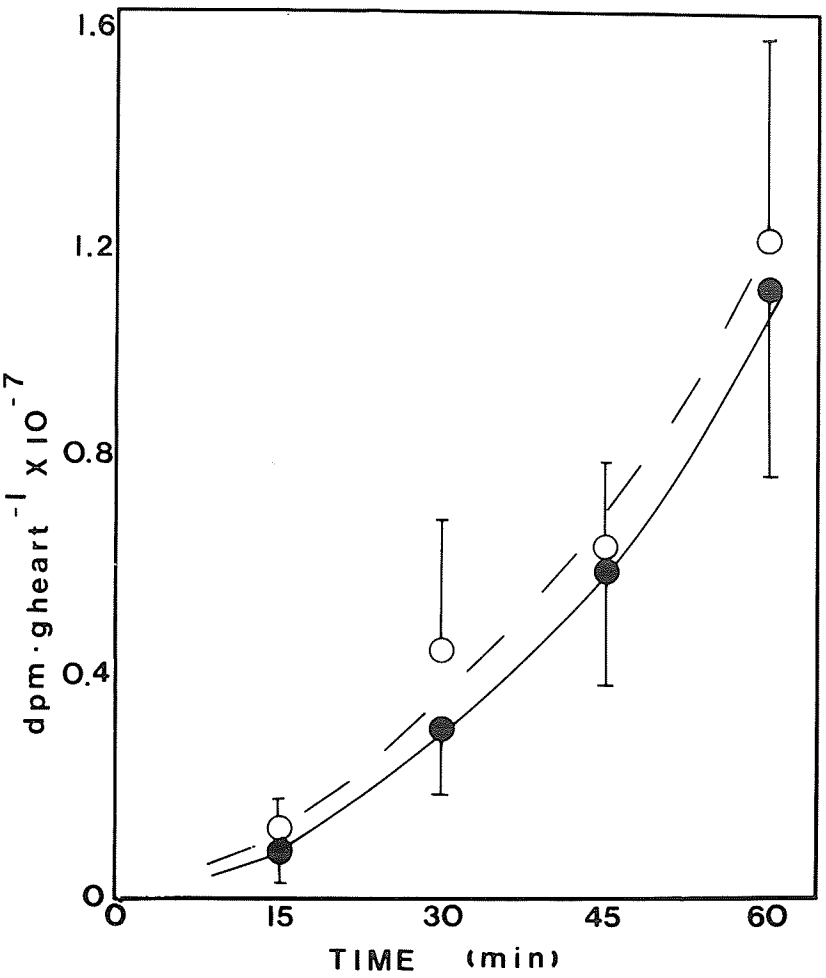

Figure 7. The incorporation of [Methyl-^{3}H]choline into phosphatidylcholine in L-alanine perfused hearts. Isolated hearts were perfused for 15,30,45 and 60 min in the absence (●) and presence (○) of 1.0 mM L-alanine and the radioactivity in phosphatidylcholine determined as described in Experimental Procedures. Points with vertical bars represent the mean of at least three separate experiments. The vertical bars are standard deviations.

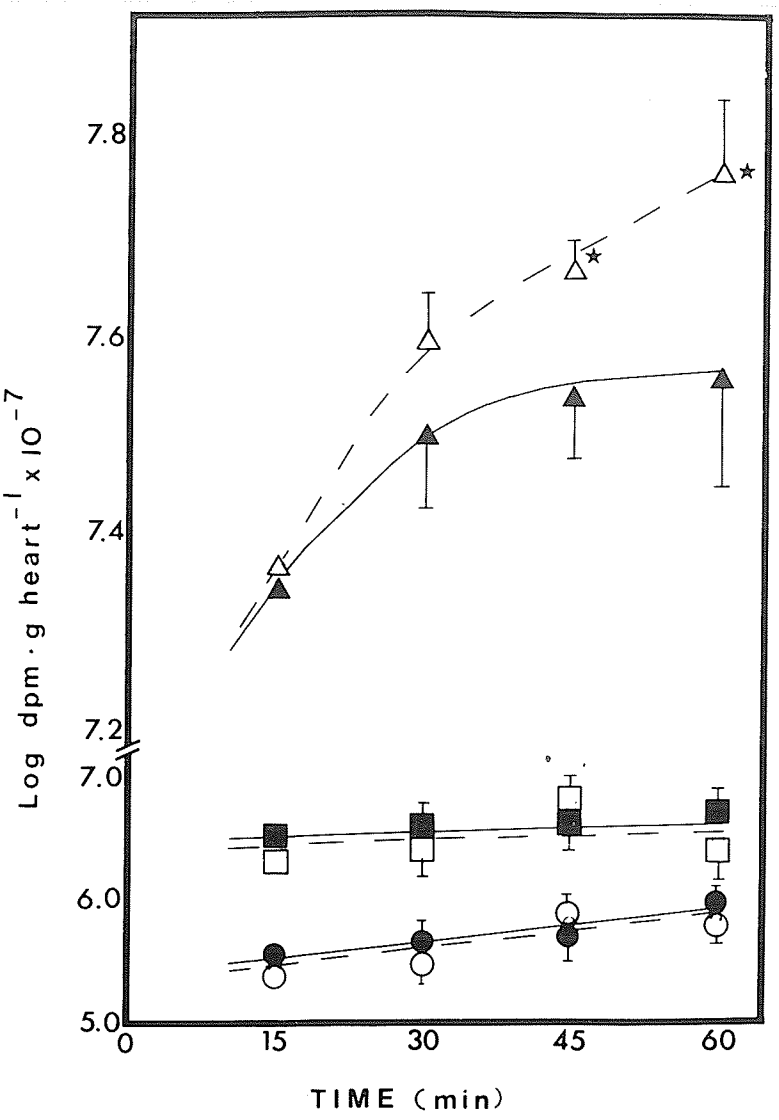

Figure 8. The incorporation of [Methyl-^{3}]choline into water-soluble choline-containing metabolites in glycine perfused hearts.

Isolated hearts were perfused for 15,30,45 and 60 min and the radioactivity in choline (■ , □), phosphocholine (▲ , △) and CDP-choline (● , ○) was determined as described in Experimental Procedures. The open symbols indicate perfusion with glycine and the closed are controls. Points with vertical bars represent the mean of at least three separate experiments. Points without vertical bars represent the mean of two separate expeiments. The vertical bars are standard deviations. $^{*}P<0.05$.

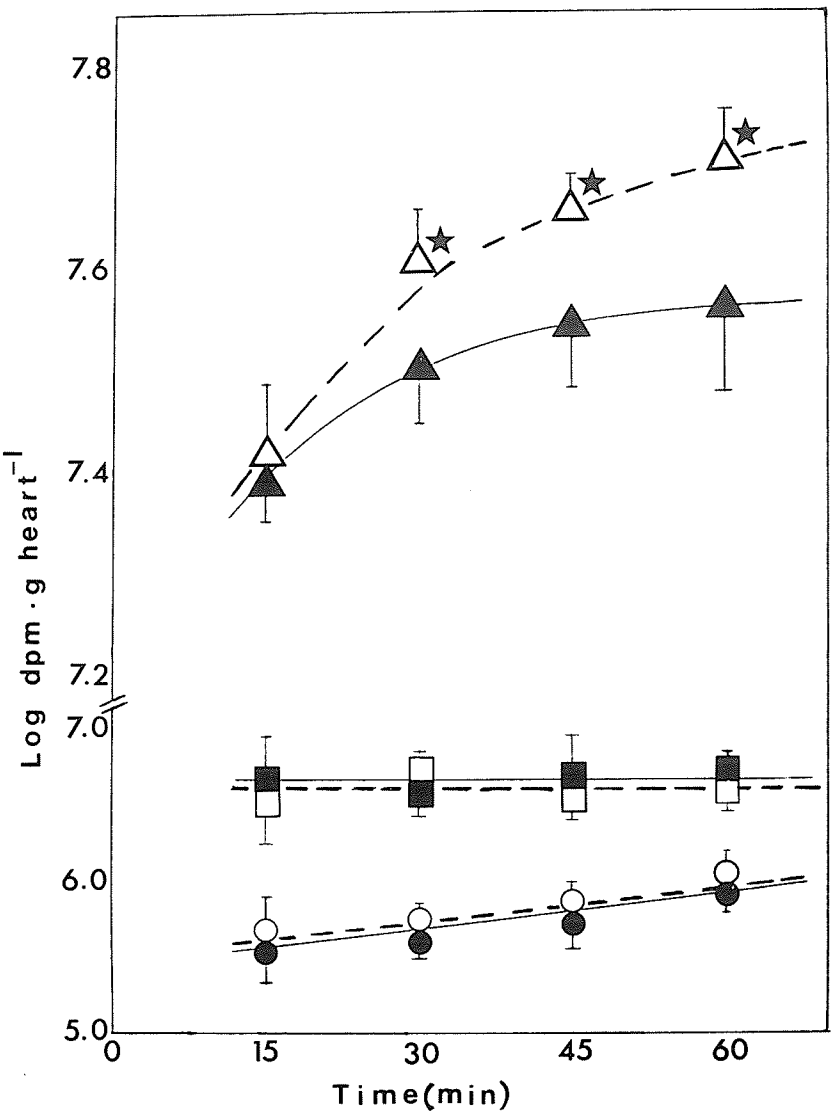

Figure 9. The incorporation of [Methyl-^{3}H]choline into water-soluble choline-containing metabolites in L-alanine perfused hearts.

Isolated hearts were perfused for 15,30,45 and 60 min and the radioactivity in choline (■ , □), phosphocholine (▲ , △) and CDP-choline (● , ○) was determined as described in Experimental Procedures. The open symbols indicate perfusion with L-alanine and the closed are controls. Points with vertical bars represent the mean of at least three separate experiments. The vertical bars are standard deviations. $^{*}P<0.05$.

L-alanine was concentration dependent, the effect on choline uptake at various concentrations of L-alanine was investigated. Choline uptake at various concentrations of L-alanine at 60 min perfusion was analyzed since at this time the enhancement of choline uptake by 1.0 mM L-alanine was most prominent. Choline uptake was not affected by 0.01-0.05 mM L-alanine but was enhanced (20-34%) in the presence of 0.1-50 mM L-alanine (Table 1). Radioactivity in the phosphatidylcholine, choline and CDP-choline fractions were not affected (Table 1, 2). An accumulation of radioactivity was detected in the phosphocholine fraction (Table 2).

To determine if the enhancement of choline uptake was a general phenomenon displayed by all amino acids, the effect of various neutral, basic and acidic amino acids on choline uptake was investigated. Both L-serine and L-phenylalanine caused the enhancement of choline uptake in the same manner as glycine and L-alanine and did not affect the amount of label incorporated into phosphatidylcholine (Table 1). An accumulation of radioactivity was found in the phosphocholine fraction of L-serine and L-phenylalanine perfused hearts (Table 2). There was no change in the amount of radioactivity incorporated into the choline or CDP-choline fractions in these hearts (Table 2). The basic amino acids (L-lysine, L-arginine) and the acidic amino acids (L-aspartate, L-glutamate) at 1.0 mM concentration had no effect on choline uptake, nor did they affect the incorporation of radioactivity into phosphatidylcholine

TABLE 1

Total uptake of [Methyl-^{3}H]choline by the isolated heart and radioactivity incorporated into phosphatidylcholine.

Hearts were perfused with [Methyl-^{3}H]choline in Krebs-Henseleit buffer for 60 min in the absence and presence of amino acids. Subsequent to perfusion, the total uptake of radioactivity and radioactivity incorporated into phosphatidylcholine were determined as described in Experimental Procedures.

