Phosphatidylcholine and Phosphatidylethanolamine Biosynthesis in Isolated Hamster Heart

by

Teresa A. Zelinski

A thesis presented to the Faculty
of Graduate Studies
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In partial fulfillment of the
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When The Journey Starts

Nobody knows when the journey starts
What he'll find along the way -
A storm - tossed sea, a thicket of thorns
Or a quiet, peaceful day . . .
Nobody knows when the journey starts
If he will travel alone
Or in the company of cherished friends
That he can call his own . . .

There is no map for the journey
To guide our footsteps along
Only our youth, our faith in ourselves
And our courage to keep us strong . ..
For youth is the time of hopes and dreams
When life's journey is only begun
Till the hills and the valleys are safely crossed
And the summit is finally won.

Jean Kyler McManus

For Mom and Dad

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ABSTRACT

The pathways for the formation of phosphatidylcholine (PC) in hamster hearts were investigated. The presence of choline kinase, phosphocholine cytidylyltransferase, phosphocholinetransferase, phosphatidylethanolamine - S - adenosylmethionine methyltransferase and base exchange enzyme activities in hamster subcellular fractions indicate that PC is synthesized via CDP-choline, by progressive methylation of phosphatidylethanolamine, (PE), and by base exchange of choline with other phospholipids. When isolated hamster hearts were perfused with [^{3}H] choline, the majority of radioactivity taken up was rapidly converted to phosphocholine and subsequently incorporated into PC. No significant amount of radioactivity was found in betaine or acetylcholine. The amount of PC formed from CDP-choline was estimated to be 39 nmol·min^{-1}· g heart^{-1}. A distinct lag in the incorporation of radioactivity into PC was observed during the first 15 min of perfusion, which suggested that the contribution of the base exchange pathway to PC formation was minor in comparison to the CDP-choline pathway. Only 2.5% of total PC in hamster heart was formed by methylation of PE.

The rate-limiting step of the CDP-choline pathway was determined in the isolated heart by pulse-chase studies. The [^{3}H] choline taken up by the heart was immediately converted into phosphocholine and throughout the chase period, about 30% of the radioactivity which

disappeared from phosphocholine was subsequently incorporated into PC. The remainder of the radioactivity was located in the effluent as choline. It is possible that CDP-choline is not an obligatory metabolite of phosphocholine, and a significant amount of phosphocholine is hydrolyzed by cardiac alkaline phosphatase. Based on the specific radioactivities of choline, phosphocholine and CDP-choline in the pulse-chase studies, the rate-limiting step for PC biosynthesis in the hamster heart is catalyzed by phosphocholine cytidylyltransferase.

The pathways leading to the formation of PE in isolated hamster hearts were also investigated. The contribution of the CDP-ethanolamine and the base exchange pathways were studied by perfusion with [^{3}H] ethanolamine. The radioactivity of ethanolamine in the heart reached maximum at 5 min of perfusion and remained constant throughout the perfusion period. Maximum labeling of phosphoethanolamine occurred at 25 min of perfusion, and labeling of CDP-ethanolamine did not reach maximum over the 30 min perfusion period. Incorporation of radioactivity into PE was marked by a lag during the first 15 min of perfusion, after which a linear increase was observed. This initial lag suggests the minor contribution of the base exchange pathway, as compared to the CDP-ethanolamine pathway, which was estimated to contribute 290 nmol·min^{-1}· g heart^{-1} to total PE formation

in hamster heart. PE formation via decarboxylation of phosphatidylserine, (PS), was studied by perfusion of hamster hearts with labeled serine. The contribution of this pathway was estimated to be 9.0 $nmol \cdot min^{-1} \cdot g\ heart^{-1}$. Hence, it was concluded that PE was synthesized by all three known pathways, and that the CDP-ethanolamine pathway was the major pathway for PE biosynthesis in the mammalian heart. The low activities of PS decarboxylase and base exchange enzyme measured *in vitro* probably reflect the minor contribution of these two pathways to PE biosynthesis.

ABBREVIATIONS

ACS	Aqueous counting scintillant
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
ANSA	1-amino-2 naphthol 4-sulfonic acid
CAPS	Cyclohexlaminopropane Sulfonic Acid
^{o}C	Degrees Celsius
CMP	Cytidine 5'-monophosphate
CTP	Cytidine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
g	Gram
g	Gravitational force
h	Hour
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
nm	Nanometre
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
s	Second
SAM	S-adenosylmethionine
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography

μl	Microlitre
U.V.	Ultraviolet
v	Volume
w	Weight

INTRODUCTION

I THE BIOLOGICAL MEMBRANE

Membranes are an integral part of any cell. In some instances, the different membrane systems may comprise up to 80% of the total dry cell mass. Membranes serve not only as barriers separating different compartments within the cell, but also as the structural basis of the cell.

The molecular organization of membranes consists of a lipid bilayer in which biologically active proteins are embedded. Most membranes contain approximately 40% lipid and 60% protein, but there is considerable variation. For example, the inner mitochondrial membrane contains 20 - 25% lipid, whereas the myelin membrane surrounding certain nerves may contain up to 75% lipid. The lipid bilayer of biological membranes is mainly composed of phospholipids and provides a fluid matrix for protein organization and movement (Singer and Nicolson, 1972). Lipids are asymmetrically distributed in plasma membranes with PE largely facing the cytoplasmic side and PC mainly orientated toward the outside (Rothman and Lenard, 1977, Chap *et al.*, 1977).

Since PC and PE are the major phospholipids of mammalian systems, the understanding of their biosynthesis is essential for understanding cellular function and membrane integrity.

II PHOSPHATIDYLCHOLINE BIOSYNTHESIS

In mammalian tissues, there are several known pathways,

for the formation of PC (Vance and Choy, 1979), (Fig. 1). The majority of the phospholipid is formed from choline via the CDP-choline pathway (Kennedy, 1962). Alternatively, PE is methylated to PC by the transfer of methyl groups from S-adenosylmethionine (SAM) (Bremer and Greenberg, 1961). Another pathway is the Ca^{++}-mediated base exchange of choline with other phospholipid head groups (Dils and Hübscher, 1961). Each pathway's contribution to total PC biosynthesis appears to be tissue specific. For example, the methylation of PE contributes significantly to PC biosynthesis in rat liver (Vance and Choy, 1979), but is completely absent in rat intestine (Mansbach and Parthasarathy, 1979). These pathways will be described in more detail in the following sections.

a) CDP-choline Pathway

The major pathway for the biosynthesis of PC was largely elucidated by Kennedy and associates. Hence, this pathway is also referred to as the Kennedy pathway or the de novo synthesis pathway.

The first step in the reaction sequence is a phosphorylation step which occurs at the expense of ATP, and is catalyzed by choline kinase. Weinhold and Rethy, (1974), described an ethanolamine kinase which also displayed choline kinase activity, and it was originally thought that both activities were shared by a single enzyme. However, studies in rat liver (Brophy et al., 1977), demonstrated unambiguously that choline kinase and

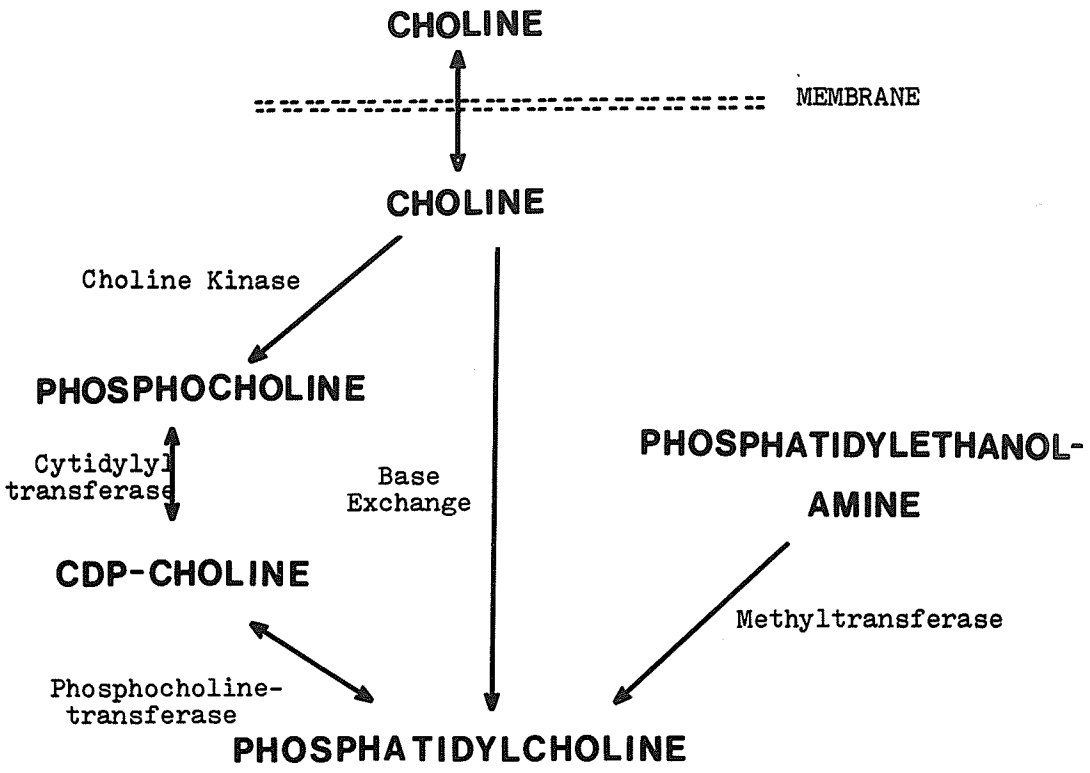


Fig. 1. Major metabolic pathways for the biosynthesis of phosphatidylcholine.

ethanolamine kinase were separate, soluble enzymes. In the same report the authors showed that choline kinase was exclusively cytosolic.

It has been firmly established that a high energy form of choline, CDP-choline, is an essential intermediate in the de novo synthesis pathway described by Kennedy. The enzyme phosphocholine cytidylyltransferase, which is responsible for the conversion of phosphocholine to CDP-choline, is widely distributed throughout nature, being found in tissues of rat, chicken, guinea pig as well as plant sources and yeast (Kennedy and Weiss, 1956). This enzyme is heat stable, requires Mg^{++} or Mn^{++} for activity and is specific for CTP or dCTP (Borkenhagen and Kennedy, 1957). Phosphocholine cytidylyltransferase activity is located in both cytosolic and microsomal fractions (Choy et al., 1977).

The final step in the reaction sequence is catalyzed by phosphocholinetransferase. This enzyme is located in microsomal fractions and is characterized by the need for Mg^{++} or Mn^{++}, inhibition by Ca^{++} and the requirement for diacylglycerol which has been emulsified in a nonionic detergent such as Tween 20. It was also shown that this enzyme is specific for CDP-choline, and other nucleotides linked to choline are ineffective in generating PC. The CMP formed in the final reaction may be rephosphorylated at the expense of ATP to form CTP, which in turn can be used again to form CDP-choline. Therefore, the cytidine

nucleotides may thus function in a catalytic cycle, with the net production of one mole of PC in each cycle.

Considerable controversy exists in the literature concerning the rate-limiting step of the CDP-choline pathway. There are two views on which enzyme catalyzes the rate-controlling step of this pathway. Infante, (1977), using theoretical arguments, suggested that choline kinase and phosphocholine cytidylyltransferase performed this function, whereas Vance and Choy, (1979), argued on the basis of experimental evidence that only phosphocholine cytidylyltransferase was employed in this capacity.