Amino acid Concentration	Total uptake of radioactivity (dpm/g heart x 10^{-7})	Radioactivity in phosphatidylcholine (dpm/g heart x 10^{-7})
Control	5.18±0.68(7)	1.11±0.36(7)
glycine 1.0 mM	6.73±0.63(3)*	1.00±0.11(3)
L-alanine 0.01 mM	5.47±0.73(4)	1.52±0.38(4)
L-alanine 0.05 mM	5.86±0.17(3)	1.36±0.18(3)
L-alanine 0.1 mM	6.63±0.43(3)*	1.20±0.52(3)
L-alanine 1.0 mM	6.24±0.69(4)*	1.25±0.25(3)
L-alanine 50.0 mM	6.96±0.50(3)*	1.50±0.89(3)
L-serine 1.0 mM	7.13±1.08(4)*	1.20±0.48(4)
L-phenylalanine 1.0 mM	6.80±0.54(4)*	1.03±0.08(3)
L-lysine 1.0 mM	5.17±0.83(4)	0.92±0.12(4)
L-arginine 1.0 mM	5.54±0.72(4)	1.25±0.44(4)
L-aspartate 1.0 mM	4.92±0.24(4)	1.04±0.04(4)
L-glutamate 1.0 mM	4.92±0.16(3)	1.20±0.40(3)
L-alanine + glycine 1.0 mM	7.03±1.04(6)*	1.16±0.19(6)

*$P<0.05$.

TABLE 2

Radioactivity incorporated into choline-containing metabolites.

Hearts were perfused with [Methyl-^{3}H]choline in Krebs-Henseleit buffer for 60 min in the absence and presence of 1.0 mM amino acids. Subsequent to perfusion, the radioactivities of the metabolites of the CDP-choline pathway were determined as described in Experimental Procedures.

Amino acid	Choline	Phosphocholine	CDP-choline
	(dpm/g heart X10^{-7})		
Control	0.21±0.11(7)	3.63±0.88(7)	0.13±0.02(7)
L-alanine	0.19±0.10(4)	5.04±0.65(4)*	0.14±0.05(4)
L-serine	0.35±0.28(4)	5.35±0.58(4)*	0.12±0.02(4)
L-phenylalanine	0.21±0.11(3)	5.14±0.14(3)*	0.12±0.02(3)
L-lysine	0.24±0.01(4)	3.40±1.04(4)	0.13±0.01(4)
L-aspartate	0.19±0.04(4)	3.51±0.45(4)	0.11±0.03(4)
L-alanine + glycine	0.34±0.11(6)	5.29±0.97(6)*	0.14±0.06(6)

*$P<0.05$.

(Table 1). In addition, the basic and acidic amino acids did not affect the amount of radioactivity incorporated into other choline-containing metabolites (Table 2).

The presence of 1.0 mM glycine and 1.0 mM L-alanine in the perfusate did not have an additive effect on choline uptake or the incorporation of radioactivity into phosphatidylcholine and aqueous choline-containing metabolites (Table 2).

The transport of L-alanine by system A in the intact heart has been demonstrated to be adaptively regulated (106,107). To study the effect of such adaptive regulation on the enhancement of choline uptake, hamster hearts were preperfused for 30 min with 1.0 mM L-alanine. Subsequently, the hearts were further perfused for 60 min with 0.01 mM labeled choline and 1.0 mM L-alanine. Prior perfusion with L-alanine had no effect on the enhancement of choline uptake nor the accumulation of labeling in the phosphocholine fraction.

II. Effect of choline on labeled amino acid uptake

To determine if choline affects the uptake of amino acids, hearts were perfused for 60 min with 1.0 mM L-[3-^{3}H]alanine (1.25 μCi/ml) or 0.01 mM [2-^{3}H]glycine in the absence and presence of 0.01 mM choline. Subsequent to perfusion, the hearts were homogenized and the protein fraction obtained. There was no difference in the labeling

of homogenates or protein fractions between control and experimental hearts (Tables 3,4). In addition, when the hearts were perfused with 0.01 mM radioactive L-alanine (a concentration of amino acid below its half saturating concentration of uptake [108]) in the absence and presence of 0.1 mM choline (a concentration at half saturating concentration of its uptake [41]), the label incorporated into the homogenates or the protein fractions were unaltered (Table 5). These results indicate that the uptake and incorporation of amino acids into protein were not affected by choline in the perfusate.

III. Effect of amino acids on the enzymes involved in the CDP-choline pathway

Hearts were perfused for 60 min with 0.01 mM choline in the absence and presence of 1.0 mM glycine or 1.0 mM L-alanine. Subsequent to perfusion, cytosolic and microsomal fractions were obtained and enzyme activities determined. No difference in enzyme activities was detected between the control and amino acid perfused hearts (Tables 6,7). Exogenous addition of 1.0 mM L-alanine in the assays did not affect enzyme activities.

The possibility that inhibition of the hydrolysis of phosphocholine to choline could lead to the accumulation of phosphocholine was investigated. Phosphocholine phosphatase activities in the microsomal fraction of hearts perfused in

TABLE 3

Effect of choline on [2-^{3}H]glycine uptake and incorporation into protein.

Hearts were perfused for 60 min with Krebs-Henseleit buffer containing 0.01 mM [2-^{3}H]glycine in the absence and presence of 0.01 mM choline. Subsequent to perfusion, the total uptake of radioactivity and the radioactivity incorporated into the protein fraction were determined as described in Experimental Procedures.

Choline concentration in perfusate	Total uptake of radioactivity	Radioactivity in protein fraction
(mM)	(dpm/g heart X10^{-6})	(dpm/mg protein X10^{-4})
(control)	6.46±0.82(4)	1.94±0.25(4)
0.01	6.25±1.32(4)	1.88±0.40(4)

TABLE 4

Effect of low choline concentration on L-[3-^{3}H]alanine uptake and incorporation into protein.

Hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM L-[3-^{3}H]alanine in the absence and presence of 0.01 mM choline. Subsequent to perfusion, the total uptake of radioactivity and the radioactivity incorporated into the protein fraction were determined as described in Experimental Procedures.

Choline concentration in perfusate	Total uptake of radioactivity	Radioactivity in protein fraction
(mM)	(dpm/g heart X10^{-6})	(dpm/mg protein X10^{-3})
(control)	3.11±0.55(3)	1.50±0.16(3)
0.01	2.97±0.32(3)	1.61±0.49(3)

TABLE 5

Effect of high choline concentration on L-[3-^{3}H]alanine uptake and incorporation into protein.

Hearts were perfused for 60 min with Krebs-Henseleit buffer containing 0.01 mM L-[3-^{3}H]alanine in the absence and presence of 0.1 mM choline. Subsequent to perfusion, the total uptake of radioactivity and radioactivity incorporated into the protein fraction were determined as described in Experimental Procedures.

Choline concentration in perfusate	Total uptake of radioactivity	Radioactivity in protein fraction
(mM)	(dpm/g heart X10^{-6})	(dpm/mg protein X10^{-4})
(control)	6.71±0.78(3)	2.59±0.26(3)
0.1	7.38±0.89(3)	2.29±0.25(3)

TABLE 6

Activities of the phosphatidylcholine biosynthetic enzymes of the CDP-choline pathway

Hearts were perfused for 60 min with Krebs-Henseleit buffer containing 0.01 mM choline in the absence and presence of 1.0 mM glycine. Subsequent to perfusion, enzyme activities were assayed in the heart subcellular fractions as described in Experimental Procedures.

	0.01 mM choline	0.01 mM choline +1.0 mM glycine
	(nmol/min/mg protein)	
Choline kinase (cytosolic)	0.31±0.07(3)	0.35±0.07(3)
CTP:phosphocholine cytidylyltransferase (microsomal)	0.68±0.28(3)	0.62±0.06(3)
(cytosolic)	0.22±0.03(3)	0.24±0.01(3)
Cholinephospho-transferase (microsomal)	1.06±0.20(3)	1.09±0.06(3)

TABLE 7

Effect of L-alanine on the activities of the enzymes involved in the CDP-choline pathway.

Hearts were perfused for 60 min with Krebs-Henseleit buffer containing 0.01 mM choline in the absence and presence of 1.0 mM L-alanine. Subsequent to perfusion, enzyme activities were assayed in subcellular fractions as described in Experimental Procedures.

	0.01 mM choline	0.01 mM choline +1.0 mM L-alanine
	(nmol/min/mg protein)	
Choline kinase (cytosolic)	0.31±0.07(3)	0.32±0.04(3)
CTP:phosphocholine cytidylyltransferase (microsomal)	0.68±0.28(3)	0.71±0.33(3)
(cytosolic)	0.22±0.03(3)	0.25±0.06(3)
Cholinephospho-transferase (microsomal)	1.06±0.20(3)	1.03±0.28(3)
Phosphocholine phosphatase (microsomal)	2.80±0.21(3)	3.08±0.18(3)

the absence and presence of 1.0 mM L-alanine were unchanged (Table 7). Exogenous addition of 1.0 mM L-alanine to the assays did not affect the enzymes activity.

IV. Effect of amino acids on phosphocholine phosphatase and alkaline phosphatase activities

In HeLa cells, phosphocholine phosphatase and alkaline phosphatase are postulated to be the same enzyme (90). The inhibition of alkaline phosphatase by high concentrations of L-alanine is well documented (109). L-alanine at 50 mM concentration did not influence the hydrolysis of phospho[Methyl-^{3}H]choline in hamster heart microsomes (Table 8). Thus, the possibility that phosphocholine phosphatase and alkaline phosphatase are different enzymes in hamster heart was investigated using amino acid inhibitors.

The majority of alkaline phosphatase (68%) and phosphocholine phosphatase (72%) activities were found to be located in the microsomal fraction with the remainder in the mitochondrial fraction. The effect of amino acids on the activities of the enzymes were assessed in the microsomal fraction. L-Phenylalanine (10-25 mM) inhibited both activities but a higher degree of inhibition was displayed by alkaline phosphatase than that of phosphocholine phosphatase (Table 9). L-Alanine at 25 and 50 mM concentration caused significant inhibitions of alkaline phosphatase activity, but did not inhibit phosphocholine phosphatase activity (Table 8).

TABLE 8

Effect of L-alanine on phosphocholine phosphatase and alkaline phosphatase activities.

The hydrolysis of 10 mM phospho[Methyl-^{3}H]choline and 10 mM p-nitrophenylphosphate by heart microsomal preparations in the absence and presence of L-alanine was determined as described in Experimental Procedures.

L-alanine concentrations (mM)	Phosphocholine phosphatase (nmol/min/mg)	Alkaline phosphatase (μmol/min/mg)
0 (control)	3.95±0.06(3)	2.17±0.21(3)
1	3.99±0.23(3)	2.12±0.39(3)
5	3.97±0.11(3)	2.16±0.18(3)
10	3.81±0.35(3)	2.13±0.10(3)
25	4.05±0.28(3)	1.71±0.04(3)*
50	3.92±0.22(3)	1.65±0.13(3)*

*$P<0.05$.

TABLE 9

Effect of L-phenylalanine on phosphocholine phosphatase and alkaline phosphatase activities.

The hydrolysis of 10 mM phospho[Methyl-^{3}H]choline and 10 mM p-nitrophenylphosphate by heart microsomal preparation in the presence of L-phenylalanine was determined as described in Experimental Procedures.

L-phenylalanine concentrations (mM)	Phosphocholine phosphatase (nmol/min/mg)	Alkaline phosphatase (μmol/min/mg)
0	3.95±0.06(3)	2.17±0.21(3)
1	3.82±0.07(3)	2.11±0.10(3)
5	3.34±0.02(3)*	1.71±0.14(3)*
10	3.32±0.09(3)*	1.22±0.08(3)*
25	2.51±0.19(3)*	0.94±0.04(3)*

*P<0.05.

V. Kinetic and physical studies of alkaline phosphatase and phosphocholine phosphatase

The nature of the inhibition of alkaline phosphatase by L-alanine was further investigated. The activities of the enzyme at different substrate levels in the absence and presence of 25 and 50 mM L-alanine were determined, and the results depicted in a double-reciprocal plot. The kinetic studies revealed that the inhibition of alkaline phosphatase by L-alanine was essentially uncompetitive (Figure 10).

The possibility that the two activities share the same active site was studied by a kinetic approach. In the presence of phosphocholine (1-50 mM), alkaline phosphatase activitites were not affected (Table 10). The presence of p-nitrophenylphosphate (1-50 mM) caused significant inhibition of phosphocholine phosphatase activities (Table 10). In the presence of 0.1 and 0.2 mM p-nitrophenylphosphate, a "mixed-type" of inhibition on phosphocholine phosphatase activity was observed (Figure 11).

As previously indicated (110), the pH optimum for alkaline phosphatase was found to be between 9.75 and 10.25 depending on the substrate concentration. In the presence of 10 mM p-nitrophenylphosphate, the enzyme displayed a pH optimum of 10.0 (Figure 12). The pH optimum of phosphocholine phosphatase was 9.0 and remained unchanged at different substrate concentrations (Figure 12).

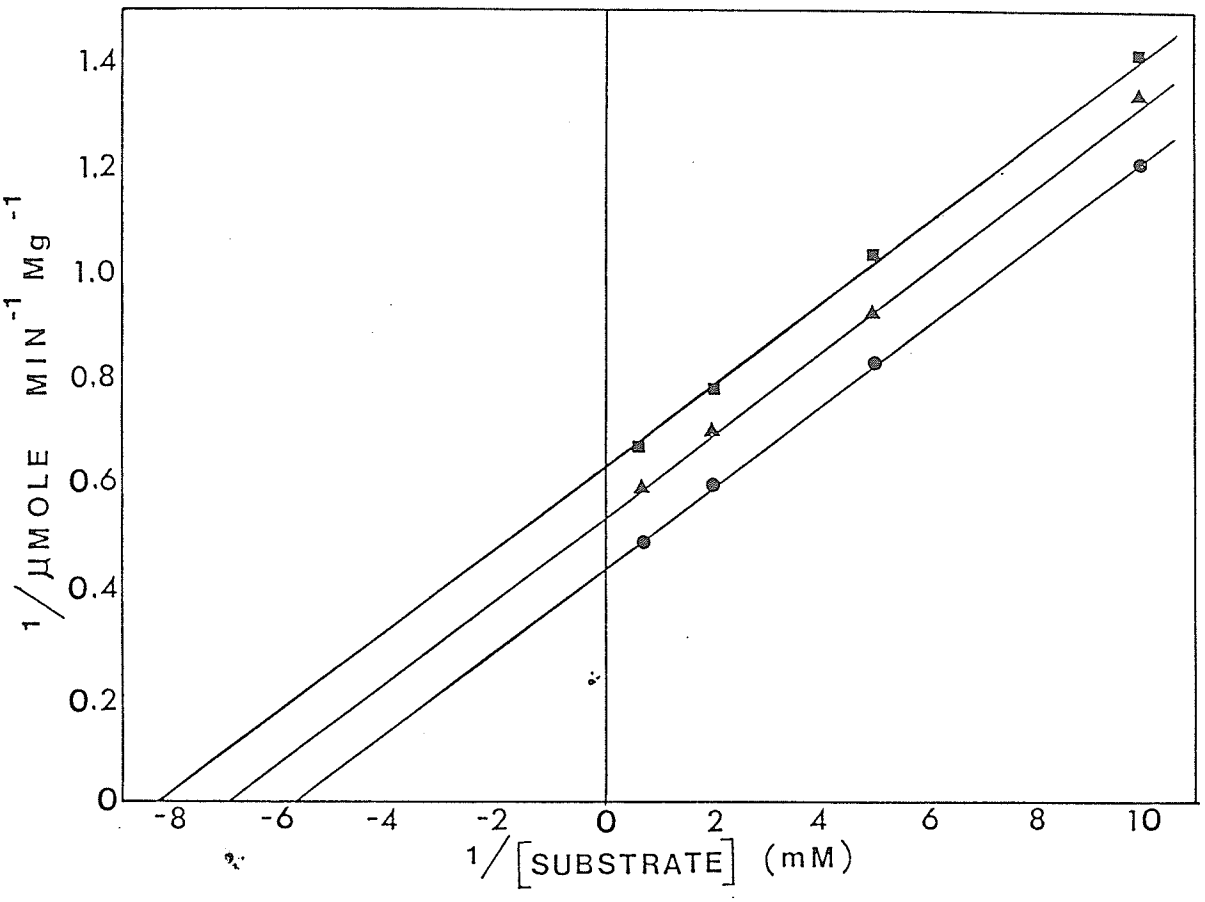

Figure 10. Double reciprocal plot of alkaline phosphatase inhibition by L-alanine.

The hydrolysis of p-nitrophenylphosphate in the absence (●) and presence of 25 mM (▲) and 50 mM (■) L-alanine were determined as described in Experimental Procedures. The points represent the mean of two separate sets of experiments, each of which was assayed in duplicate. The lines depicted were obtained from least squares analysis. The correlation coefficient was 0.99 for each line.

TABLE 10

Effect of p-nitrophenylphosphate on phosphocholine phosphatase and phosphocholine on alkaline phosphatase activities.

The hydrolysis of phospho[Methyl-^{3}H]choline in the presence of p-nitrophenylphosphate and the hydroysis of p-nitrophenylphosphate in the presence of phosphocholine in microsomal preparations were determined as described in Experimental Procedures.

p-Nitrophenyl-phosphate (mM)	Phospho-choline (mM)	Phosphocholine phosphatase (nmol/min/mg)	Alkaline phosphatase (μmol/min/mg)
0 (control)	0	3.95±0.06(3)	2.17±0.21(3)
1	–	1.07±0.26(3)*	
5	–	0.51±0.06(3)*	
10	–	0.52±0.20(3)*	
25	–	0.23±0.09(3)*	
–	1		2.44±0.12(3)
–	5		2.33±0.31(3)
–	10		2.41±0.10(3)
–	25		2.40±0.11(3)
–	50		2.30±0.04(3)

*$P<0.05$.

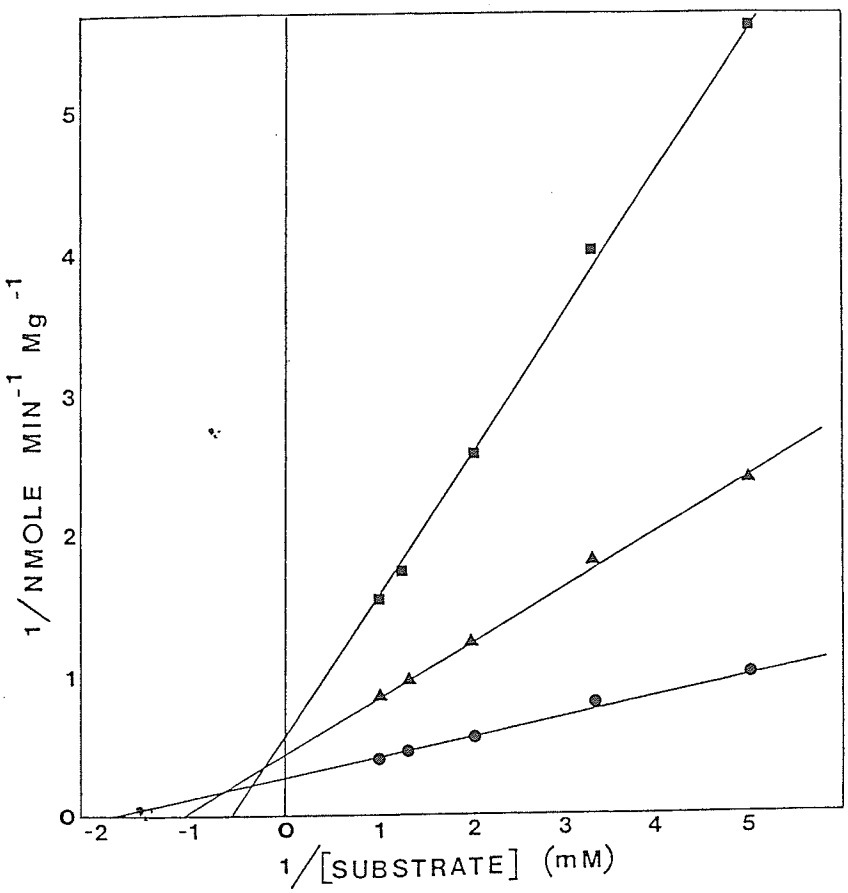

Figure 11. Double reciprocal plot of phosphocholine phosphatase inhibition by p-nitrophenylphosphate. The hydrolysis of phospho[Methyl-^{3}H]choline in the absence (●) and presence of 0.1 mM (▲) and 0.2 mM (■) p-nitrophenyl-phosphate were determined as described in Experimental Procedures. The points represent the mean of two separate sets of experiments, each of which was assayed in duplicate. The lines depicted were obtained from least squares analysis. The correlation coefficient was 0.99 for each line.

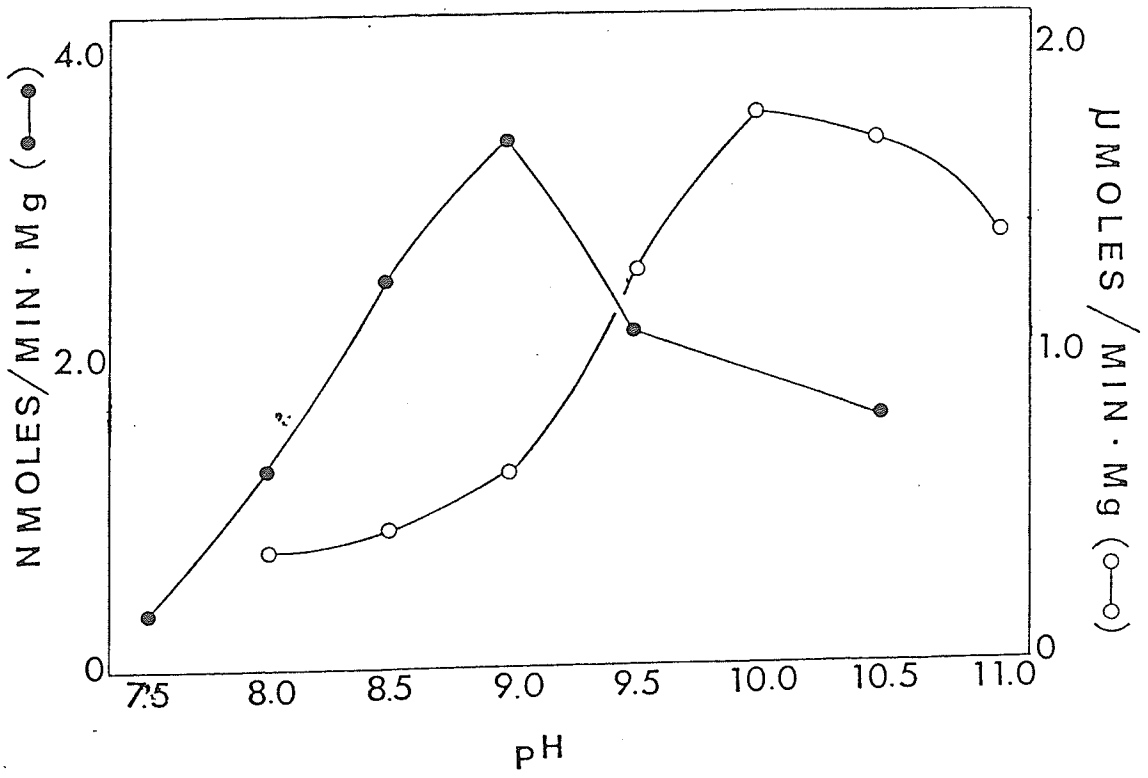

Figure 12. The pH profiles of alkaline phosphatase and phosphocholine phosphatase.

The pH profiles of phosphocholine phosphatase (●) and alkaline phosphatase (O) were determined as described in Experimental Procedures. The points represent the mean of two separate sets of experiments, each of which was assayed in duplicate.

The effect of temperature on the activities of alkaline phosphatase and phosphocholine phosphatase was investigated. Microsomal preparations were incubated at 55^{o}C for different time periods, and the enzyme activities were subsequently determined. Treatment at 55^{o}C caused a differential loss of activities between these two enzymes (Figure 13). Incubation of the microsomal preparation for 5 min caused a 30% loss of phosphocholine phosphatase activity, whereas a 10% loss of alkaline phosphatase activity was observed (Figure 13).