Infante stated that analysis of available data, coupled to theoretical principles of metabolic regulation, lead to his conclusion that choline kinase and phosphocholine cytidylyltransferase might catalyze rate-limiting steps. He stated that phosphocholinetransferase catalyzed a near-equilibrium reaction and thus was not rate-limiting. Only those reactions far from equilibrium can be rate-limiting, as the flux through these steps is highly sensitive to changes in enzyme activity and therefore these sites have the greatest potential for control of flux through a metabolic sequence. Near-equilibrium reactions are extremely resistant to even large changes in enzyme activity and are therefore poor sites for flux regulation.

The extent to which a reaction is rate-limiting can be quantitatively determined by the value of its

disequilibrium ratio:

$$\rho = \frac{\text{mass action ratio}}{K^{eq.}_{obs.}}$$

where ρ is the disequilibrium ratio and K^{eq}_{obs} is the observed equilibrium constant for the reaction measured under physiological conditions. If the value of ρ is ≤ 0.05 this is taken as an indication of a rate-limiting step, since the rate of the backward reaction is less than 5% of the forward velocity, therefore, the flux of this reaction, that is the forward velocity - the backward velocity, through that step is dependent on the forward rate of the reaction. Using this rationale, Infante calculated a disequilibrium ratio of 2.77×10^{-4} for choline kinase and 1.35×10^{-2} for phosphocholine cytidylyltransferase. Since the ρ value for choline kinase was 49-fold less than that obtained for phosphocholine cytidylyltransferase, he concluded that the rate-limiting step of the CDP-choline pathway occurred at the first reaction, catalyzed by choline kinase.

However, Vance and Choy, (1979), maintain that only phosphocholine cytidylyltransferase catalyzes the rate-controlling step in this reaction sequence. This view is contrary to the general biochemical principle that the first committed reaction in a pathway is under metabolic control and is rate-limiting.

The evidence to support this theory is that the total pool size of choline is 5 - 10 fold lower than phospho-

choline in liver (Sundler et al., 1972), and HeLa cells (Vance et al., 1980). These relative pool sizes indicate a rapid conversion of choline to phosphocholine, with the rate-limiting step occurring subsequent to the choline kinase catalyzed reaction. Tracer studies have demonstrated that labeled choline is rapidly converted to phosphocholine (Sundler et al., 1972, Vance et al., 1980). Additional evidence was provided by Sundler et al., (1972), who reported that the phosphocholine pool in rat liver was 150 times higher than the CDP-choline pool. Since CDP-choline is the only known metabolite of phosphocholine, these results suggest that once CDP-choline is formed, it is rapidly converted to PC. Similar results were also reported by Vance et al., (1980), in HeLa cells.

More definitive evidence for the rate-limiting role of this reaction was obtained from pulse-chase studies in HeLa cells (Vance et al., 1980). The radioactivity of the exogenously added [^{3}H]-choline was quickly converted to phosphocholine (97% at the end of 1 h). Subsequently, as the radioactivity disappeared from phosphocholine, it was transiently associated with CDP-choline and then immediately converted to PC.

Further support was obtained by Choy et al., (1980), studying polio infected HeLa cells. These cells, when infected with virus, tend to stimulate the incorporation of [^{3}H]-choline into PC. Subsequent pulse-chase studies showed that the observed increase in PC biosynthesis was

due solely to an acceleration of the phosphocholine cytidylyltransferase reaction.

There appears to be a discrepancy between the theoretical prediction that both choline kinase and phosphocholine cytidylyltransferase catalyze rate-limiting steps in the CDP-choline pathway, and the experimental results which suggest only phosphocholine cytidylyltransferase in this capacity. Since the evidence is still inconclusive, more experimentation is required to resolve this controversy.

b) Progressive Methylation of Phosphatidylethanolamine

Bremer and Greenberg, (1961), were the first to demonstrate that PC could be formed from PE via the addition of methyl groups from SAM, liberating S-adenosyl-homocysteine. This process is a stepwise one, in which one methyl group is transferred to the base moiety at a time (Hirata et al., 1978), yielding phosphatidyl-N-monomethylethanolamine, phosphatidyl-N, N-dimethyl-ethanolamine and PC. These researchers, in the same report, identified two enzymes in the adrenal medulla that converted PE to PC by successive methylations with SAM.

The two phospholipid methyltransferases in the adrenal medulla have different properties. The first enzyme (methyltransferase I) converts PE to phosphatidyl-N-monomethylethanolamine, requires Mg^{++}, has an optimal pH about 7.0 and a low Km for SAM. The second enzyme (methyltransferase II), which was described previously

by Bremer and Greenberg, (1961) and Gibson et al., (1961), catalyzes the stepwise methylation of phosphatidyl-N-monomethylethanolamine to PC. This enzyme does not require Mg^{++}, has a high Km for SAM and an optimal pH of 10.0. The two methyltransferases are located in the microsomes and mitochondria of the adrenal medulla and were found in all tissues examined so far including brain (Crews et al., 1980), red cells (Hirata and Axelrod, 1978a), lymphocytes (Hirata et al., 1980), mast cells (Hirata et al., 1979a), and basophils and neutrophils (Hirata et al., 1979b).

The ability to prepare erythrocyte ghosts either inside-out or right-side-out with respect to cellular contents (Steck and Kant, 1974), allowed Hirata and Axelrod, (1978a), to study the localization of these enzymes in membranes. It was concluded in this study, that methylation of phospholipids began on the cytoplasmic side of the membrane, where the substrate PE for methyltransferase I was located. Subsequent to initial methylation, methyltransferase II added an additional two methyl groups to phosphatidyl-N-monomethylethanolamine producing PC, facing the outside of the membrane (Kahlenberg et al., 1974). This enzymatically facilitated translocation of phospholipids was very rapid (less than 2 min) and was also reported in membranes of Bacillus megatherium (Rothman and Kennedy, 1977).

The methylation and rapid rearrangement of phospho-

lipids has an influence on membrane fluidity. When phospholipids are methylated, there is a measured decrease in microviscosity of the membrane (Hirata and Axelrod, 1978b).

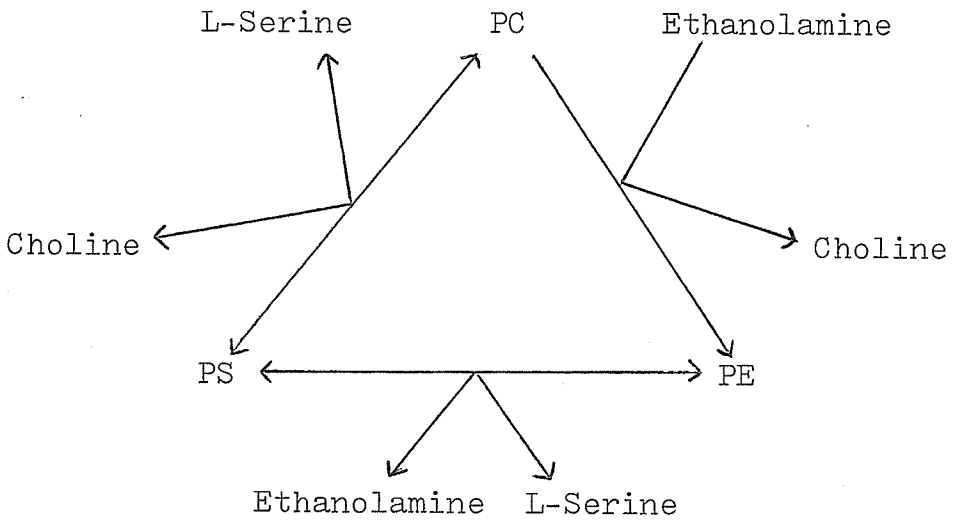
It is this translocation of phospholipids in the membranes, with the decreased microviscosity of the membrane, which Hirata and Axelrod, (1980), believe to play an important role in the transduction of receptor-mediated signals through the membranes of a variety of cells. In this report, the authors cite numerous references and they conclude that catecholamine neuro-transmitters and peptides and immunoglobulins interacting with cell surface receptors initiate a cascade of biochemical and physical changes in local domains of the membrane. This leads to increased mobility of receptors, elevated phospholipid methylation, generation of cyclic AMP, histamine release, mitogenesis and chemotaxis, all of which ultimately impart specialization of function and regulation to a variety of tissues and cell types. The authors point to phospholipid methylation as an initial common pathway for the transduction of many receptor mediated biological signals through membranes.

c) Base Exchange of Choline with other Phospholipids

The third pathway for the formation of PC, is the Ca^{++}-mediated exchange of choline for another phospholipid head group, described by Dils and Hübscher, (1961), in rat liver microsomes. This reaction is often simply called

base exchange and appears to be of minor importance in the liver, contributing less than 10% to the newly synthesized PC pool.

Bjerve (1973) also investigated Ca^{++}-stimulated base exchange in liver microsomes. He demonstrated that PC, PE and PS could all act as substrates in this reaction. Furthermore, choline, ethanolamine and serine could exchange with PC and PS, but only serine and ethanolamine could exchange with PE. In the same report, Bjerve suggested that more than one enzyme was involved in these reactions, since choline incorporation was competitively inhibited by ethanolamine and L-serine; L-serine incorporation was competitively inhibited by choline and ethanolamine, with ethanolamine incorporation inhibited noncompetitively by L-serine and uncompetitively by choline. Bjerve also proposed that the phospholipids of rat liver microsomes could be interconverted by the Ca^{++}-stimulated exchange scheme shown below:



It should be mentioned here that although base exchange accounts for only a small percentage of net PC biosynthesis in liver, this pathway is considered to be the major pathway for PS biosynthesis in this same tissue (Bjerve, 1973).

d) Reacylation Systems

Studies of pulmonary lipid composition and distribution imply that lung has the capacity for biosynthesis of many different complex lipid classes. The major emphasis in the study of lung lipid metabolism has been directed toward elucidation of the mechanisms for the biosynthesis of dipalmitoyl-PC, the major component of lung surfactant.

The primary physiological function of the lung is the exchange of respiratory gases, a process that requires structurally intact and open alveoli. The maintenance of alveolar structural integrity is dependent on the reduction of surface tension forces in the alveolar layer lining, by pulmonary surfactant (Scarpelli, 1968a, 1968b).

The preponderance of evidence indicates that lung PC biosynthesis occurs primarily through the CDP-choline pathway (Weinhold, 1968, Akino et al., 1971, Epstein and Farrell, 1975). Since this pathway was already discussed, attention in this section will focus on the synthesis of the unique phospholipid species, dipalmitoyl-PC. This phospholipid, in terms of positional distribution of its fatty acyl residues, is both symmetrical and highly nonrandom. This is in direct contrast to the situation

which exists in most other tissues, where the phospholipids are highly asymmetric, with a preponderance of saturated residues at position 1 and unsaturated residues at position 2. Also, while the distribution of fatty acids at each position is characteristic for each tissue, the degree of randomness is typically greater in nonpulmonary tissues.

The putative role of dipalmitoyl-PC in the maintenance of alveolar structural integrity and its high concentration in lung suggests specific mechanisms for its biosynthesis in pulmonary tissue. Consequently, an important aspect of pulmonary biochemistry is the determination of which point or points in the metabolic pathways leading to PC biosynthesis, may be responsible for imparting the unique fatty acid distribution of dipalmitoyl-PC.

There are two mechanisms by which dipalmitoyl-PC can be formed in the lung:

1) Initial acylations of sn-glycerol-3-phosphate are highly nonrandomn, thereby producing large amounts of dipalmitoyl-phosphatidic acid whose acyl distribution pattern would be maintained through the subsequent steps to the PC level.

2) Initial acylations may produce phosphatidic acid and subsequently PC species with varying degrees of randomness and assymmetry. The non-specific fatty acid distribution pattern of these PC species would then be modified to give dipalmitoyl-PC through the

action of other enzymatic reacylation systems.

If a significant portion of dipalmitoyl-PC was produced by the CDP-choline pathway, the acyltransferase system would show considerable preference for palmitoyl-CoA as a donor substrate. In vitro studies by Hendry and Possmayer, (1974), showed that the relative rates of acyl group incorporation, did not account for the amount of dipalmitoyl-PC in the lung. Therefore it seemed that the majority of this unique phospholipid was formed by a reacylation reaction.

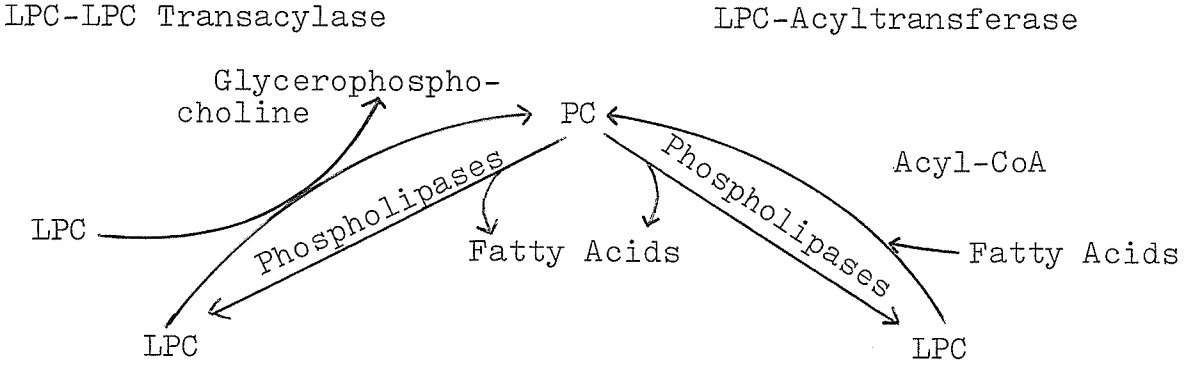
Generally, the most important alternative mechanisms for pulmonary PC biosynthesis, involve reactions of lysophosphatidylcholine, (LPC), which serve as receptor substrates for acylating enzyme systems that have high degrees of donor-substrate specificity for palmitoyl and other saturated acyl groups. Since there is no evidence to suggest that LPC is the major biosynthetic product of pulmonary metabolism, these pathways rely on the deacylation of PC produced via the CDP-choline pathway, via the enzyme phospholipase A_2. There are two important reacylation reactions.

The first reacylation reaction involves the enzyme LPC-acyltransferase. In contrast to results obtained with liver microsomes, and other nonpulmonary tissues, where acylations with saturated acyl-CoA at the 2-LPC position were slow compared to those at the 1-LPC position, the palmitoyl-CoA derivative had equal and marked reactivity for both lyso positions in lung microsomes (Frosolono

et al., 1971). Therefore, when LPC molecules were available to this enzyme, it had the capacity to reacylate the lysolipid with the transfer of palmitoyl residues to either the 1 or 2 position with similar effectiveness. Hence, there seemed to be cooperation between phospholipases and LPC-acyltransferase.

A second reacylation reaction is catalyzed by LPC-LPC acyltransferase. This enzyme catalyzes acyl group transfer from one molecule of LPC to another, producing PC and sn-glycerol-3-phosphorylcholine (Akino et al., 1971, 1972). This reaction occurs in the cytosol (Van den Bosch et al., 1965), and has no requirement for ATP, CoA or palmitoyl-CoA (Abe et al., 1972). These energy poor conditions suggest that this mechanism of synthesizing dipalmitoyl-PC is not as important as the LPC-acyltransferase pathway in vivo. In this context, the endoplasmic reticulum, the site of CDP-choline pathway and LPC-acyltransferase activity, is generally considered quantitatively more important in complex phospholipid biosynthesis, than the cytosol, site of LPC-LPC transacylase activity.

The general schemes of these two reacylation systems are shown below:



III PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

The pathways responsible for the formation of PE in mammalian tissues are shown in Fig. 2. PE can be synthesized from ethanolamine via CDP-ethanolamine (Kennedy, 1962), by Ca^{++}-mediated base exchange of ethanolamine with other phospholipids (Dils and Hübscher, 1961) and by decarboxylation of PS (Borkenhagen et al., 1961). Since the CDP-ethanolamine and base exchange pathways are analogous to reactions with choline described previously, they will not be discussed in this section.

a) Decarboxylation of Phosphatidylserine

Borkenhagen and co-workers initially studied the incorporation of labeled serine and ethanolamine into phospholipids in rat liver homogenates. Analysis of the labeled lipids revealed that the principal labeled phospholipid derived from ethanolamine was PE. However, when labeled serine was used, the principal radioactive phospholipid recovered was also PE.

It became clear that serine must be decarboxylated at some step in the reaction sequence. By using dipalmitoyl-L-α-glycerophosphoryl-DL-serine-1-C^{14} as a substrate, these researchers located a PS decarboxylase enzyme in the mitochondrial fraction of various rat tissues. The reaction was monitored by determination of the labeled CO_2 which was liberated during the reaction. The reaction was characterized by a slightly acidic pH optimum (6.5), and the ability to be strongly activated by certain

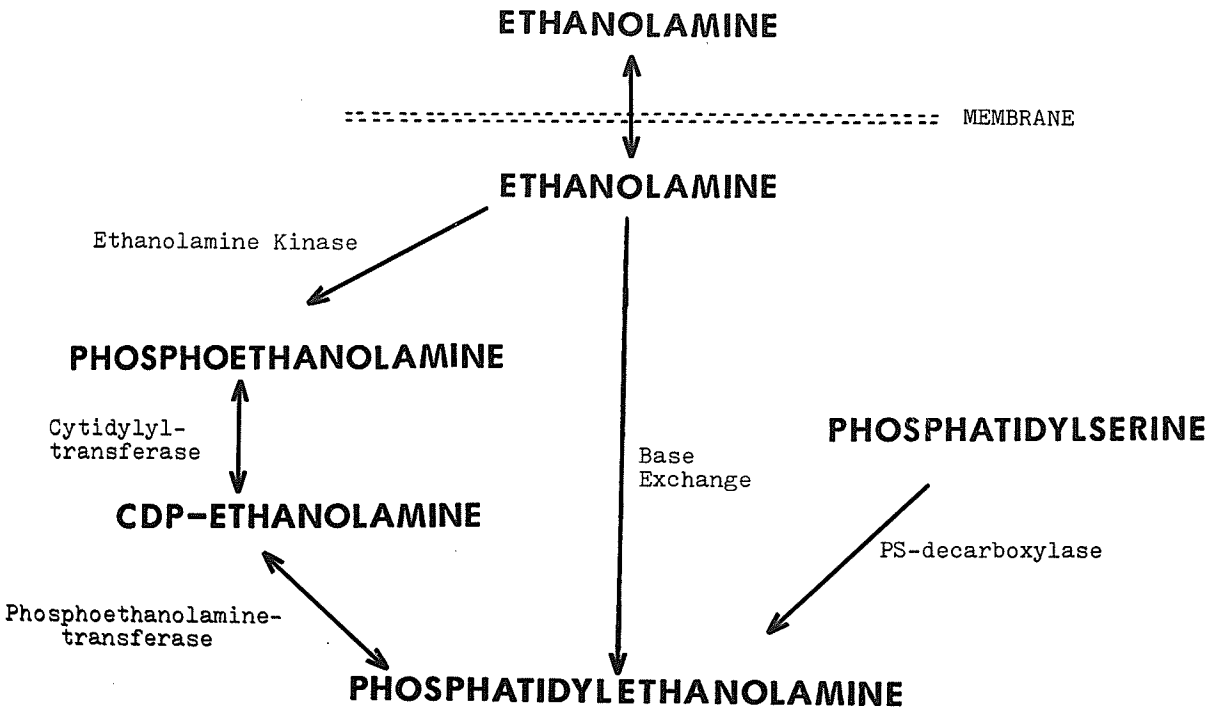


Fig. 2. Major metabolic pathways for phosphatidylethanolamine biosynthesis.

organic solvents, such as toluene. The identification of a decarboxylase enzyme, along with the results obtained by Wilson et al., (1960), and Bremer et al., (1960), confirmed the existence of this pathway for PE formation.

IV RESEARCH AIMS

PC and PE are major phospholipids in mammalian heart (White, 1973). They are not only structural components of the membranes, but PC is also known to modulate a number of membrane-bound enzymes (Coleman, 1973). Although the biosynthesis of PC (Van Golde and Van den Bergh, 1977) and PE (Eibl, 1980), have been extensively studied in several mammalian tissues, the pathways leading to their formation in mammalian hearts remain largely unknown.

The heart is a tissue which beats constantly. With every beat morphological alterations occur. These changes may directly or indirectly affect the membranes of the heart, of which PC and PE are integral components. Therefore, the basic aim of this research was to elucidate the routes by which PC and PE were formed in the heart, and to investigate whether or not the heart synthesized phospholipids differently than a non-beating tissue, such as liver.

As previously described there are three known pathways for the biosynthesis of PC and PE. One goal was to establish whether or not all of these pathways were present in the heart. Subsequently, the major pathway for PC and

PE biosynthesis would then be elucidated. If the CDP-choline or the CDP-ethanolamine pathways were found to be the major routes for the synthesis of PC and PE, respectively, the rate-controlling step(s) of these reaction sequences could then be studied.

The proposed research may also contribute to our understanding of the origin of cardiomyopathies. Once the major pathways for PC and PE biosynthesis have been established, investigations may be directed to a comparative study of PC and PE biosynthesis in normal and cardiomyopathic hamsters. A strain of hamsters genetically predisposed to cardiomyopathy is available for such studies. Since cardiomyopathies have been linked to phospholipid alterations in membrane structure (Bajouz, 1971), and since the animal model closely mimics the diseased state in humans, in depth knowledge of phospholipid biosynthesis in the normal and diseased states may help us understand the primary defect in cardiomyopathies.

MATERIALS AND METHODS

I MATERIALS

a) Chemicals

Choline kinase, alkaline phosphatase (Type III S), phosphodiesterase (Type II), choline chloride, phosphocholine, CTP, ATP, S-adenosylmethionine, and CDP-ethanolamine were obtained from Sigma Chemical Company. CDP-choline, diacylglyceride, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and other phospholipid standards were the products of Serdary Research Laboratories. [Me-^{3}H]-S-adenosylmethionine, [Me-^{3}H] choline, [Me-^{14}C] CDP-choline, and [Me-^{14}C] choline were purchased from New England Nuclear. [γ-^{32}P] ATP, [1-^{3}H] ethanolamine, [2-^{14}C] ethanolamine, L-[3-^{3}H] serine, [2-^{14}C] phosphoethanolamine, [U-^{14}C] phosphatidylserine and aqueous counting scintillant, (ACS), were obtained from Amersham Corporation. [Me-^{3}H] phosphocholine was prepared enzymatically as described by Paddon and Vance, (1977). CDP-[1,2-^{14}C] ethanolamine was a generous gift from Dr. D.E. Vance. Phosphoethanolamine and 2-aminoethanol were purchased from Fisher Chemical Company. Dowex resins AG-1-X8 in chloride form and in formate form were the products of Bio-Rad Laboratories. Thin-layer chromatography, (TLC), plates (Sil-G25) were purchased from Brinkmann and (Redi-Plate Silica Gel G) from Fisher Chemical Company. All other chemicals were of reagent grade and were obtained from Fisher Chemical Company. All solutions were prepared with distilled water and were adjusted to

the desired pH.

b) Experimental Animals

Syrian Golden Hamsters, 100 - 150 g were maintained on Purina Hamster Chow and tap water, ad libitum, in a light and temperature-controlled room.