B. The Effect Of Hypoxia On Phosphatidylcholine Biosynthesis

I. Effect of hypoxia on the electrophysiology and morphology of the hamster heart

The viability of the hearts was monitored via electrocardiac recording during the perfusion and histological analysis subsequent to perfusion. The electrocardiogram was found to differ between hearts perfused with 95% O_2-saturated buffer (Figure 14a) and 95% N_2-saturated buffer (Figure 14b). Perfusion of the heart with 95% N_2-saturated buffer for 60 min caused a complete atrioventricular block (111). However, subsequent perfusion with the 95% O_2-saturated buffer for another 60 min resulted in an electrocardiogram which resembled the control, indicating that the electrophysiological abnormalities generated under hypoxic insult were reversible. Further hypoxic exposure (>60 min) caused irreversible

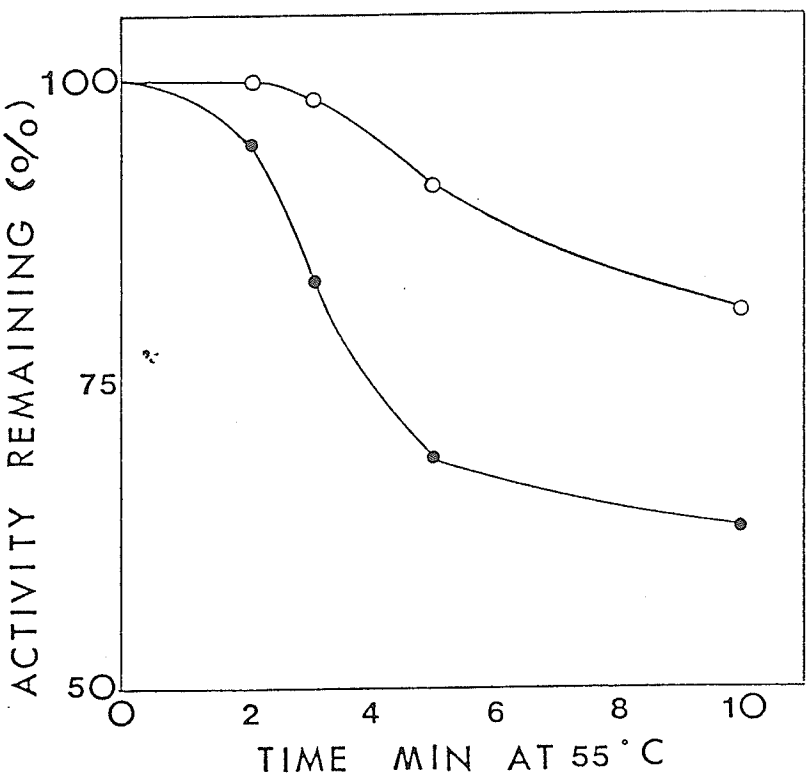

Figure 13. The effect of heat inactivation at 55°C on phosphocholine phosphatase and alkaline phosphatase activities.

Microsomal preparations were incubated at 55°C for 0-10 min and the ability of the incubated microsomal preparation to hydrolyze p-nitrophenylphosphate (O) and phospho[Methyl-^{3}H]choline (●) determined as described in Experimental Procedures. The points represent the mean of two separate sets of experiments, each of which was assayed in duplicate.

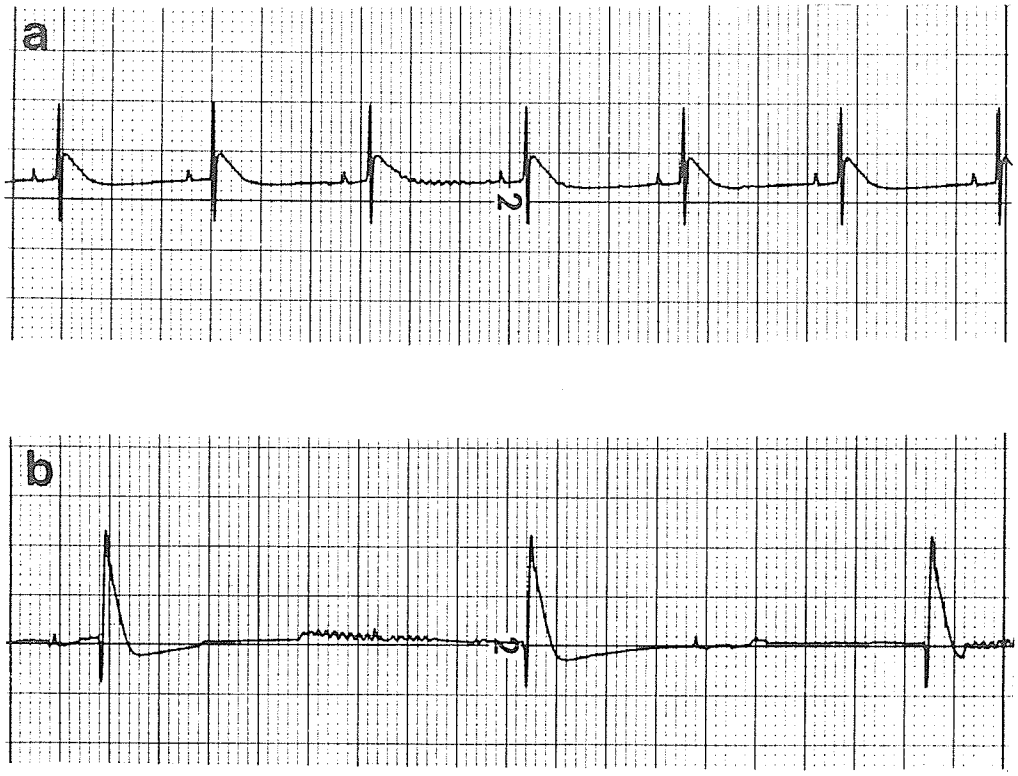

Figure 14. Electrocardiograms of control and hearts perfused under hypoxic conditions.

Hamster hearts were perfused for 60 min as described in Experimental Procedures. A. Perfusion with 95% O_2-saturated buffer (control). B. Perfusion with 95% N_2-saturated buffer (hypoxic).

electrophysiological damage. Thus, all experiments were limited to hypoxic exposure for 60 min or less. Electron micrographic analysis of the heart revealed some degree of mitochondrial swelling (arrows) after 60 min of perfusion with 95% N_2-saturated buffer (Figure 15b) when compared to the control (Figure 15a). The mitochondrial swelling was shown to be extensively reversible upon reoxygenation (112). There were no significant changes in the morphology of the other organelles.

II. Effect of hypoxia on the labeling of choline-containing metabolites of the CDP-choline pathway

Hamster hearts were perfused with [Methyl-^{3}H]choline for 30 min in 95% O_2-saturated Krebs-Henseleit buffer. Subsequently, the hearts were perfused with 95% N_2-saturated buffer for 30,45 and 60 min. Hamster hearts perfused with 95% O_2-saturated buffer after pulse-labeling were used as controls. There was no significant difference in the amount of labeling of choline, phosphocholine, CDP-choline and phosphatidylcholine between the hearts perfused with 95% N_2-saturated buffer (hypoxic) and 95% O_2-saturated buffer (control) during 30 or 45 min of chase (Figures 16,17). Perfusion of hamster hearts for 60 min under hypoxic conditions did not change the total amount of radioactivity in the heart, but resulted in a decrease in the labeling of phosphatidylcholine (Figure 16). Analysis of the aqueous choline-containing metabolites revealed that the labeling of

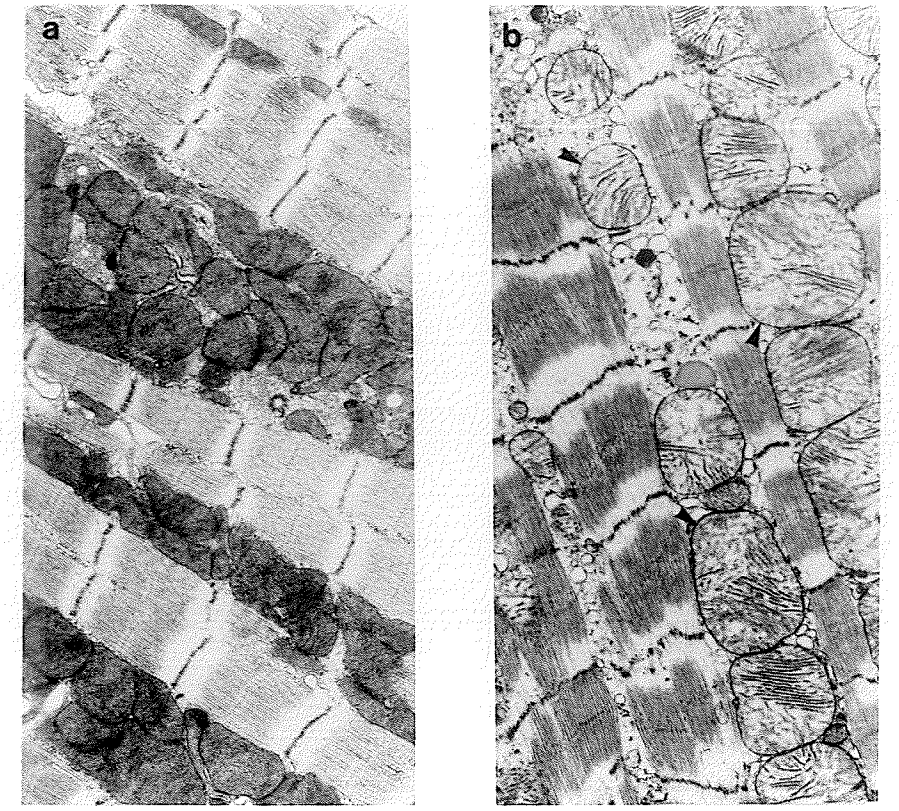

Figure 15. Electron micrographs of control and hearts perfused under hypoxic conditions.

Hamster hearts were perfused for 60 min as described in Experimental Procedures. A. Perfusion with 95% O_2-saturated buffer (control). B. Perfusion with 95% N_2-saturated buffer (hypoxic). The magnification was 19,300 X.

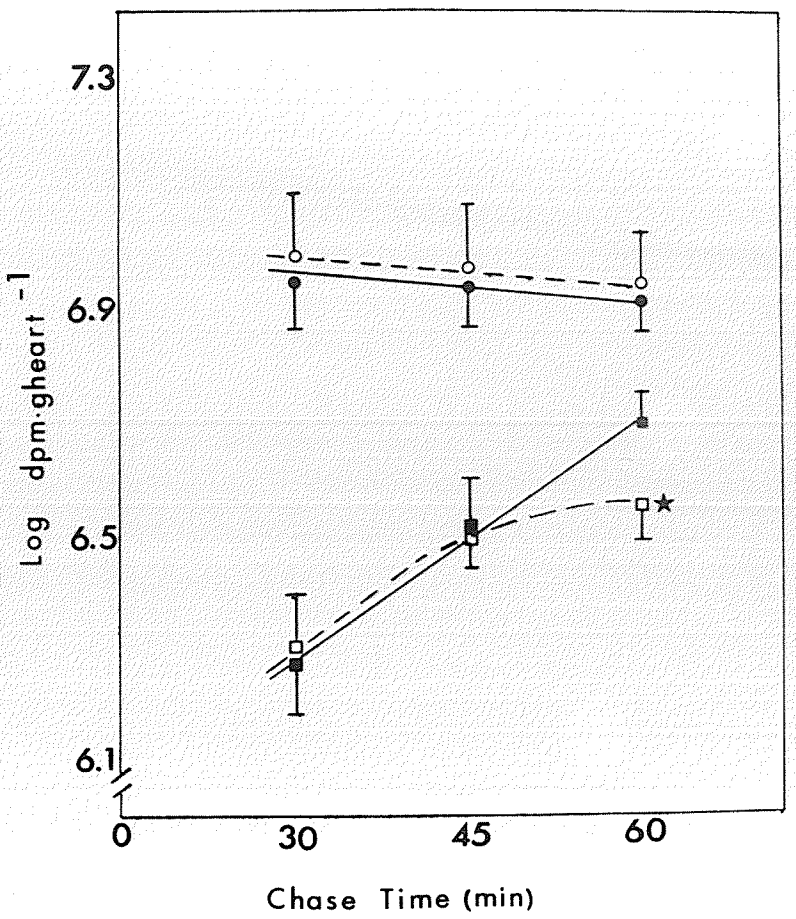

Figure 16. Total radioactivity and radioactivity in phosphatidylcholine in control and hearts perfused under hypoxic conditions.