II METHODS

a) Preparation of Subcellular Fractions for Phosphatidylcholine Studies

Hamsters were sacrificed by decapitation and the hearts were rapidly removed and placed in ice-cold 0.145 M NaCl. The hearts were weighed and homogenized by blending for 15 s. The homogenates were centrifuged at 12,000 x g for 15 min and the supernatant was subsequently centrifuged at 150,000 x g for 60 min. The resulting supernatant was designated the cytosolic fraction. The microsomal pellet obtained from the high speed centrifugation was resuspended in 0.145 NaCl - 5 mM Tris-HCl (pH 7.4). Since cytosolic cytidylyltransferase activity might change with the incubation of cytosol at 4°C (Choy et al., 1977), the enzyme activities in the subcellular fractions were routinely assayed at 4 h after the tissue was homogenized.

b) Preparation of Subcellular Fractions for Phosphatidylethanolamine Studies

Hamsters were sacrificed and their hearts placed in 0.25 M sucrose. The hearts were weighed, and a 10%

homogenate was prepared by blending for 20 s. The homogenates were centrifuged at 3,000 x g for 10 min and the supernatant was then centrifuged at 12,000 x g for 20 min. The pellet obtained was designated the mitochondrial fraction and was resuspended in 0.25 M sucrose. The supernatant from the 12,000 x g spin was subsequently centrifuged at 150,000 x g for 60 min. The resulting supernatant was designated the cytosolic fraction and the pellet obtained (microsomal) was resuspended in 0.25 M sucrose.

c) Protein Determinations

Protein concentrations were determined by the method of Lowry et al., (1951). Aliquots of subcellular fractions were incubated in 0.5 ml of 0.66 N NaCl at 37°C overnight. A mixture of 13% Na_2CO_3 - 2% Cu SO_4 - 4% NaK Tartrate, (1.5 ml), was added to each tube and the entire mixture was vortexed vigorously. The mixture was allowed to sit at room temperature for 10 min after which 0.5 ml of 2N phenol reagent was added. The mixture was again vortexed vigorously and allowed to sit at room temperature for 1 h. Absorbance was measured at 625 nm against albumin standards, (1 mg/ml).

d) Determination of Lipid Phosphorous Without Acid Digestion

The phospholipid concentrations were determined by the method of Raheja et al., (1973). In this procedure, 16 g of ammonium molybdate was added to 120 ml of water

to yield solution I. Subsequently, 40 ml of concentrated HCl and 10 ml Hg were shaken with 80 ml of solution I for 30 - 60 min to yield, after filtration, solution II. 200 ml of concentrated H_2SO_4 was then added to the remainder of solution I, after which solution II was added to this solution to yield solution III. 45 ml of methanol, 5 ml of chloroform and 20 ml of H_2O were added to 25 ml of solution III to give the chromogenic solution. Aliquots of heart homogenates were taken, and an equal volume of 0.1 N KCl was added. The mixture was vortexed well, the organic phase removed, the volume reduced, and subsequently the sample was spotted on a TLC plate with phospholipid standards. The plate was developed in $CHCl_3$/MeOH/H_2O acetic acid (70/30/4/2; v/v/v/v) which results in excellent separation of PC and PE. The corresponding phospholipid bands were removed from the plate and eluted with $CHCl_3$/MeOH (2/1; v/v). The phospholipid samples or standards were subsequently dried under a stream of N_2. Chloroform, (0.4 ml), and an aliquot of the chromogenic solution, (0.1 ml), were added to each tube. The tubes were placed in a boiling water bath for 1 - 1.5 min. After cooling to room temperature, 5 ml of chloroform was added and each tube was vortexed vigorously. The chloroform layer was removed and absorbance was measured at 710 nm. Phospholipid concentrations in heart samples were calculated from the standard phospholipid curve.

e) Determination of Lipid Phosphorous by Acid Digestion

In order to increase the sensitivity of the assay for lipid phosphorous, phospholipid concentrations were also determined by the method of Bartlett, (1959). In these determinations, aliquots of heart homogenates were placed on TLC plates with phospholipid standards. The plate was developed in $CHCl_3/MeOH/H_2O/NH_4OH$ (70/30/4/2; v/v/v/v) which yields excellent separation of PE and PS. The corresponding bands were placed in test tubes and 0.5 ml of H_2SO_4 was added. The mixture was incubated at 160°C overnight and then allowed to cool to room temperature. At this time 0.2 ml of H_2O_2 was added to each tube. The tubes were heated for an additional 2 h at 160°C, and subsequent to cooling 3.0 ml of H_2O and 6.0 ml of 0.4% ammonium molybdate were added. After vortexing, 0.4 ml of freshly prepared ANSA reagent was added to each tube. These tubes were then placed in a boiling H_2O bath for 10 min and absorbance was measured at 820 nm against a silica gel blank. Phospholipid concentrations in heart homogenates were similar to those described in section (d).

f) Determination of Enzyme Activities for Phosphatidylcholine Biosynthesis

i) Choline Kinase

Choline kinase activity is found exclusively in the cytosolic fraction (Brophy et al., 1977), and was

assayed as described by Weinhold and Rethy, (1974). The typical reaction mixture contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM ATP, 5 mM [Me-^{3}H] choline, heart cytosol and H_2O in a final volume of 200 μl. The mixture was incubated for 15 min at 37°C, after which the reaction was stopped by boiling. The mixture was placed on an AG-1-X8 (OH^- form) ion-exchange column (0.5 cm x 3.0 cm), and washed with 10 ml H_2O. The column was subsequently washed with 1.5 ml of 1.0 N NaOH, and then 1.5 ml of 0.1 N NaOH for the elution of phosphocholine from the resin. Radioactivity associated with phosphocholine was determined by liquid scintillation counting. The formation of product under these assay conditions was linear up to 20 min.

ii) Phosphocholine Cytidylyltransferase

Phosphocholine cytidylyltransferase is located in both the cytosolic and microsomal fractions (Choy et al., 1977). Enzyme assays were performed as described by these investigators. A typical reaction mixture contained 100 mM Tris-Succinate (pH 6.0), 12 mM MgAcetate, 2 mM CTP, 1 mM [Me-^{3}H] phosphocholine and either heart cytosol or heart microsomes in a total volume of 100 μl. The mixture was incubated for 30 min at 37°C and the reaction was stopped by immersing the tubes in boiling H_2O for 2 min. An aliquot of the reaction mixture was placed on a TLC plate with CDP-choline carrier. The plate was developed in MeOH/0.6% NaCl/NH_4OH (50/50/5; v/v/v). CDP-choline spot was visualized by U.V. light, and the spot was removed

and analyzed for radioactivity associated with CDP-choline. Under these assay conditions, product formation was linear up to 40 min.

iii) Phosphocholinetransferase

Phosphocholinetransferase is associated with the microsomal fraction and was assayed by the method of Vance and Burke, (1974) with exogenous diacylglyceride added to the assay mixture. Diacylglyceride was prepared by emulsifying in 1% Tween 20 in H_2O, followed by sonication. Other assay components were 25 mM Tris-HCl (pH 8.5), 1.33 mM $MgCl_2$, 1 mM [Me-^{14}C] CDP-choline, 4 mM glutathione, H_2O and heart microsomes. The assay was performed at 37^{o}C for 15 min and was stopped by the addition of 1.0 ml $CHCl_3$/MeOH (1/1; v/v). 0.1 N KCl, (3.0 ml), was added to the mixture and the aqueous phase removed. The mixture was washed two additional times with 2.0 ml of 0.1 N KCl, and the remaining organic phase placed in a scintillation vial, dried under N_2, and counted for radioactivity associated with PC. The reaction rate in this case was linear for at least 20 min under these assay conditions.

iv) Base Exchange of Choline with other Phospholipids

Base exchange reaction was assayed with microsomal fraction as described previously by Saito et al., (1975), without addition of exogenous phospholipids. The reaction mixtures contained, 10 μM [Me-^{3}H] choline, 8 mM CAPS buffer

(pH 9.5), 0.1 mM $CaCl_2$, H_2O and heart microsomes in a final volume of 500 μl. The assay was performed at 37°C for 30 min, and was stopped by addition of 5.0 ml $CHCl_3$/MeOH (2/1; v/v), 1.0 ml of 0.1 N KCl and 1.2 ml of 1 N HCl. The aqueous phase was aspirated off, followed by three additional washings with 2.0 ml of 0.1 N KCl. The organic phase was placed in a scintillation vial, dried under N_2 and counted for radioactivity associated with PC. Under these conditions the reaction was linear up to 30 min.

v) Phosphatidylethanolamine Methyltransferase

Phosphatidylethanolamine methyltransferase is exclusively associated with the microsomal fraction (Bremer and Greenberg, 1960), and was assayed without addition of exogenous lipid, as described by Rehbinder and Greenberg, (1965). The reaction mixture contained, 100 mM Tris-HCl (pH 9.0), 1 mM cysteine, 68 μM [Me-^{3}H]-S-adenosyl-methionine, 0.1% Triton x-100, and microsomal fraction in a total volume of 1.0 ml. The reaction was stopped after 1 h at 37°C with 12 M HCl. Analysis of the reaction products by TLC demonstrated that 90% of the radioactivity was associated with methyl-derivatives of PE. The reaction rate was linear up to 1 h with 1.0 mg microsomal protein.

vi) Phosphocholine Phosphatase (Alkaline Phosphatase)

This enzyme was assayed according to the procedure of Paddon and Vance, (1977), at pH 7.4 with 1 mM [Me-^{14}C]

phosphocholine. A typical reaction mixture contained, 3.33 mM Tris-HCl (pH 7.4), 3.33 μM [Me-^{3}H] phosphocholine, 10 mM $MgCl_2$, H_2O and heart microsomes in a final volume of 100 μl. The reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by boiling for 2 min. An aliquot of the reaction mixture was placed on a TLC plate with choline and phosphocholine carrier. The plate was developed in MeOH/0.6% NaCl/NH_4OH (50/50/5; v/v/v), the bands were visualized with iodine vapor and were subsequently removed for radioactivity determinations.

g) Determination of Enzyme Activities for Phosphatidylethanolamine Biosynthesis

i) Ethanolamine Kinase

Ethanolamine kinase activity is found exclusively in the cytosolic fraction (Brophy et al., 1977) and was assayed as described by Schneider and Vance, (1978). A typical reaction mixture contained 80 mM Sodium Glycylglycine (pH 8.5), 1 mM [1-^{3}H] ethanolamine, 3 mM $MgCl_2$, 3 mM ATP, H_2O and heart cytosol in a final volume of 100 μl. The reaction mixture was incubated for 30 min at 37°C, and the reaction was stopped by boiling. The protein was pelleted down by centrifugation and an aliquot of the mixture was spotted on a TLC plate with phosphoethanolamine carrier. The plate was developed in 96% ethanol/2% NH_4OH (1/2; v/v). The phosphoethanolamine spot was visualized with ninhydrin spray, removed and analyzed for radioactivity associated with phosphoethanolamine. Under these assay conditions

product formation was linear to 30 min.

ii) Ethanolaminephosphate Cytidylyltransferase

Ethanolaminephosphate cytidylyltransferase is exclusively located in the cytosolic fraction (Sundler, 1975), and was assayed as described by this investigator. The reaction mixture contained 20 mM Tris-HCl (pH 7.8), 0.5 mM [1,2-^{14}C] phosphoethanolamine, 10 mM $MgCl_2$, 2 mM CTP and heart cytosol in a final volume of 100 μl. Upon addition of enzyme, the mixture was incubated at 37°C for 20 min. The reaction was stopped by boiling for 5 min, and protein was pelleted by centrifugation. Subsequently an aliquot of the reaction mixture was placed on a TLC plate with CDP-ethanolamine carrier. The same solvent system was used for this assay as for ethanolamine kinase. The product was visualized by U.V. light, and the corresponding spot removed for radioactivity determination.

iii) Phosphoethanolaminetransferase

This enzyme was assayed identically to phosphocholinetransferase, with 1 mM CDP-[1,2-^{14}C] ethanolamine as substrate (Vance and Dahlke, 1975).

iv) Base Exchange of Ethanolamine with other Phospholipids

This assay was performed under the same conditions as the choline base exchange assay, substituting 6 μM [1-^{3}H] ethanolamine for 10 μM [Me-^{3}H] choline (Saito et al., 1975).

v) Phosphatidylserine Decarboxylase

This assay was performed as a modification of the procedure described by Borkenhagen et al., (1961). The reaction mixture contained 10 mM Phosphate buffer (pH 7.0), 1 mM pyridoxal phosphate, 1 mM EDTA (pH 7.0), 1 mM [U-^{14}C] phosphatidylserine, dispersed in Triton X-100 (10 mg/ml) and heart mitochondria, in a final volume of 100 µl. After addition of substrate the mixture was incubated for 2 h at 37°C. The addition of 2.0 ml $CHCl_3$/MeOH (2/1; v/v) stops the reaction. 0.1 N KCl, (1.0 ml), was added to each reaction, and the upper (aqueous) phase removed. The mixture was washed three additional times with 2.0 ml of 0.1 N KCl, with subsequent removal of the aqueous phase. The entire organic phase was dried under a stream of N_2 and reconstituted in $CHCl_3$/MeOH (2/1; v/v). This mixture was placed on a TLC plate and developed in $CHCl_3$/MeOH/H_2O/NH_4OH (70/30/4/2; v/v/v/v) with carrier PS and PE. The bands were visualized with iodine vapor and were subsequently removed for radioactivity determinations via liquid scintillation counting.

h) Perfusion of Isolated Hamster Heart

Hamsters were sacrificed by decapitation and the hearts were rapidly removed and placed in Krebs-Henseleit buffer (Krebs and Henseleit, 1932), saturated with 95% O_2 - 5% CO_2 at room temperature. The heart was cannulated via the aorta in the Langendorff mode (Langendorff, 1895), and the pulmonary artery was incised to ensure adequate

coronary drainage. Perfusion at 37°C was initiated at a constant pressure of 90 - 120 mm Hg, with a coronary flow rate of 2.5 - 4.0 $ml \cdot min^{-1}$. A model of isolated heart perfusion in the Langendorff mode was described recently by Manning et al., (1980). Since it is desirable to monitor the cardiac rhythm of the heart in order to assess its viability during perfusion, electrocardiac recording of the isolated heart was also obtained. One electrode was attached to the aortic cannula and the other was placed in the solution bathing the heart. The placement of electrodes in this manner allowed assessment of atrial and ventricular activities simultaneously. The signals were amplified and recorded by a Gould Brush 2400 paper recorder. No significant differences were observed in the electrocardiac recording from 5 - 60 min of perfusion with [Me-^{3}H] choline or 5 - 30 min of perfusion with [1-^{3}H] ethanolamine. Irregular electrocardiac recording was observed during the first 3 min of perfusion, indicating that several min were required for the isolated heart to adjust to the temperature change at the commencement of perfusion.

i) Uptake of Labeled Compounds by the Isolated Heart

i) General

The isolated heart was allowed to perfuse for 5 min in Krebs-Henseleit buffer saturated with 95% O_2 - 5% CO_2. The heart was then perfused with Krebs-Henseleit

buffer containing [Me-^{3}H] choline for 5 - 60 min or [1-^{3}H] ethanolamine for 5 - 30 min. Subsequently, the heart was reperfused with 5 ml Krebs-Henseleit buffer to remove any labeled compound trapped in the vascular space. After perfusion, 10 ml of air was forced through the cannula to remove the buffer. The heart was cut open, blotted dry and the wet weight determined. The tissue was homogenized in 20 ml $CHCl_3$/MeOH (2/1; v/v) and an aliquot of the homogenate was taken for radioactivity determination. The uptake of labeled compound was calculated from the specific radioactivity of the compound in the perfusate.

ii) Analysis of Phospholipids

The homogenate obtained after perfusion was separated into 2 phases by the addition of 10 ml of 0.1 N KCl to the homogenate. In studies where [Me-^{3}H] choline or [1-^{3}H] ethanolamine were the labeled precursors, phospholipids in the organic phase were separated by TLC with $CHCl_3$/MeOH/H_2O/acetic acid (70/30/4/2; v/v/v/v). This system allows for excellent separation of PC, PE and LPC. Unfortunately, PC and LPE did not separate well by this solvent system, therefore quantitation of these two lipids were confirmed by TLC with a solvent system of $CHCl_3$/MeOH/H_2O/NH_4OH (70/30/4/1; v/v/v/v) as described by Choy and Vance, (1978). In experiments where L-[3-^{3}H] serine was used as substrate, PS and PE were separated by TLC with $CHCl_3$/MeOH/H_2O/NH_4OH (70/30/4/2; v/v/v/v). In this case PS, PC and PE were clearly separated from one

another. The lipid phosphorous content in each phospholipid was determined as described previously by the method of Raheja et al., (1973), or Bartlett, (1959). The amount of radioactivity incorporated into the phospholipids was separately determined by liquid scintillation counting.

iii) Analysis of Choline-containing Metabolites

The aqueous phase of the total homogenate was lyophilized and reconstituted in H_2O. The metabolites choline, phosphocholine, CDP-choline, acetylcholine, glycerophosphocholine and betaine were separated by TLC with appropriate carriers, as described by Yavin, (1976). The locations of these metabolites on the thin-layer plate were visualized by exposure of the plate to iodine vapor. The spots were taken from the plate and placed in scintillation vials. Water and ACS were added to each vial, and the amount of radioactivity was determined. Choline, phosphocholine and CDP-choline were also separated by TLC with MeOH/0.6% NaCl/NH_4OH (50/50/5; v/v/v). The amounts of radioactivity in these compounds were comparable to those obtained previously. Since CDP-choline and betaine were separated quite poorly by TLC, the amount of radioactivity in these two compounds was routinely confirmed by further separation with Dowex AG-1-X8 (OH^- form) ion exchange chromatography.

iv) Analysis of Ethanolamine-containing Metabolites

The aqueous soluble components, ethanolamine, phosphoethanolamine and CDP-ethanolamine were separated

by TLC with appropriate carriers in 96% ethanol/2% NH_4OH (1/2; v/v) as described by Sundler, (1975). CDP-ethanolamine was visualized by U.V. light, whereas ethanolamine and phosphoethanolamine were located by ninhydrin spray. The corresponding spots were removed from the plates, placed in scintillation vials with water and ACS, and the amount of radioactivity associated with each intermediate determined.

j) Pulse-Chase Studies on the Metabolism of Choline

After a stabilization period of 5 min, the isolated hamster heart was perfused with Krebs-Henseleit buffer containing 0.1μM [Me-^{3}H] choline for 5 min. The heart was subsequently perfused with Krebs-Henseleit buffer containing 0.1μM choline for 1 - 60 min. After perfusion, the heart was homogenized as previously described, and the labeled compounds of the organic and aqueous phases were analyzed by TLC.

k) Measurement of Choline, Phosphocholine and CDP-choline in Hamster Hearts

Three groups of hamster hearts were employed for this study: (a) normal, unperfused hearts (b) hearts perfused with 0.1μM choline for 60 min (c) hearts perfused with 50μM choline for 60 min. Immediately after their removal from the animals or after perfusion, two hearts from each group were weighed and homogenized in $CHCl_3$/MeOH (2/1; v/v). 0.1 M KCl was added to the homogenate and the aqueous phase was removed and evaporated under reduced pressure. Yields

of the aqueous soluble choline-containing compounds were estimated by the recovery of [Me-^{3}H] choline, [Me-^{3}H] phosphocholine and [Me-^{14}C] CDP-choline which were added to the extract.

The procedure for quantitation of the choline-containing compounds in the aqueous extracts was adapted from Vance et al., (1980). The aqueous phase was redissolved in H_2O and applied to a column (1 cm x 30 cm) that contained Dowex AG-1-X8, (OH^- form), which had been equilibrated with CH_3OH/H_2O (1/1; v/v). Choline was eluted from the column with CH_3OH/H_2O (1/1; v/v) followed by H_2O. Phosphocholine and CDP-choline were eluted with 0.4 M NH_4HCO_3. These fractions were evaporated under reduced pressure.

The phosphocholine and CDP-choline samples were redissolved in H_2O and applied to a Norit A-celite (1/1; v/v) charcoal bed (1 cm x 5 cm). The column was washed with H_2O and 2% ethanol. Phosphocholine was eluted and subsequently evaporated under reduced pressure. CDP-choline was eluted from the column with further addition of 40% ethanol containing 1% NH_4OH. This solution was evaporated under reduced pressure. The fractions containing choline, phosphocholine and CDP-choline were reconstituted in H_2O, and an aliquot from each fraction was taken for radioactivity determination. The fraction containing phosphocholine was digested with 6.5 units of E.coli alkaline phosphatase for 2 h at 37°C. The fraction

containing CDP-choline was digested with 6.5 units of E.coli alkaline phosphatase and 0.5 units of Crotalus adementeus phosphodiesterase for 3 h at 37°C. Subsequently, both groups of reaction mixtures were lyophilized and the residue was extracted with 70% ethanol. The ethanol in the extracted sample was evaporated under a stream of N_2.

The choline, the digested phosphocholine and CDP-choline were separately dissolved in H_2O. Tetraphenylboron in 3-heptanone was used to extract choline from each pool. A second extraction was performed with a second aliquot of tetraphenylboron in 3-heptanone. The choline in each sample was back-extracted from the tetraphenylboron solution with 0.4 N HCl followed by a second extraction with 1.0 N HCl. The HCl solutions were combined and lyophilized. Each pool was redissolved in H_2O and an aliquot from each sample was taken for radioactivity determination. The yield was calculated from the total radioactivity recovered in each pool.

The reaction of each sample with choline kinase and [γ-^{32}P] ATP, as described by Choy et al., (1978), allowed quantitation of the amount of extracted choline, by the production of [^{32}P] phosphocholine. A standard choline curve was established, and the choline concentration in each pool was calculated from the standard curve.

1) Measurement of Ethanolamine, Phosphoethanolamine and CDP-ethanolamine in Hamster Heart

In this study, normal unperfused hearts were used

for determination of the pool sizes of the various ethanolamine-containing metabolites. As in the measurement of choline-containing metabolites, the hearts were rapidly removed from the animal, weighed and homogenized in $CHCl_3$/MeOH (2/1; v/v). After the addition of 0.1 N KCl the aqueous phase was separated from the organic phase and was evaporated under reduced pressure. [1-^{3}H] ethanolamine was added to estimate the recovery of the ethanolamine-containing compounds. The aqueous phase was subsequently redissolved in H_2O. An aliquot of the aqueous phase was combined with trichloroacetic acid, (TCA), to make a final concentration of 55% TCA. This solution was centrifuged at 3,000 x g for 20 min to pellet any protein in the sample. An aliquot of the supernatant was taken for radioactivity determination and a separate aliquot was applied to amino acid analyzer. Analysis of ethanolamine-containing compounds was adapted from the procedure of van Sande and van Camp, (1979). The amino acid analyzer was equipped with a W-2 (30 cm x .75 cm) column. The samples were eluted with 0.2 M Na Citrate buffer (pH 4.75) at a constant flow rate of 78 ml/h at 40°C. The column was standardized against norleucine, ethanolamine, phosphoethanolamine and CDP-ethanolamine. Ethanolamine was retained under these conditions (32 min), whereas phosphoethanolamine and CDP-ethanolamine co-eluted at 10 min. Since these two compounds were poorly resolved, an identical aliquot of the aqueous phase was taken and digested with 4 units of alkaline

phosphatase in 50 mM Tris-HCl buffer (pH 8.5) for 2 h at 37°C. Subsequent to digestion, TCA was added to precipitate the protein material in the sample. An aliquot of this mixture was taken for calculation of recovery and another aliquot for application to the analyzer. In this case, the peak at 10 min is CDP-ethanolamine, and the other peak (32 min) is ethanolamine, which originates from phosphoethanolamine hydrolysis and that which was in the original (i.e. non-digested) sample. The decrease in the peak area at 10 min, corresponded to the increase in the peak area at 32 min. By this procedure, the pool sizes of ethanolamine, phosphoethanolamine, and CDP-ethanolamine were calculated by the simple equation:

$$\text{Concentration} = \frac{\text{area under the peak}}{\text{KF} \times 10^{-4}}$$

m) Measurement of Choline in Hamster Plasma

Blood was obtained from the hamster by cardiac puncture with a heparinized syringe. Plasma was separated from other blood components by centrifugation. A small amount of $\left[\text{Me-}^{3}\text{H}\right]$ choline was added to the plasma, followed by the addition of $CHCl_3$/MeOH (2/1; v/v). The precipitate was filtered, and the aqueous phase was removed. The organic phase was re-extracted with 0.1 N KCl. The aqueous phase from the re-extraction was pooled with the original aqueous phase, and the solution was lyophilized. The isolation and determination of choline content in the lyophilized sample was identical to the procedure for choline determination in hamster hearts.

n) Measurement of Ethanolamine in Hamster Plasma

Blood was obtained by heart puncture with a heparinized syringe and plasma was separated from other blood components by centrifugation. A small amount of [1-^{3}H]ethanolamine was added to the plasma sample to allow recovery calculations. An aliquot of the plasma sample was taken, the proteins precipitated with TCA and applied to the amino acid analyzer. The ethanolamine content of plasma was determined as previously described.