Hamster hearts were perfused for 30,45 and 60 min with 95% O_2-saturated buffer (control) or 95% N_2-saturated buffer (hypoxic) as described in Experimental Procedures. Total radioactivity (●, ○) and radioactivity in phosphatidylcholine (■, □) were determined. The open symbols indicate hearts perfused with 95% N_2-saturated buffer and the closed symbols represent hearts perfused with 95% O_2-saturated buffer. The vertical bars represent the standard deviation of at least three hearts. *$P<0.05$.

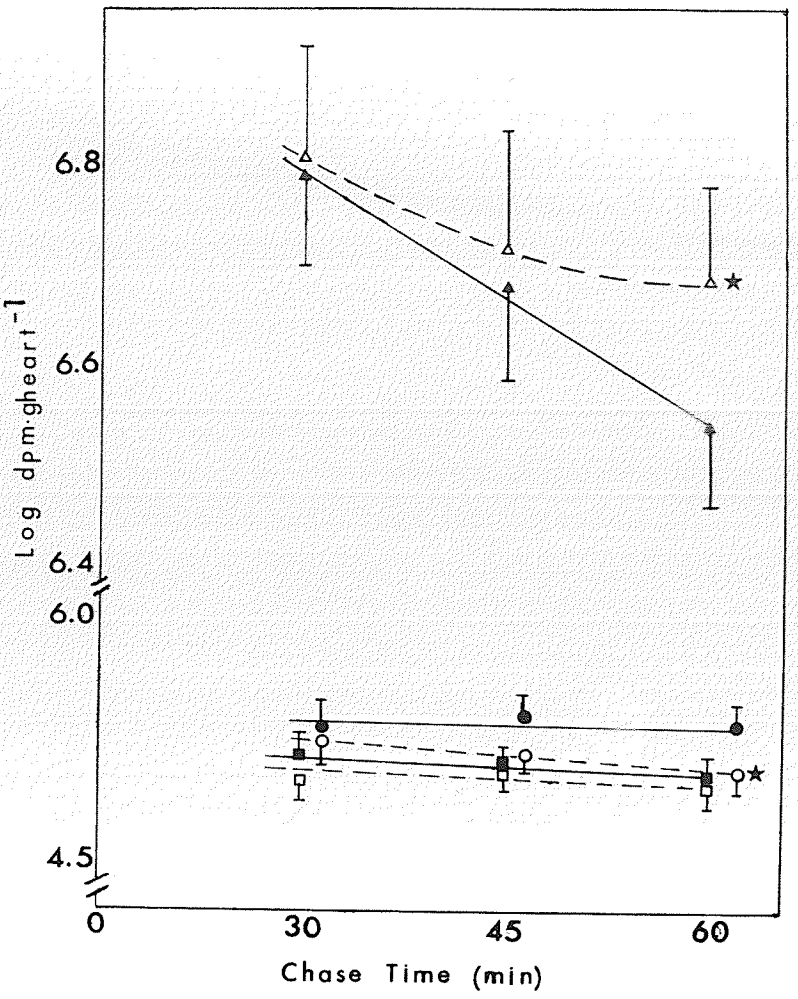

Figure 17. Radioactivity in the choline, phosphocholine and CDP-choline fractions in control and hearts perfused under hypoxic conditions.

Hamster hearts were perfused for 30, 45 and 60 min with 95% O_2-saturated buffer (control) or 95% N_2-saturated buffer (hypoxic) as decribed in Experimental Procedures. Radioactivity in choline (■, □), phosphocholine (▲, △) and CDP-choline (●, ○) determined. The open symbols indicate hearts perfused with 95% N_2-saturated buffer and the closed symbols represent hearts perfused with 95% O_2-saturated buffer. The vertical bars represent the standard deviation of at least three hearts. *$P<0.05$.

phosphocholine was increased during hypoxic treatment with a simultaneous decrease in the labeling of CDP-choline (Figure 17). There was no change in the labeling of choline at all times of perfusion (Figure 17). The increased radioactivity in the phosphocholine fraction quantitatively accounted for the decrease in radioactivity in the CDP-choline and phosphatidylcholine fractions (Figure 16,17). The sum of the radioactivities recovered from the choline, phosphocholine, CDP-choline and phosphatidylcholine fractions accounted for over 98% of the total radioactivity in the homogenate. Significant but equal amounts of radioactivity were found in the perfusate of both groups of hearts during the chase. The loss of some labeled choline into the perfusate has been previously demonstrated in the isolated perfused hamster heart (41).

III. Effect of hypoxia on the enzymes of the CDP-choline pathway

The reduction in the labeling of CDP-choline, phosphatidylcholine and the increase in phosphocholine labeling at 60 min of perfusion under hypoxic conditions might be caused by some change(s) in the activities of the enzymes of the CDP-choline pathway. Hence, the specific activities of these enzymes were examined. No difference was detected in the specific activities of choline kinase, and cholinephosphotransferase between control and hearts perfused under hypoxic conditions for 60 min (Table 11). In

Table 11

Activities of the enzymes of the CDP-choline pathway and phosphocholine phosphatase in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95% O_2-saturated buffer (control) or 95% N_2-saturated buffer (hypoxic). Subsequent to perfusion enzyme activities in the appropriate subcellular fraction were determined as described in Experimental Procedures.

	Enzyme activities	
	Control	Hypoxic
	nmol/min/mg protein	
Choline kinase (cytosol)	0.52±0.09(4)	0.47±0.09(4)
Phosphocholine transferase (microsomal)	1.04±0.04(4)	1.01±0.09(4)
Phosphocholine cytidylyltransferase (microsomal)	0.63±0.24(11)	1.31±0.52(10)*
Phosphocholine cytidylyltransferase (cytosol)	0.57±0.21(11)	0.48±0.24(10)
Phosphocholine cytidylyltransferase (cytosol + lipid)	1.76±0.29(11)	1.23±0.24(10)*
Phosphocholine phosphatase (microsomal)	2.94±0.17(3)	2.88±0.10(3)

Table 11 (continued)

	nmol/min/g heart	
Phosphocholine cytidylyltransferase (microsomal)	9.30±2.86(11)	16.63±6.59(10)*
Phosphocholine cytidylyltransferase (cytosolic)	23.04±3.37(11)	16.31±3.66(10)*

*P<0.05.

addition, the specific activity of phosphocholine phosphatase for the hydrolysis of phosphocholine was unchanged (Table 11). The specific activity of CTP:phosphocholine cytidylyltransferase was increased in the microsomal fraction but not changed in the cytosolic fraction. of hypoxic hearts compared to control (Table 11). Since the cytosolic cytidylyltransferase has lipid requirements (82,83) the addition of lipids to the assay produced a general increase in the specific activity of the enzyme. In the presence of these lipids a smaller degree of increase in the cytosolic cytidylyltransferase activity was observed in the hearts perfused under hypoxic conditions as compared to control. To determine whether there was a redistribution of the cytidlyltransferase from the cytosolic to the microsomal fraction, the total cytidylyltransferase activity in both the cytosolic and microsomal fractions were estimated. The total cytosolic cytidylyltransferase activity was calculated based upon complete activation of the enzyme with the addition of lipids. The microsomal enzyme activity was calculated based upon yield of the microsomal fraction from the homogenate. Under hypoxic conditions the total activity of cytidylyltransferase was found to increase in the microsomal fraction with a corresponding decrease in the cytosolic fraction (Table 11). However, the sum of the enzyme activities in these two fractions was similar to the control. These results clearly indicate that a redistribution of cytidylyltransferase occurred in hearts

perfused under hypoxic conditions. Since the microsomal form of cytidylyltransferase has been regarded as the active form of the enzyme (72), an increase in total microsomal enzyme activity would imply a corresponding increase in CDP-choline synthesis. Obviously, such change in cytidylyltransferase activity during hypoxia did not explain the increase in the labeling of phosphocholine nor the decrease in labeling of CDP-choline and phosphatidylcholine.

IV. Effect of hypoxia on the pool sizes of aqueous choline-containing metabolites

Changes in the pool sizes of aqueous choline-containing metabolites might affect the specific radioactivities of these intermediates which may subsequently affect the labeling of phosphatidylcholine. Thus, the pool sizes of choline, phosphocholine and CDP-choline were determined in unperfused hearts, hearts perfused with 95% O_2-saturated buffer and 95% N_2-saturated buffer. There was no significant change in the pool sizes of the choline-containing metabolites between the three groups of hearts (Table 12). Clearly the decrease in labeling of phosphatidylcholine in hearts perfused under hypoxic conditions was not caused by a change in the pool size of the choline-containing metabolites of the CDP-choline pathway.

V. Effect of hypoxia on CDP-choline catabolism

Since CDP-choline is the immediate precursor of

Table 12

Choline, phosphocholine and CDP-choline concentration in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95% O_2-saturated buffer or 95% N_2-saturated buffer. The pool sizes of the choline-containing metabolites were determined as described in Experimental Procedures.

	Before perfusion	Perfusion with 95% O_2 saturated buffer	Perfusion with 95% N_2 saturated buffer
		nmol/g heart	
Choline	151±10(3)	137±21(4)	141±11(3)
Phosphocholine	230±20(3)	203±19(3)	201±24(4)
CDP-choline	100±12(4)	106±8(4)	105±25(4)

phosphatidylcholine, the cause for the decrease in the labeling of CDP-choline in hearts perfused under hypoxic conditions was investigated. One possible explanation for the decrease in CDP-choline labeling was that the catabolism of CDP-choline might be different under hypoxic conditions. To investigate this possibility, homogenates from the control and hearts perfused under hypoxic conditions were incubated with labeled CDP-choline. The percent radioactivity in the aqueous and organic metabolites were determined. There were no significant differences in the labeling of these metabolites between the two groups (Table 13). These results indicate that the decrease in labeling of CDP-choline during hypoxia was not caused by a change in its catabolism.

VI. Effect of hypoxia on ATP and CTP level

Since ATP and CTP are required co-factors for phosphatidylcholine biosynthesis via the CDP-choline pathway, a change in the levels of these nucleotides may affect the labeling of phosphocholine and CDP-choline. Thus, ATP and CTP levels in hearts perfused under hypoxic conditions were determined and compared with control. There was a decrease with time in the level of both ATP and CTP under hypoxic conditions (Table 14). Although the level of ATP was reduced (61%) at 60 min of hypoxia, the conversion of choline to phosphocholine was not affected since the labeling in choline was not affected (Figure 17). However,

Table 13

CDP-choline metabolism in homogenates from control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95% O_2-saturated (control) or 95% N_2-saturated buffer (hypoxic). Subsequent to perfusion, a 10% tissue homogenate (w/v) in 0.25 M sucrose was prepared. Aliquots of the homogenates (1.5 mg protein) were incubated with CDP-[Me-^{14}C]choline for 15 min. After incubation 2 ml of $CHCl_3$:CH_3OH (2:1 v/v) was added to stop the reaction. Phase separation was caused by the addition of 1 ml water to the mixture. The aqueous phase was separated from the organic phase by centrifugation. The metabolites in each phase were analyzed by thin-layer chromatography as described in Experimental Procedures. Results are the average of two separate experiments.

	Control	Hypoxic
	% radioactivity recovered	
CDP-choline	90.0	89.4
Phosphocholine	0.6	0.8
Choline	0.2	0.3
Others in aqueous phase	2.2	2.2
Phosphatidylcholine	0.2	0.1
Sphingomyelin	<0.1	<0.1
Others in organic phase	0.2	0.1

Table 14

ATP and CTP concentrations in control and hypoxic hearts

Hamster hearts were perfused for 30 or 60 min with 95% O_2-saturated buffer (control) or 95% N_2-saturated buffer (hypoxic). Subsequent to perfusion, ATP and CTP levels were determined as described in Experimental Procedures.

	Control	Hypoxic
ATP concentration		
	μmoles/ g heart	
30 min	2.70±0.79(3)	1.27±0.41(3)*
60 min	2.54±0.16(3)	0.99±0.09(3)*
CTP concentration		
	nmoles/ g heart	
30 min	11.80±1.84(3)	7.03±0.55(3)*
60 min	11.49±2.24(3)	3.25±1.12(3)*

*$P<0.05$.

the reduction in CTP (72%) at 60 min of hypoxia (Table 14), might be one of the factors which caused the reduced conversion of labeled phosphocholine to CDP-choline and consequently, resulted in the accumulation of radioactivity in phosphocholine.