A second sample of plasma was taken and applied to a AG-1-X8, (OH^- form), column, and ethanolamine was eluted with 13.0 ml of H_2O. The solution was lyophilized and redissolved in H_2O. An aliquot was taken for radioactivity determination and a separate aliquot was spotted on a TLC plate and developed in 96% ethanol/2% NH_4OH (2/1; v/v). The ethanolamine spot was removed, placed in a pasteur pipet and ethanolamine was eluted from the silica gel with 15.0 ml of 75% ethanol. An aliquot of this solution was taken for recovery calculations and then the sample was dried under a stream of N_2. The sample was reconstituted in 2.0 ml ethanol and placed in a boiling water bath for 5 min with 100 μl ninhydrin solution prepared according to the procedure of Hirs et al., (1956). Ethanol, (1.5 ml), was added to each tube and absorbance was measured at 570 nm. The amount of ethanolamine in plasma was determined by comparison with a series of ethanolamine standards.

o) Extracellular Choline and Ethanolamine in Hamster Heart

The amount of choline and ethanolamine trapped in the extracellular space was calculated based on the following assumptions:

a) Extracellular volume = 19.0% wet weight of the heart, Polimeni, (1974).

b) The amount of choline and ethanolamine in the perfusate or plasma was at equilibrium with the extracellular fluid.

The amount of choline and ethanolamine in extracellular space was determined by the concentration of choline and ethanolamine in plasma or perfusate x volume of extracellular space calculated.

EXPERIMENTAL RESULTS

I PHOSPHATIDYLCHOLINE BIOSYNTHESIS

a) In vitro Enzyme Activities Involved in Phosphatidylcholine Biosynthesis

The initial approach to identify the known pathways leading to the formation of PC in hamster heart was to assay for the enzymes responsible for these pathways. A 5% hamster heart homogenate was prepared in 0.145 M NaCl and subcellular fractions were obtained by differential centrifugation. The subcellular localization of these enzymes was determined, and the activities of these enzymes assayed at optimal conditions are shown in Table I. The presence of enzyme activities for PC formation via CDP-choline pathway, by progressive methylation of PE and by base exchange of choline with other phospholipids suggest that all these pathways may contribute to PC formation in hamster heart.

b) Phosphatidylcholine Biosynthesis from Choline

Since choline is the common precursor for PC formation via CDP-choline pathway and base exchange, the contributions of these two pathways were evaluated in the isolated hamster heart by perfusion with [Me-^{3}H] choline as described in "Materials and Methods". Total uptakes of radioactivities by the hearts were linear from 5 - 60 min of perfusion with 0.1 or 50 μM [Me-^{3}H] choline (4 μCi/ml). Linearity of uptake was also observed with 0.1 - 50 μM choline at 30 min of perfusion. (Fig. 3A and B). From the double reciprocal plot of choline uptake vs choline

Table I

The activities of phosphatidylcholine biosynthetic enzymes from hamster heart.

Enzyme activities were assayed under optimal conditions as described in "Materials and Methods".

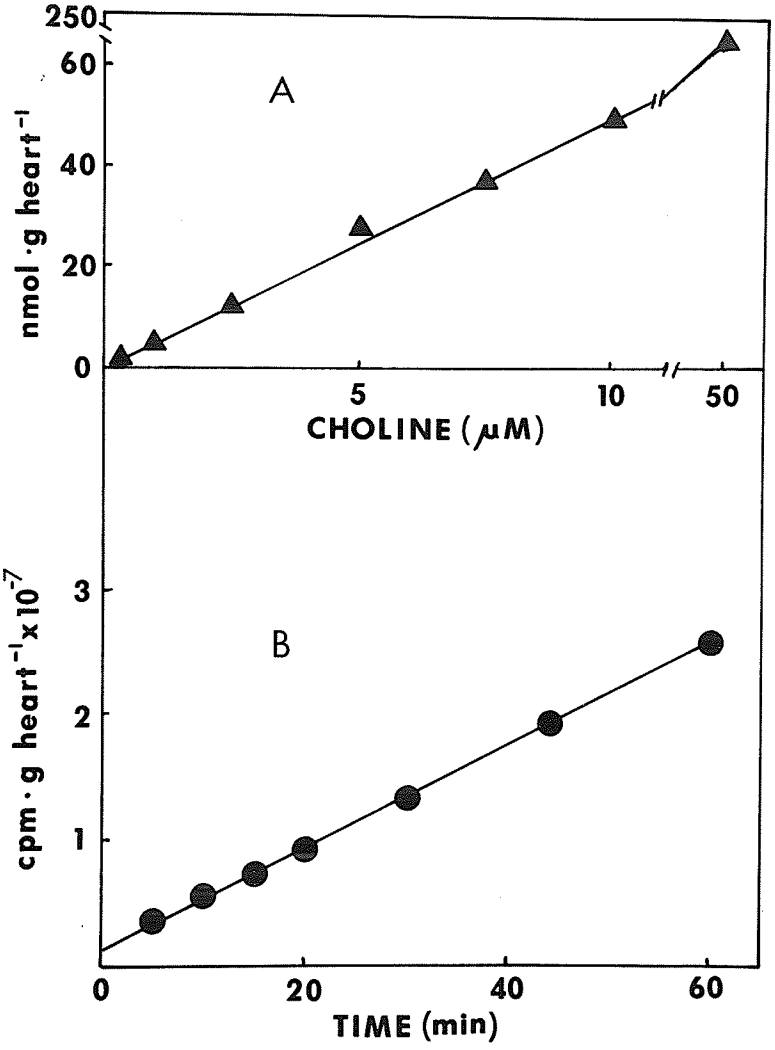
Enzyme Activities	
	($nmol \cdot min^{-1} \cdot mg^{-1}$)
Choline kinase (Cytosolic)	0.67[a] ± 0.14[b] (8)[c]
Phosphocholine cytidylyltransferase (Microsomal)	0.17 ± 0.06 (6)
Phosphocholine cytidylyltransferase (Cytosolic)	0.13 ± 0.05 (9)
Phosphocholinetransferase (Microsomal)	0.014 ± 0.005 (9)
	($nmol \cdot 30\ min^{-1} \cdot mg^{-1}$)
PE-SAM methyltransferase (Microsomal)	0.012 ± 0.008 (4)
Base exchange of phospholipids with choline (Microsomal)	0.020 ± 0.003 (3)

[a] Mean

[b] Standard deviation

[c] Number of experiments

Fig. 3. Total choline uptake in isolated hamster hearts. Isolated hamster hearts were canulated via the aorta and perfused with Krebs-Henseleit buffer containing [Me-^{3}H] choline. After perfusion the hearts were homogenized in chloroform/methanol (2/1; v/v), and the total radioactivities in the homogenate were determined. (A), hearts were perfused with 0.1 - 50 μM [Me-^{3}H] choline (4 μCi/ml) for 30 min. The total choline uptake by the isolated hearts was calculated from the specific radioactivities of choline in the perfusate. (B), hearts were perfused with 0.1 or 50 μM [Me-^{3}H] choline (4 μCi/ml) for 5 - 60 min. The amounts of radioactivity taken up by the hearts at both choline concentrations were identical.


250
60
40
20
0
A
nmol · g heart⁻¹
5
10
50
CHOLINE (μM)
3
2
1
0
B
cpm · g heart⁻¹ x 10⁻⁷
20
40
60
TIME (min)

concentrations, the Km for choline uptake by the isolated heart was estimated to be 0.1 mM of choline. The Km value obtained was quite different from the results obtained with brain cell cultures as described by Yavin, (1976).

The labeled metabolites in the aqueous phase and organic phase of the heart homogenate after perfusion were analyzed by TLC. More than 85% of the total radioactivity in the aqueous phase was found in phosphocholine. Unlike the results obtained in rat liver studies Sundler et al., (1972), less than 5% of the label was recovered from betaine. No significant amount of labeled glycerolphosphocholine was detected. Over 90% of the radioactivity in the organic phase was recovered in PC and less than 2% was found in sphingomyelin, PE or PS. A small but consistant amount of radioactivity (3 - 5%) was detected in LPC at all time points of perfusion. The counts incorporated into choline, phosphocholine, CDP-choline and PC in the heart perfused with 0.1 or 50μM [Me-^{3}H] choline from 5 - 60 min are depicted in Fig. 4 and 5. The counts in choline reached maximum within 5 min of perfusion and remained constant, whereas the counts in phosphocholine and CDP-choline reached maxima at 30 and 45 min of perfusion, respectively. A distinct lag in the incorporation of label into PC was apparent during the first 15 min of perfusion with 0.1 or 50μM [Me-^{3}H] choline. Since the specific radioactivity of labeled choline in the heart remained constant (Table II), this apparent lag

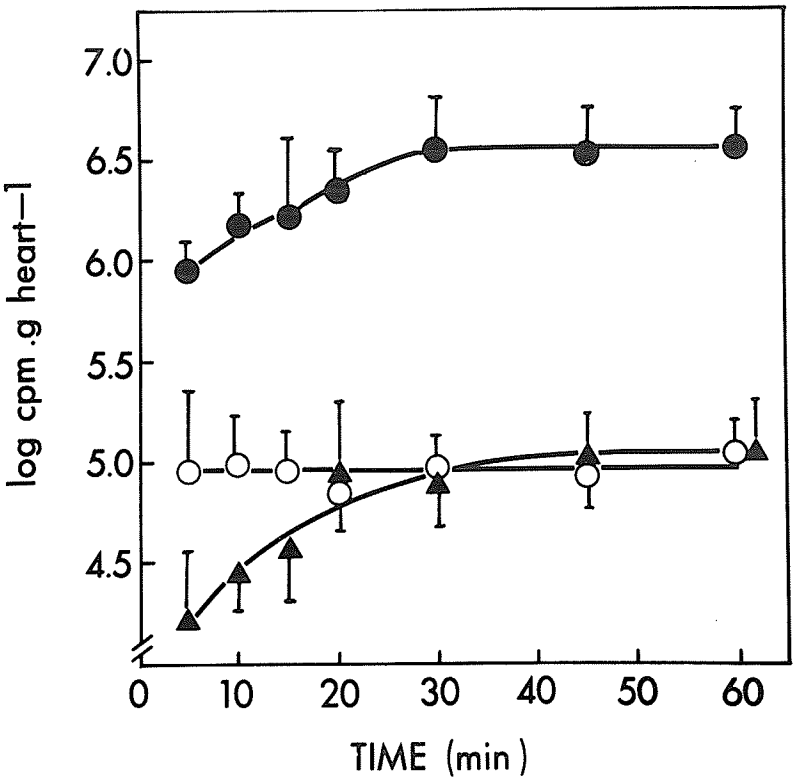


Fig. 4. Time course of [Me-^{3}H] choline incorporation into the major choline-containing compounds.

Hamster hearts were perfused with [Me-^{3}H] choline in Krebs-Henseleit buffer from 5 - 60 min. Subsequent to perfusion, hearts were homogenized in chloroform/methanol (2/1; v/v), to which an equal volume of 0.1 N KCl was added. The aqueous phase was analyzed by thin-layer chromatography for labeled metabolites, (o—o) choline; (●—●) phosphocholine and (▲—▲) CDP-choline. Each point represents the mean of four separate experiments. The vertical bars are standard deviations.

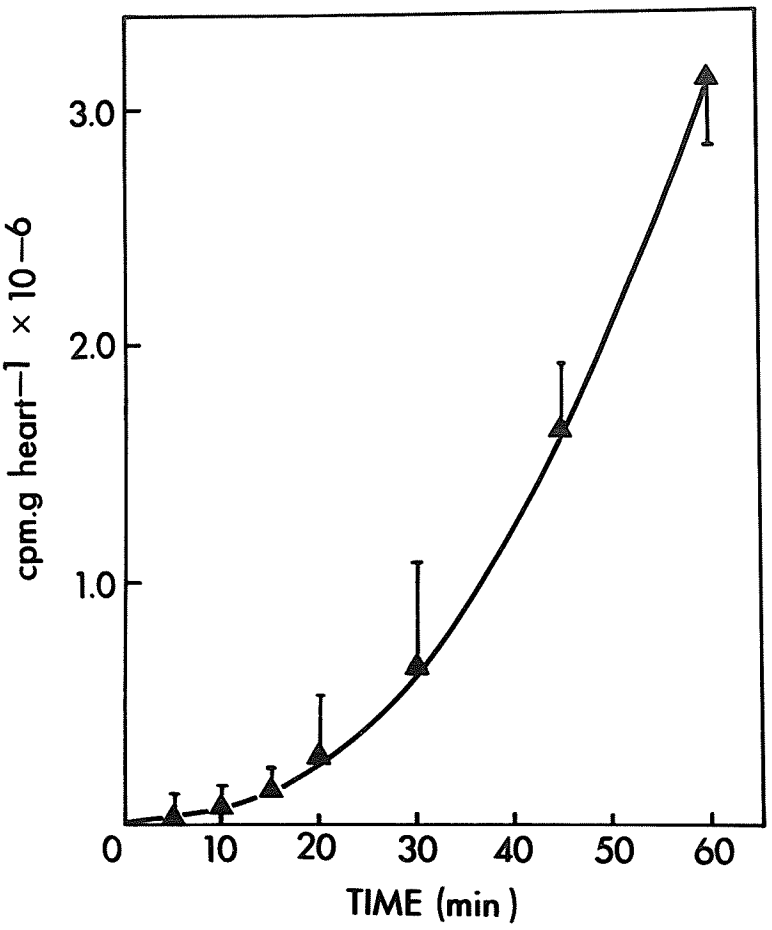


Fig. 5. Time course of [Me-^{3}H] choline incorporation into phosphatidylcholine. The lower (chloroform) phase from Fig. 4 was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine. Each point represents the mean of four separate experiments, the vertical bars indicate standard deviations.

of label incorporation into PC indicates that the majority of the labeled phospholipid was not formed by the base exchange reaction. From the average specific radioactivity of CDP-choline at 30 - 60 min of perfusion, the rate of PC formation via the CDP-choline pathway was estimated to be 39 nmol·min^{-1}· g heart^{-1}.

c) Pool Sizes of Precursors to Phosphatidylcholine

A change in the pool size of one of the choline-containing precursors during perfusion may change the specific radioactivity of the metabolite, and may subsequently affect the rate of radioactivity incorporation into PC. Hence the amount of choline, phosphocholine and CDP-choline in hamster heart were determined before and after perfusion with 0.1 or 50 μM choline (Table II). Since some choline was trapped in the extracellular space before or after perfusion, the choline in the extracellular compartment was estimated as described in "Materials and Methods". The amount of intracellular choline was calculated by subtracting the extracellular choline from the total amount of choline in tissue homogenate. No significant changes in choline, phosphocholine and CDP-choline concentrations were observed (Table II), ($p < .05$). The amount of choline in hamster plasma was estimated to be 180 $\pm$ 41 μM and the amount of PE in hamster heart was 4.8 $\pm$ 0.8 μmol· g wet weight^{-1}.

Table II

Concentration of choline, phosphocholine and CDP-choline in hamster heart.

The pool sizes of the choline-containing metabolites in hamster hearts before and after 60 min of perfusion were measured as described in "Materials and Methods". Each value represents the mean of three separate experiments.

	Before Perfusion	Perfusion with 0.1 μM choline	Perfusion with 50 μM choline
	(nmol/g heart)		
Choline	283[a] ± 44[b]	245 ± 22	285 ± 59
Phosphocholine	236 ± 22	188 ± 35	176 ± 32
CDP-choline	91 ± 2	71 ± 16	92 ± 9

[a] Mean

[b] Standard deviation

d) Phosphatidylcholine Biosynthesis from Phosphatidylethanolamine

The contribution to PC formation by the methylation of PE was also investigated in the hamster heart. The isolated hearts were perfused with 0.005 μM [1-^{3}H] ethanolamine (Specific radioactivity 19.5 Ci/mmol) for 10 - 60 min and the radioactivity incorporated into PE and PC were determined (Fig. 6). From the average specific radioactivity of PE at 30 - 60 min of perfusion, the rate of PC formed via progressive methylation was estimated to be 1.0 nmol$\cdot$min$^{-1}\cdot$ g heart^{-1}.

e) Rate-Limiting Step for Phosphatidylcholine Biosynthesis

Since the CDP-choline pathway appeared to be the major pathway for the formation of PC in isolated hamster heart, the rate-limiting step for this pathway was studied by pulse-chase experiments. Isolated hamster hearts were perfused with 0.1 μM [Me-^{3}H] choline (10 μCi/ml) for 5 min, and subsequently perfused with 0.1 μM non-radioactive choline for 1 - 60 min. The hearts were homogenized after perfusion and the labeled metabolites were analyzed (Fig. 7). The radioactivity in choline varied from one experiment to the other during the first 5 min of chase and this variation was attributed to the varying amounts of labeled choline trapped in the extracellular space. Maximum labeling of phosphocholine was observed immediately after pulse and the counts decreased rapidly during the

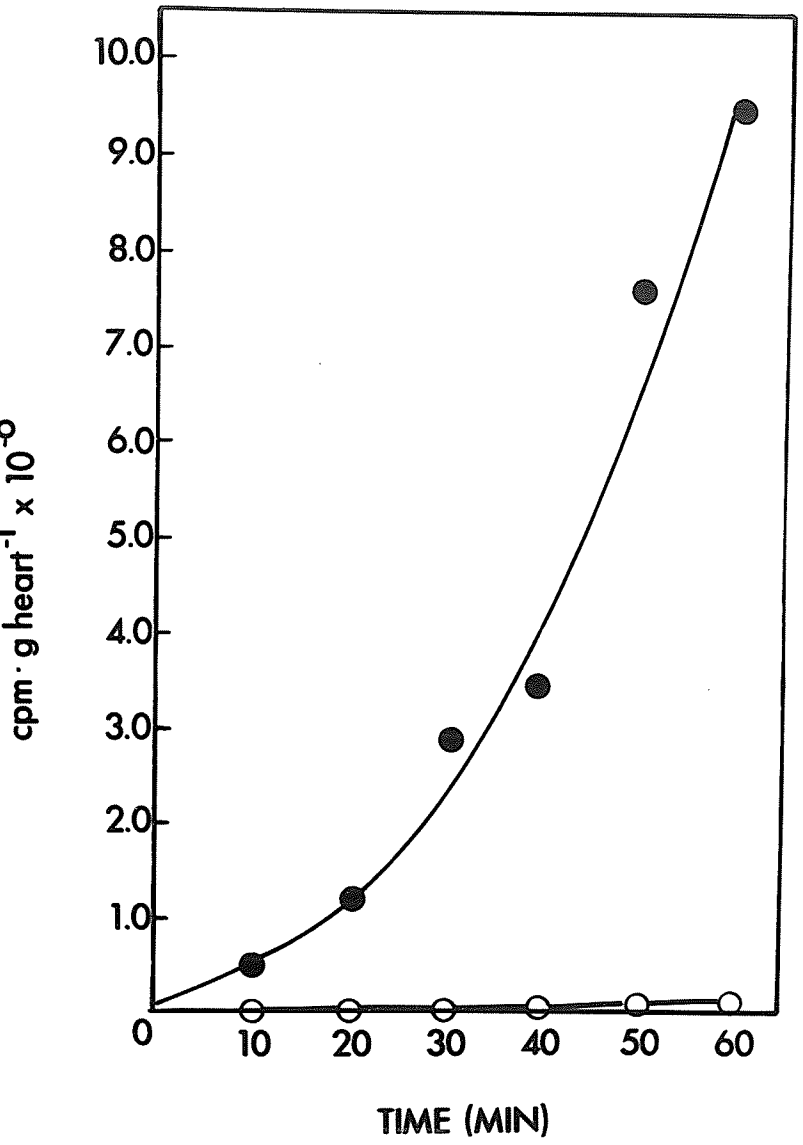


Fig. 6. The incorporation of [1-^{3}H] ethanolamine into phosphatidylcholine.

Isolated hamster hearts were perfused with [1-^{3}H] ethanolamine as described in Fig. 4. Phosphatidylcholine (o—o) and phosphatidylethanolamine (●—●) were separated by thin-layer chromatography. Each point represents the mean of two separate experiments.

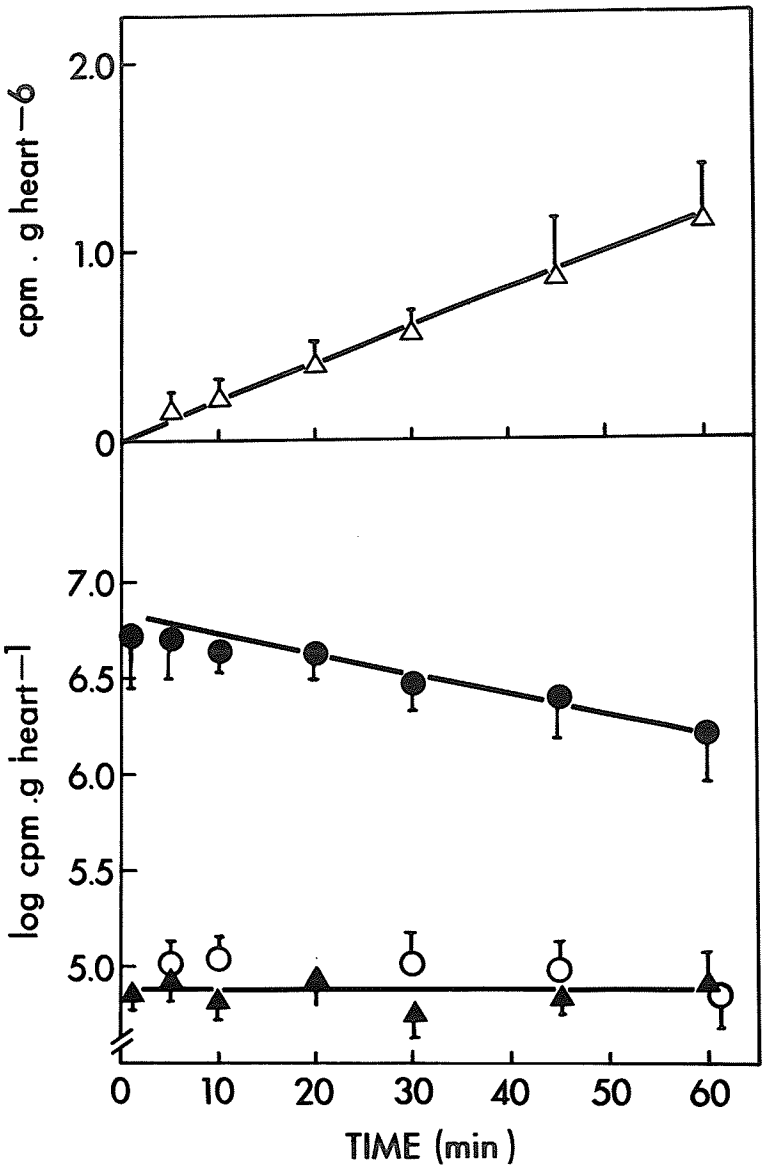


Fig. 7. Pulse-chase study on the metabolites of [Me-^{3}H] choline in hamster hearts.