VII. Effect of hypoxia on the rate of phosphatidylcholine biosynthesis

The rate of phosphatidylcholine biosynthesis in the heart was estimated from the specific radioactivity of CDP-choline and the radioactivity incorporated into phosphatidylcholine within a period of time. From the average labeling of CDP-choline during a 30-60 min chase and the pool sizes of CDP-choline, the specific radioactivities of CDP-choline from control and hearts perfused under hypoxic conditions were determined. The specific radioactivity of CDP-choline in hearts perfused under hypoxic conditions was found to be substantially lower than that of the control (Table 15). The rate of phosphatidylcholine biosynthesis in the control hearts was estimated to be 41.0 nmoles/min/g and that of hearts perfused under hypoxic conditions 42.6 nmoles/min/g (Table 15). Clearly, the overall rate of phosphatidylcholine biosynthesis was not changed during hypoxia. The lowered amount of label incorporated into phosphatidylcholine in the hypoxic hearts was a direct result of the lowered specific radioactivity of CDP-choline.

Table 15

Rate of phosphatidylcholine biosynthesis in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95% O_2-saturated (control) or 95% N_2-saturated buffer (hypoxic). The rate of phosphatidylcholine biosynthesis was calculated from the average specific radioactivity of CDP-choline and the radioactivity incorporated into phosphatidylcholine during a 30-60 min chase.

	Specific radioactivity of CDP-choline	Amount of Phosphatidyl-choline formed
	dpm/nmol/g heart	nmol/min/g heart
Control	2264	41.5
Hypoxic	1142	42.6

VIII. Effect of hypoxia on phospholipid and fatty acid content

The mechanism for the increase in microsomal cytidylyltransferase activity in hearts perfused under hypoxic conditions was investigated. Cytidylyltransferase has been shown to be activated by a number of lipids (82,83,113,114). The possibility that hypoxia caused a change in the cardiac lipid content was investigated. The hearts were perfused with 95% O_2-saturated buffer or 95% N_2-saturated buffer for 60 min, and the phospholipid contents were determined. There was no significant difference in phospholipid content between the two experimental groups (Table 16). The increase in microsomal cytidylyltransferase activity under hypoxic conditions could not be attributed to a change in the cardiac phospholipid content.

Fatty acids have been shown to activate cytidylyltransferase (114) and long chain fatty acids (>C8:0) may also promote the translocation of the enzyme from cytosolic to microsomal fraction (74). The change in fatty acid content in the homogenate and the cytosol of control and hearts perfused under hypoxic conditions were investigated. There was no significant change in the fatty acid content in the homogenate between the two groups. Further analysis revealed a 2-fold increase in fatty acid content in the cytosolic fractions of the hearts perfused under hypoxic conditions compared to control (Table 17). The

Table 16

Phospholipid content in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95% O_2-saturated buffer (control) or 95% N_2-saturated buffer (hypoxic). Subsequent to perfusion, the phospholipid content was determined as described in Experimental Procedures.

Phospholipid species	Control	Hypoxic
	μmol lipid-Pi/g heart	
Phosphatidylcholine	13.14±1.39(3)	12.85±1.12(3)
Phosphatidylethanolamine	10.41±1.18(3)	9.61±0.55(3)
Sphingomyelin	1.85±0.09(4)	1.97±0.23(4)
Lysophosphatidylcholine	0.18±0.07(4)	0.16±0.06(4)
Lysophosphatidyl-ethanolamine	0.12±0.04(4)	0.13±0.03(4)
Phosphatidylserine + Phosphatidylinositol	1.32±0.06(4)	1.41±0.17(4)
Phosphatidylglycerol + Cardiolipin	3.58±0.69(4)	3.74±0.62(4)

Table 17

Fatty acid content and composition in cytosol of control and hypoxic hearts.

Hamster hearts were perfused for 60 min with 95% O_2-saturated (control) or 95% N_2-saturated buffer (hypoxic). Subsequent to perfusion, the hearts were homogenized and subcellular fractions prepared. The fatty acid content and composition of the cytosolic fraction were determined as described in Experimental Procedures.

Fatty acid	Control	Hypoxic
	nmol/g heart	
	18.9±6.2(3)	37.5±5.2(3)*
Acyl groups	% total (n=3)	
10:0	23.3±5.1	18.7±3.1
12:0	13.2±2.7	13.3±0.6
14:0	0.7±0.4	0.8±0.4
16:0	14.9±3.1	13.7±2.6
16:1	0.7±0.3	0.7±0.4
18:0	8.1±2.7	11.0±2.1
18:1	7.5±1.1	5.0±1.9
18:2	1.4±0.2	1.4±0.8
18:3	0.2±0.1	0.3±0.1
20:4	<0.1	<0.1
Others	29.9±8.2	35.0±6.4

*$P<0.05$

composition of the fatty acid species in the cytosolic fraction of the two groups were examined. There was no change in the percent distibution of acyl species between the two groups (Table 17). The higher fatty acid content in the cytosolic fraction of hearts perfused under hypoxic conditions results from a general increase in all acyl species.

DISCUSSION

A. Effect Of Amino Acids On Choline Uptake And Phosphatidylcholine Biosynthesis In Hamster Heart

In mammalian tissues, choline is actively taken up by a low-affinity sodium-independent system (115). In the hamster heart choline is actively taken up and the rate of uptake is linear from 5-60 min and between 0.1 and 50 uM choline (41). Exogenous ethanolamine in the perfusate competitively inhibited choline uptake in the hamster heart (61). The amino acid glycine, found in significant quantities in hamster plasma (0.4-0.5 mM) (87) , was postulated to inhibit choline uptake in a fashion similar to ethanolamine since it is structurally analogous to ethanolamine. Clearly, glycine did not inhibit choline uptake nor its subsequent conversion to phosphatidylcholine. Glycine at 1.0 mM concentration actually caused an increase in choline uptake but did not stimulate phosphatidylcholine biosynthesis. This is the first demonstration of enhanced choline uptake in the heart. This increase in choline uptake was most apparent at 45 and 60 min of perfusion. The inability of glycine to stimulate phosphatidylcholine biosynthesis was further substantiated by analysis of the enzyme activities of the CDP-choline pathway. The activities of the three biosynthetic enzymes were not altered in hearts perfused with glycine.

The enhanced amount of radioactivity taken up by the heart in the presence of glycine was quantitatively accounted for in the phosphocholine fraction. Choline, upon

entering the heart is rapidly phosphorylated to phosphocholine by choline kinase (41). The accumulation of radioactivity at the phosphocholine level is not surprising, as CTP:phosphocholine cytidylyltransferase, the enzyme that converts phosphocholine to CDP-choline, has been shown to be the rate limiting step of the CDP-choline pathway in hamster heart (41) and many mammalian systems (67).

The effect of other amino acids on choline uptake was investigated to determine whether the enhancement of choline uptake was specific for glycine or a general phenomenon displayed by all amino acids. Neutral amino acids were found to enhance choline uptake and the presence of more than one neutral amino acid (glycine and L-alanine) did not produce a synergistic effect. L-alanine enhanced choline uptake at 0.1-50 mM concentration and the enhancement of choline uptake was significant at 30, 45 and 60 min of perfusion. Neutral amino acid transport system A has been demonstrated to be adaptively regulated by the presence of a neutral amino acid added to the incubation medium of chick embryo heart cells prior to the measurement of transport activity (106,107). Adaptive regulation of the amino acid transport system A did not influence the enhancement of choline uptake by L-alanine. The basic and acidic amino acids did not influence choline uptake or phosphatidylcholine biosynthesis. As the neutral amino acids are found in significant concentrations (0.1-1.0 mM) in hamster plasma (116), these amino acids may play a role in the regulation

of choline uptake in the heart. Although the neutral amino acids have no immediate effect on phosphatidylcholine biosynthesis, the long-term effect on such synthesis is not known. Such an investigation could be performed in isolated cardiac myocytes.

Neutral amino acids at 0.1 mM but not 0.05 mM concentration are effective in the modulation of choline uptake. It was intriguing that above the 0.1 mM concentration threshold, enhancement of choline uptake was no longer dependent on the concentration of amino acid present in the perfusate. These observations, together with the fact that choline did not alter the rate of labeled glycine or L-alanine uptake, suggests that the amino acids and choline are not cotransported by the same transport site. Maximal enhancement of choline uptake by neutral amino acids was typically between 20-35%, and the typical standard deviations between experiments were 10-15% of the mean. A dose-dependent response between the narrow 0.05 and 0.1 mM concentration range could not be demonstrated, owing to the limitations of these experimental conditions. The isolated cardiac myocyte model may again not be subject to these limitations, and could provide further insight.

At present, the precise biochemical mechanism for the modulation of choline uptake by the neutral amino acids is not known. Amino acids are transported across the membrane by a large number of sodium-dependent and sodium-independent

systems (117). These systems are widespread and considerable crossover for amino acid transport by these systems has been demonstrated (117). It is possible that the modulation of choline uptake is facilitated by coupling of the choline transport site with one of the amino acid transport systems, since the degree of enhancement of choline uptake by different neutral amino acids was remarkably similar. However, the demonstration that choline did not influence labeled glycine or L-alanine uptake, together with the finding that adaptive regulation of system A did not influence the enhancement of choline uptake, makes this possibility unlikely. The direct modulation of the choline transport site by neutral amino acids to enhance choline uptake is a more intriguing explanation. The elucidation of the exact mechanism may be dependent upon purification of the choline transporter. Many heroic attempts have been made to solubilize and purify the choline transport system with limited success. The choline transport system has been solubilized from rat brain synaptosomes and reincorporated into artificial membranes (118). However, the major drawback of such experimental approach is that it is unknown what precisely is in the solubilized material. In addition, a reliable assay for the choline transport system is unavailable at the present time.

The influence of amino acids on phosphatidylcholine biosynthesis has been documented (119). L-Lysine was shown

to stimulate the incorporation of [^{14}C]choline into phosphatidylcholine in renal cortical slices. Amino acids clearly have no immediate effect on phosphatidylcholine biosynthesis in the hamster heart. As in the case of glycine, the increase in choline uptake in neutral amino acid perfused hearts was found to accumulate in the phosphocholine fraction. This accumulation was not caused by an increase in the phosphorylation of choline since the radioactivity in choline and choline kinase activity remained unchanged. Nor was the conversion of phosphocholine to CDP-choline inhibited since cytidylyltransferase activity was unchanged. Finally, the accumulation was not caused by the inhibition of dephosphorylation of phosphocholine since L-alanine did not affect phosphocholine phosphatase activity.

B. Phosphocholine Phosphatase And Alkaline Phosphatase Are Different Enzymes In Hamster Heart

Some of the phosphocholine formed in the isolated perfused hamster heart was postulated to be hydrolyzed by an undefined phosphatase (41,62). In HeLa cells phosphocholine phosphatase and alkaline phosphatase were postulated to be the same enzyme (90). It was intriguing that 50 mM L-alanine did not inhibit the hydrolysis of labeled phosphocholine as high concentrations of this amino acid are known to inhibit alkaline phosphatases (109). Thus, the possibility that the alkaline phosphatase and phosphocholine phosphatase are different enzymes in hamster

heart was investigated.

It is clear that the two activities in hamster heart microsomes are modulated differently *in vitro*. This was demonstrated by the the fact that L-alanine inhibited alkaline phosphatase but not phosphocholine phosphatase activity. In addition, the degree of inhibition of the two enzymes in the presence of L-phenylalanine was different. Finally, the differences in pH profiles and responses to heat treatment suggest that the two activities originate from two separate and distinct enzymes.

Alkaline phosphatase from mammalian sources exists in at least three distinct categories: the placenta type, the intestinal type and the liver-bone-kidney type (120). They differ from each other by their responses to uncompetitive inhibition by specific amino acids, heat stability and pH optima at specific substrate concentrations. The cardiac alkaline phosphatase belongs to the liver-bone-kidney type (121,122). It has also been reported that the liver-bone-kidney type in the heart may exist in multiple molecular forms, and these forms have different properties (120-122). Hence, it can be argued that the observed differences between phosphocholine phosphatase and alkaline phosphatase result from a broad specificity of one specific form of alkaline phosphatase. However, this is not supported by the kinetic data of phosphocholine phosphatase in the presence of p-nitrophenylphosphate where this compound produced a

"mixed-type" of inhibition. In addition, the fact that phosphocholine did not inhibit alkaline phosphatase activity does not support this supposition. Definitive proof that the two enzymes are distinct will await the purification of the enzymes to homogeneity.

The ability to hydrolyze phosphocholine has been reported in the liver (123), intestinal cells (124) and HeLa cells (90), but such a reaction was postulated to be of limited importance in the regulation of the phosphocholine pool or phosphatidylcholine biosynthesis in these tissues (90). However, the pool of phosphocholine in the heart (0.23 mM) is much lower than that of the liver (1.3 mM) or HeLa cells (1.8 mM) (67). In the heart, the diminished pool size of phosphocholine implies that the metabolism of this important metabolite of the CDP-choline pathway may be regulated differently. In view of the fact that the apparent Km (0.56 mM) of the enzyme for phosphocholine is much greater than the pool of phosphocholine in the heart, it is possible that one of the major functions of the cardiac phosphocholine phosphatase is to prevent the accumulation of a large phosphocholine pool. However, only a small fraction of labeled phosphocholine is actually hydrolyzed back to choline in the perfused hamster heart (41). The apparent discrepancy between the present <u>in vitro</u> and the perfusion studies (41) might be explained by the finding that the enzyme displayed high activity only at alkaline pH, and its

activity was substantially attenuated at physiological pH. These results suggest that the *in vivo* dephosphorylation of phosphocholine by phosphocholine phosphatase may only be a minor pathway for the metabolism of phosphocholine.

C. The Effect Of Hypoxia On Phosphatidylcholine Biosynthesis

The objective of this study was to investigate the effect of a lowered energy status on phosphatidylcholine biosynthesis in the heart. To produce a rapid decrease in the level of ATP and CTP, the "hypoxic" model was employed for this study (88). The isolated heart was perfused under hypoxic conditions for up to 60 min. These conditions resulted in some electophysiological and morphological changes which were reversible upon reoxygenation (112). Perfusion under hypoxic conditions for more than 60 min would inevitably produce further reductions in ATP and CTP levels, but would also generate irreversible damages to the cardiac tissue (125).

A significant decrease in cardiac ATP level was observed at 30 and 60 min of hypoxic perfusion. However, the conversion of choline to phosphocholine was not affected since there was no change in the labeling of choline in all instances. In addition, the pool sizes of choline and phosphocholine and choline kinase activity were unchanged at 60 min of hypoxic perfusion. Although it can be predicted that a severe reduction in ATP level would certainly curtail the formation of phosphocholine, it is clear that such

formation was not affected by up to a 61% reduction in the level of ATP.

A 40% reduction in the levels of CTP was observed at 30 min of hypoxic perfusion, yet the conversion of phosphocholine to CDP-choline (and the synthesis of phosphatidylcholine) were unaffected. This was surprising since a 34% reduction of the CTP level in the myopathic hamster heart was shown to have a profound effect on the formation of CDP-choline and triggered the activation of cytidylyltransferase (65). The difference between these two findings remains undefined. One plausible explanation is that in cardiomyopathy the lowering of the CTP was instilled through a prolonged period of time. The prolonged decrease in CTP level might contribute to a significant decrease in the synthesis of CDP-choline and might thereby initiate the adaptive changes in cytidylyltransferase activity. Although the level of CTP was also reduced at 30 min of hypoxic perfusion, such reduction took place within a relatively short period and did not produce any significant effect on the synthesis of CDP-choline. Since the formation of CDP-choline was not affected, no compensatory action of cytidylyltransferase was required. When the level of CTP was further deminished for a longer period of time (at 60 min hypoxic perfusion), CDP-choline synthesis was significantly reduced. Consequently, cytidylyltransferase activity was increased as a compensatory mechanism to maintain the

synthesis of CDP-choline.

The mechanism for the increase in cytidylyltransferase activity at 60 min of hypoxic perfusion was investigated. It is generally accepted that the microsomal form of the enzyme is the active form and the cytosolic form may serve as a storage form (72). The determination of the specific and total activities of the cytidylyltransferase in the subcellular fractions clearly indicates that there was a translocation of the enzyme from the cytosolic to the microsomal fraction in the hypoxic heart. This view was further supported by the fact that the sum of the enzyme activities of the microsomal and cytosolic fractions of the hypoxic heart was very similar to the control heart. Two related mechanisms have been proposed for the translocation of cytidylyltransferase activity: enzyme phosphorylation-dephosphorylation (85) and translocation induced by lipids (74). At present, direct evidence for enzyme phosphorylation-dephosphorylation is still lacking, but enzyme translocation mediated by fatty acids has been repeatedly demonstrated (74-77,126,127). To determine whether fatty acids promoted enzyme translocation of cytidylyltransferase in the hypoxic heart, the fatty acid content in the hearts were determined. There was no significant change in the total fatty acid content in the homogenate. Further analysis revealed that there was a significant increase in fatty acid content in the cytosol. Due to the fact that the cytosolic fatty acid content was

very low when compared with total fatty acid in the homogenate, changes in fatty acid content in the cytosol represent a very small change within the total fatty acid content in the homogenate. Hence, it is not surprising to detect changes in cytosolic fatty acid contents while no detectable changes was observed in the homogenate. These results suggest that the translocation of the enzyme activity during hypoxia was probably caused by the elevated levels of the fatty acids in the cytosolic compartment. The elevation of fatty acid level during hypoxia has been well documented (89,128).

Analysis of the cytosolic fatty acid compositions in the control and hypoxic hearts indicates that the distribution of the fatty acid species were similar between the two groups. If the elevation of the fatty acid levels was indeed the mechanism which caused the translocation of the cytidylyltransferase, it would have been interesting to determine the specific fatty acid(s) which might have caused the enzyme translocation in the hypoxic heart. It was not possible to identify the fatty acid(s) responsible for the translocation process since there was a general increase in all fatty acid species during hypoxia. In addition, attempts to study enzyme translocation by the fatty acids <u>in vitro</u> were not successful. Previously, it was demonstrated that exogenous addition of fatty acids has no effect on the activity of the hamster heart enzyme when assayed <u>in vitro</u>

(86).

It was interesting to note that in the isolated hamster heart, the rate of phosphatidylcholine biosynthesis was maintained for at least 60 min when there was a major reduction of the cardiac triphosphate nucleotide levels. This study amply illustrated the compensatory ability of the CDP-choline pathway under short term hypoxia. It is quite likely that the irreversible membrane damage during prolonged hypoxia was partly contributed by the inability of the organ to sustain the synthesis of phosphatidylcholine and other phospholipids. Further reduction of the ATP levels beyond 60 min of hypoxia might have exceeded the adaptive ability of the CDP-choline pathway, and consequently, resulted in the impairment of phosphatidylcholine biosynthesis.

SUMMARY AND FUTURE PERSPECTIVES

At present, at least three tiers of control of phosphatidylcholine biosynthesis in the heart have been identified:-(1) at the level of choline uptake, (2) the energy status of the organ and (3) modulation of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase. The work presented in this thesis dealt with all three of these control mechanisms. The results of the amino acid perfusion studies clearly showed that choline uptake by the heart can be enhanced. Despite the enhancement of choline uptake, phosphatidylcholine biosynthesis in the heart was not affected. In addition, using amino acid inhibitors, the existence of a separate and distinct enzyme for the hydrolysis of phosphocholine was identified in the hamster heart. The results of the hypoxic study clearly demonstrated that the rate of phosphatidylcholine biosynthesis was maintained in the short term when the energy status of the heart was lowered. The translocation of cytidylyltransferase from the cytosolic to the microsomal fraction represents a mechanism for such maintanance.

It must be stressed that the amino acids constitute only a minute fraction of compounds present in the serum, many of which have escaped investigation as to their effect on choline uptake and phosphatidylcholine biosynthesis. These compounds range from small molecules to macromolecules and hormones which may involve signal transduction and thus may

affect many processes in concert with phospholipid biosynthesis. Such compounds would have their specific effect at the level of the cell membrane. In view that the content of phosphatidylcholine rarely changes in a specific membrane, the biosynthetic and catabolic processes must be highly coordinated. Thus, during hypoxia there must be some compensatory changes in the catabolism of phosphatidylcholine, in addition to those of biosynthesis, if the heart is to maintain the composition of the plasma membrane under such an insult for a prolonged period of time.

Over the last several decades since the discovery of the CDP-choline pathway by Kennedy and co-workers, considerably exciting information has been obtained regarding the regulation of phosphatidylcholine biosynthesis. Such information has provided both insight and a framework for future investigation.

REFERENCES

1. Lehninger, A.L. (1976) in: Biochemistry 2nd ed. Worth Pub. Inc., New York. pp. 304-305.

2. Cullis, P.R. and Hope, M.J. (1985) in: Biochemistry of Lipids and Membranes. (Vance, D.E. and Vance, J.E. eds.) Benjamin/Cummings Pub. Co. Inc., California. pp. 25-72.

3. Ansell, G.B. and Spanner, S. (1982) in: Phospholipids. (Hawthorne, J.W. and Ansell, G.B. eds.) Elsevier Biomedical Press., Amsterdam. pp. 1-49.

4. Arthur, G., Mock, T., Zaborniak, C. and Choy, P.C. (1985) Lipids 20, 693-698.

5. Wientzek, M., Man, R.Y.K. and Choy, P.C. (1987) Biochem. Cell Biol. 65, 860-868.

6. White, D.A. (1973) in:Form and Function of Phospholipids. (Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C., eds.) Elsevier Sci. Pub. Co., Amsterdam. pp. 441-482.

7. Stryer, L. (1981) in: Biochemistry 2nd ed. W.H. Freeman and Co. Pub., San Francisco. pp. 205-231.

8. Coleman, R. (1973) Biochim. Biophys. Acta 300, 1-30.

9. Rooney, S.A., Canavan, P.M. and Motoyama, E.K. (1974) Biochim. Biophys. Acta 360, 56-67.

10. Clements, J.A. (1977) Amer. Rev. Resp. Dis. 115, 67-71.

11. Trewhella, M.A. and Collins, F.D. (1973) Biochim. Biophys. Acta 296, 51-61.

12. Young, D.L. and Schersten, T. (1969) J. Lipid Res. 13,

244-252.

13. Sekuzu, I, Jurtshuk, P. and Green, D.E. (1963) J. Biol. Chem. 238, 975-981.

14. Vance, D.E. and Choy, P.C. (1979) Trends Biochem. Sci. 4, 145-148.

15. Kennedy, E.P. (1962) Harvey Lect. 57, 143-171.

16. Bremer, J. and Greenberg, J. (1961) Biochim. Biophys. Acta 46, 205-216.

17. Mansbach, C.M. and Parthasarathy, S. (1979) J. Biol. Chem. 254, 9688-9694.

18. Dils, R.R. and Hubscher, G. (1961) Biochim. Biophys. Acta 46, 205-216.

19. Lands, W.E.M. (1960) J. Biol. Chem. 253, 2233-2237.

20. Erbland, J.E. and Marinetti, G.V. (1965) Biochim. Biophys. Acta 106, 128-138.

21. Schneider, W.C. (1963) J. Biol. Chem. 238, 3572-3578.

22. Wilson, J.E. (1978) Trends Biochem. Sci. 3, 124-125.

23. Choy, P.C., Schneider, W.J. and Vance, D.E. (1978) Eur. J. Biochem. 85, 185-193.

24. Weinhold, P.A., Rounsifer, M.E. and Feldman, D.A. (1986) J. Biol. Chem. 261, 5104-5110.

25. Vance, D.E., Choy, P.C., Farren, S.B., Lim, P.H. and Schneider, W.J. (1977) Nature 270, 268-269.

26. Weiss, S.B., Smith, S.V. and Kennedy, E.P. (1958) J. Biol. Chem. 231, 53-64.

27. Sundler, R., Arvidson, G. and Akesson, B. (1972) Biochim. Biophys. Acta 280, 554-568.

28. Ridgeway, N.D. and Vance, D.E. (1987) J. Biol. Chem. 262, 17231-17239.

29. Sundler, R. and Akesson, B. (1975) J. Biol. Chem. 250, 3354-3367.

30. Hirata, F. and Axelrod, J. (1978) Nature 275, 219-220.

31. Vance, D.E. and de Kruijff, B. (1980) Nature 288, 277-278.

32. Bjerve, K.S. (1973) Biochim. Biophys. Acta 306, 396-402.

33. Miura, T. and Kanfer, J.N. (1976) Arch. Biochem. Biophys. 175, 654-660.

34. Buchanan, A.G. and Kanfer, J.N. (1980) J. Neurochem. 34, 720-725.

35. Lands, W.E.M. and Merkl, I. (1963) J. Biol. Chem. 238, 898-904.

36. Van Golde, L.M.G. and Van den Bergh, S.G. (1977) in: Lipid Metabolism in Mammals. Vol. I. (Snyder, F., ed.) Plenum Press, New York. pp. 1-33.

37. Hendy, A.T. and Possmayer, F. (1974) Biochim. Biophys. Acta 369, 156-172.

38. Van Hensden, G.P.H., Ruestow, B., Van der Mast, M.A. and Van den Bosch, H. (1981) Biochim. Biophys. Acta 666, 313-321.

39. Van den Bosch, H., Boute, H.A. and Van Deenen, L.L.M. (1965) Biochim. Biophys. Acta 98, 648-651.

40. Vance, D.E. (1985) in:Biochemistry of Lipids and Membranes. (Vance, D.E. and Vance, J.E., eds.) Benjamin/Cummings Pub. Co. Inc., California. pp. 242-270.

41. Zelinski, T.A., Savard, J.D., Man, R.Y.K. and Choy, P.C. (1980) J. Biol. Chem. 255, 11423-11428.

42. Strecker A. (1862) Ann. Chem. Pharmacie 123, 353-360.

43. Baeyer, A. (1866) Ueber. Neurin. Ann. Chem. Liebigs. 140, 306-313.

44. Best, C.H. and Huntsman, M.E. (1932) J. Physiol. 75, 405-412.

45. Wilson, J.D., Gibson, K.D. and Udenfriend, S. (1960) J. Biol. Chem. 235, 3213.

46. Harper, H.A. (1969) in: Review of Physiological Chemistry, 2nd ed. Lange Med. Pub., Los Altos, California. pp. 123-127.

47. Zeisel, S.H., Story, D.L. Wurtman, R.J. and Brunengraber, H. (1980) Proc. Nat'l. Acad. Sci. 77, 4417-4419.

48. Hirsch, M.J., Growdon, J.H. and Wurtman, R.J. (1978) Metabolism 27, 953-960.

49. Zeisel, S.H. (1981) Ann. Rev. Nutr. 1, 95-121.

50. Sung, C.P. and Johnstone, R.N. (1963) Biochim. Biophys. Acta 173, 548-553.

51. Cao, Y-Z., O. K., Choy, P.C. Chan, A.C. (1987) Biochem. J. 247, 135-140.

52. Boupaep, E. (1962) Arch. Int. Physiol. Biochem. 71, 623-624.

53. Bosteels, S., Vlengels, A. and Cameliet, E. (1970) J. Gen. Physiol. 55, 602-619.

54. Haines , P.S.M. and Rose, C.I. (1970) Can. J. Biochem. 48, 885-892.

55. Blumenstein, J. (1964) Can. J. Biochem. 42, 1183-1194.

56. Haeffner, E.W. (1975) Eur. J. Biochem. 51, 219-228.

57. Plagemann, P.G.W. (1971) J. Lipid Res. 12, 715-724.

58. Pelech, S.L., Pritchard, P.H. and Vance, D.E. (1982) Biochim. Biophys. Acta 713, 260-269.

59. Diamond, I. and Kennedy, E.P. (1969) J. Biol. Chem. 244, 3258-3263.

60. Zelinski, T.A. and Choy, P.C. (1984) Can. J. Biochem. 60, 817-823.

61. Zelinski, T.A. and Choy, P.C. (1984) Biochim. Biophys. Acta 794, 326-332.

62. Li, H.-C., Hsiao, K.-J. and Sampathkumar, S. (1979) J. Biol. Chem. 254, 3368-3374.

63. Choy, P.C., Paddon, H.B. and Vance, D.E. (1980) J. Biol. Chem. 255, 1070-1073.

64. Pritchard, P.H., Chiang, P.K., Cantoni, G.L. and Vance, D.E. (1982) J. Biol. Chem 257, 6362-6367.

65. Choy, P.C. (1982) J. Biol. Chem. 257, 10928-10933.

66. Bajusz, E. (1971) Recent Adv. Stud. Card. Struct. Met. 2, 291-292.

67. Pelech, S.L. and Vance, D.E. (1984) Biochim. Biophys. Acta 779, 217-251.

68. Vance, D.E., Trip, E.M. and Paddon, H.B. (1980) J. Biol. Chem. 255, 1064-1069.

69. Sleight, R. and Kent, C. (1980) J. Biol. Chem. 255, 10644-10650.

70. Weinhold, P.A., Feldman, D.A., Quade, M.M., Miller, J.C. and Brooks, R.L. (1981) Biochim. Biophys. Acta 665, 134-144.

71. Pelech, S.L., Power, E. and Vance, D.E. (1983) Can. J. Biochem. Cell Biol. 61, 1147-1152.

72. Vance, D.E. and Pelech, S.L. (1984) Trends Biochem. Sci. 9, 17-20.

73. Pritchard, P.H., Chiang, P.K., Cantoni, G.L. and Vance, D.E. (1983) J. Biol. Chem. 257, 6362-6367.

74. Pelech, S.L., Pritchard, P.H., Brindley, P.N. and Vance, D.E. (1983) J. Biol. Chem. 258, 6782-6788.

75. Cornell, R. and Vance, D.E. (1987) Biochim. Biophys. Acta 919, 26-36.

76. Pelech, S.L., Cook, H.W., Paddon, H.B. and Vance, D.E. (1984) Biochim. Biophys. Acta 795, 433-440.

77. Weinhold, P.H., Rounsifer, M. William, S., Brubaker, P. and Feldman, D.A. (1984) J. Biol. Chem. 259, 10315-10321.

78. Sleight, R. and Kent, C. (1983) J. Biol. Chem. 258, 824-830.

79. Sleight, R. and Kent, C. (1983) J. Biol. Chem. 258, 831-835.

80. Sleight, R. and Kent, C. (1983) J. Biol. Chem. 258, 836-839.

81. Weinhold, P.A., Feldman, D.A., Quade, M.M., Miller, J.C. and Brooks, R.L. (1981) Biochim. Biophys. Acta 665, 134-144.

82. Choy, P.C. and Vance, D.E. (1978) J. Biol. Chem. 253, 5163-5167.

83. Choy, P.C., Farren, S.B. and Vance, D.E. (1979) Can. J. Biochem. 57, 605-612.

84. Pelech, S.L. and Vance, D.E. (1982) J. Biol. Chem. 257, 14198-14202.

85. Pelech, S.L. and Vance, D.E. (1984) in: Enzyme Regulation by Reversible Protein Phosphorylation - Further Advances. (Cohen P., ed.) Elsevier Sci. Pub., Amsterdam. pp. 63-80.

86. Mock, T., Slater, T.L., Arthur, G., Chan, A.C. and

Choy, P.C. (1986) Biochem. Cell Biol. 64, 413-417.

87. Balis, M.E. and Samarth, K. (1962) Arch. Biochem. Biophys. 99, 517-521.

88. de Leiris, J., Harding, D.P. and Pestre, S. (1984) Basic Res. Cardiol. 79, 313-321.

89. Scheucer, J. (1967) Amer. J. Cardiol. 19, 325-292.

90. Paddon, H.B. and Vance, D.E. (1977) Biochim. Biophys. Acta 488, 181-189.

91. Krebs, H.A. and Henseleit, K. (1932) Hoppe. Seylers. Z. Physiol. Chem. 210, 33-66.

92. Langendorff, O. (1895) Pfleugers Arch. 61, 291-332.

93. Arthur, G. and Choy, P.C. (1984) Biochim. Biophys. Acta 795, 221-229.

94. Cox, R.P. and Griffin, M.J. (1967) Arch. Biochem. Biophys. 122, 552-562.

95. Weinhold, P.A. and Rethy, V.B. (1974) Biochem. 13, 5135-5141.

96. Choy, P.C., Lim, P.H. and Vance, D.E. (1977) J. Biol. Chem. 252, 7673-7677.

97. Inui, Y. and Ishioku, H. (1983) Gen. Comp. Endocrinol. 51, 208-212.

98. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

99. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.

100. Moore, J.D. (1971) Meth. Enzymol. 22, 130-148.

101. Zelinski, T.A. (1984) PhD. Thesis. University of Manitoba.

102. Conner, A.M., Brimble, P.D. and Choy, P.C. (1981) Prep. Biochem. 11, 91-97.

103. McCaman, R.E. and Stetzler, J. (1977) J. Neurochem. 28, 669-671.

104. Shimizu, S., Taui, Y., Yamada, H., Tabata, M. and Murachi, T. (1980) Anal. Biochem. 107, 193-198.

105. Metcalfe, L.D. and Schnitz, A.A. (1961) Anal. Biochem. 33, 363-364.

106. Gazzola, G.C., Franchi, R., Saibene, P., Ronchi, P. and Guidotti, G.G. (1972) Biochim. Biophys. Acta 266, 407-421.

107. Guidotti, G.G., Borghetti, A.F. and Gazzola, G.C. (1978) Biochim. Biophys. Acta 515, 329-366.

108. Kristensen, L.O. and Folke, M. (1986) Biochim. Biophys. Acta 885, 49-57.

109. Morton, R.K. (1955) Meth. Enz. Vol II, 533-539.

110. Moss, D.W. (1982) Clin. Chem. 28, 2007-2016.

111. Jensen, D. (1980) in: The Principles of Physiology. 2nd ed. Appleton-Century-Crofts Pub. Co., New York. pp. 537-584.

112. Piper, H.M., Schwartz, P., Hutter, J. and Spiekermann, P.G. (1984) J. Mol. Cell. Cardiol. 16, 995-1007.

113. Feldman, D.A., Kovac, C.R., Dranginis, P.L. and

Weinhold, P.A. (1978) J. Biol. Chem. 253, 4980-4986.

114. Feldman, D.A., Brubaker, P.G. and Weinhold, P.A. (1981) Biochim. Biophys. Acta 665, 53-59.

115. Jenden, D.J. (1979) in:Nutrition and the Brain. (Barbeau, A., Growden, J.H. and Wurtman, R.J., eds.) Raven Press, New York. pp. 13-24.

116. Anda, Von L.P. and Liappis, N. (1976) Z. Tierphysiol. Tierernaehr. Futtermittelkde. 37, 70-74.

117. Christensen, H.N. (1984) Biochim. Biophys. Acta 779, 255-269.

118. King, R.G. and Marchbanks, R.M. (1980) Nature 287, 64-65.

119. Havener, L.J. and Tobak, F.G. (1980) J. Clin. Invest. 65, 741-745.

120. Moss, D.W. (1982) Clin. Chem. 28, 2007-2016.

121. Moss, D.W., Muller, E. Pearce, A.G.E. and Thomas, D.M. (1973) J. Mol. Cell. Cardiol. 5, 191-196.

122. Muller. E., Pearce, A.G.E. and Moss, D.W. (1971) Biochem. J. 123, 895-900.

123. Lumeng, L. and Li, T.-K. (1975) J. Biol. Chem. 250, 8126-8131.

124. Guynn, R.W. (1976) J. Biol. Chem. 251, 7162-7167.

125. Lowe, J.E., Hawkins, H.K. and Jennings, R.B. (1978) Surg. For. 29, 247-250.

126. Whilton, D.S., Anderson, K.E. and Mueller, G.C. (1985) Biochim. Biophys. Acta 835, 369-377.

127. Aebehard, E., Barret, C. Kaplan, S. and Scott, M. (1986) Biochim. Biophys. Acta 875, 6-11.

128. Katz, A.M. and Messineo, F.C. (1981) Circ. Res. 48, 1-16.