Isolated hearts were pulsed for 5 min with 0.1 μM [Me-^{3}H] choline, and subsequently chased with 0.1 μM choline for 1 - 60 min. The amount of radioactivity in choline (o—o); phosphocholine (●—●); CDP-choline (▲—▲) and phosphatidylcholine (△—△) were determined. Each point represents the mean of three separate experiments. The vertical bars are standard deviations.

Table III

Distribution of radioactivity in the tissue and effluent after an initial 5 min pulse with [Me^{3}H] choline.

Isolated hearts were pulse labeled with [Me-^{3}H] choline for 5 min and were chased with non-radioactive choline for 10 - 60 min. In a typical set of experiments the amount of radioactivity in each heart and the effluent collected during the chase period was determined. More than 95% of the radioactivity in the effluent was recovered as choline.

Chase period	Radioactivity in the heart	Radioactivity in the effluent
min	cpm x 10^{-6} (cpm x 10^{-6}.g wet weight^{-1})	cpm x 10^{-6} (cpm x 10^{-6}.g wet weight^{-1})
10	3.9 (9.8)	3.3 (8.3)
30	3.3 (8.0)	5.0 (12.2)
45	2.6 (6.4)	5.0 (12.2)
60	2.3 (6.0)	5.1 (13.4)

chase period (t 1/2 = 35 min). A concomitant increase in PC labeling was observed, and the labeling of CDP-choline remained constant during the chase. The results clearly indicated that the rate-limiting step occurs at the conversion of phosphocholine to CDP-choline. However, there was a discrepancy between the amount of radioactivity lost from phosphocholine and the amount of radioactivity gained in PC. The difference in radioactivity was subsequently recovered in the effluent as labeled choline (Table III). This labeled choline may originate from the hydrolytic cleavage of phosphocholine by phosphocholine phosphatase (alkaline phosphatase) in the hamster heart (11 $nmol \cdot min^{-1} \cdot mg^{-1}$).

II PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

a) Phosphatidylethanolamine Biosynthesis from Ethanolamine

From the previous study of PC biosynthesis, it was shown that labeled ethanolamine was taken up by the heart and some of the label was incorporated into PE. Therefore, the initial approach in this study was to perfuse the isolated hamster heart with [1-^{3}H] ethanolamine, thereby simultaneously evaluating the contribution of both the CDP-ethanolamine and base exchange pathways for the new formation of PE. Total uptakes of radioactivities by the hearts were linear from 5 - 30 min of perfusion (Fig. 8) with 0.1 or 100 μM [1-^{3}H] ethanolamine (4 μCi/ml).

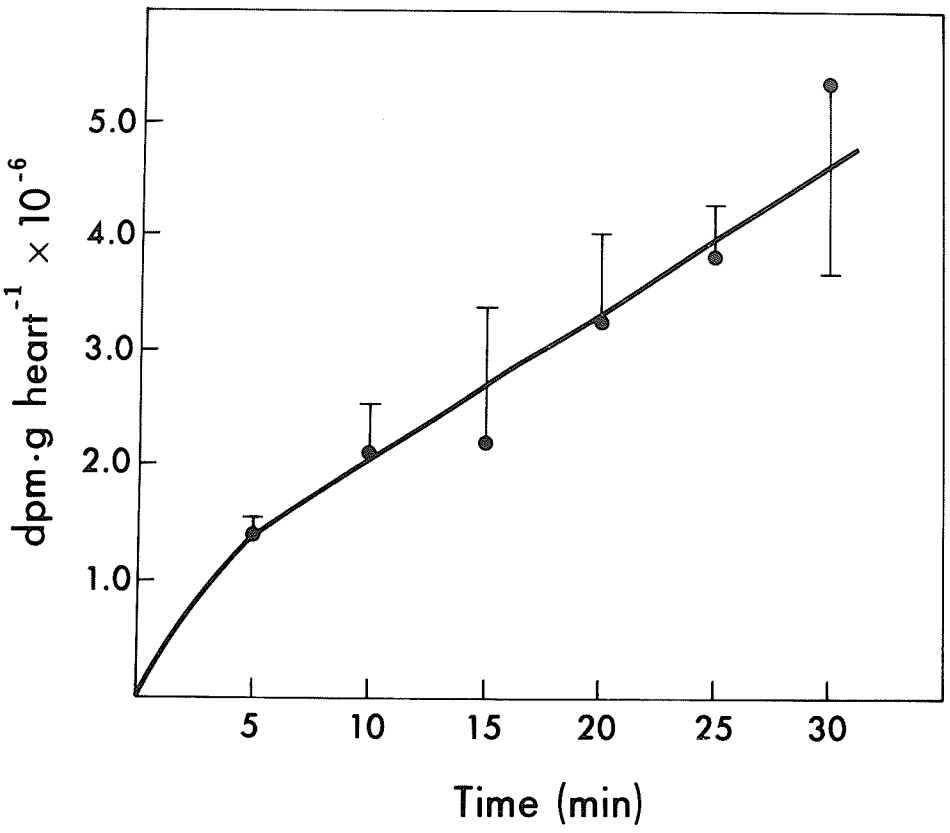


Fig. 8. Time course of [1-^{3}H] ethanolamine uptake in isolated hamster hearts.

Isolated hamster hearts were perfused with 0.1 μM [1-^{3}H] ethanolamine for 5 - 30 min as described in Fig. 4. After perfusion the hearts were homogenized in chloroform/methanol (2/1; v/v), and the total radioactivities in the homogenates were determined. Uptake of radioactivity (●—●) was linear from 5 - 30 min at 0.1 μM [1-^{3}H] ethanolamine. Each point represents the mean of four separate experiments. The vertical bars are standard deviations.

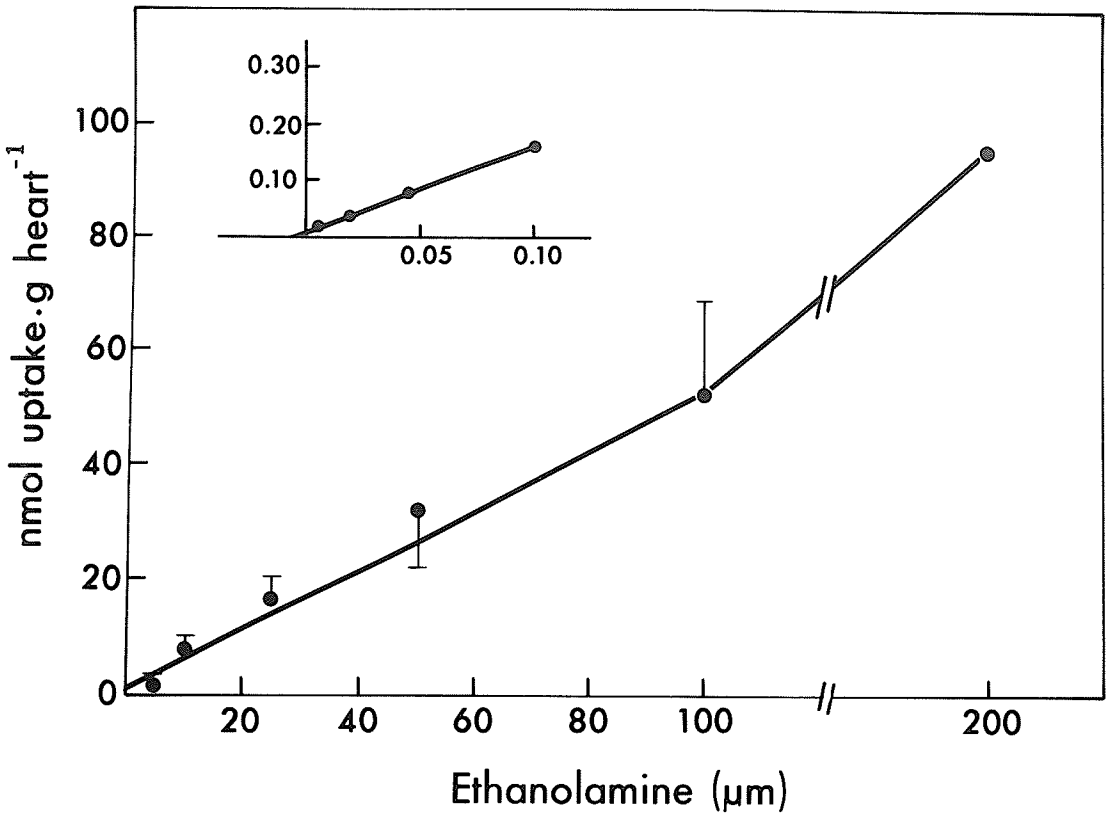


Fig. 9. Total ethanolamine uptake in isolated hamster hearts.

Hamster hearts were perfused with Krebs-Henseleit buffer containing 0.1 - 200 μM [1-^{3}H] ethanolamine, (4 μCi/ml), for 30 min. The total uptake of ethanolamine was calculated as described in Fig. 3A. Uptake of ethanolamine was linear from 0.1 - 100 μM ethanolamine (●—●). Each point represents the mean of four separate experiments, the vertical bars indicate standard deviations. The Km ethanolamine uptake was determined by double reciprocal plot (inset). The Km was found to be = 0.17 mM.

Linearity of uptake was also observed with 0.1 - 100 μM ethanolamine at 30 min of perfusion (Fig. 9). From the double reciprocal plot of ethanolamine uptake vs ethanolamine concentrations, the Km for ethanolamine uptake by the isolated heart was estimated to be 0.17 mM of ethanolamine (Fig. 9 inset).

The labeled metabolites in the aqueous and organic phases of the heart homogenate after perfusion were analyzed by TLC. The majority of the radioactivity in the aqueous phase was recovered in phosphoethanolamine, but a significant amount of radioactivity was also recovered in CDP-ethanolamine. Most of the radioactivity in the organic phase was recovered in PE and only a small amount of radioactivity was recovered in LPE or PC. The counts incorporated into ethanolamine, phosphoethanolamine, CDP-ethanolamine and PE in the heart perfused with 0.1 μM [1-^{3}H] ethanolamine from 5 - 30 min are depicted in Fig. 10 and 11. The radioactivity of ethanolamine in the heart reached its maximum at 5 min of perfusion and remained constant throughout the perfusion period. Maximum labeling of phosphoethanolamine occured at 25 min of perfusion, and labeling of CDP-ethanolamine did not reach maximum over the 30 min perfusion period. Incorporation of radioactivity into PE was marked by a lag during the first 15 min of perfusion, after which a linear increase was observed. This initial lag suggested the minor contribution of the base exchange pathway. From the average specific radio-

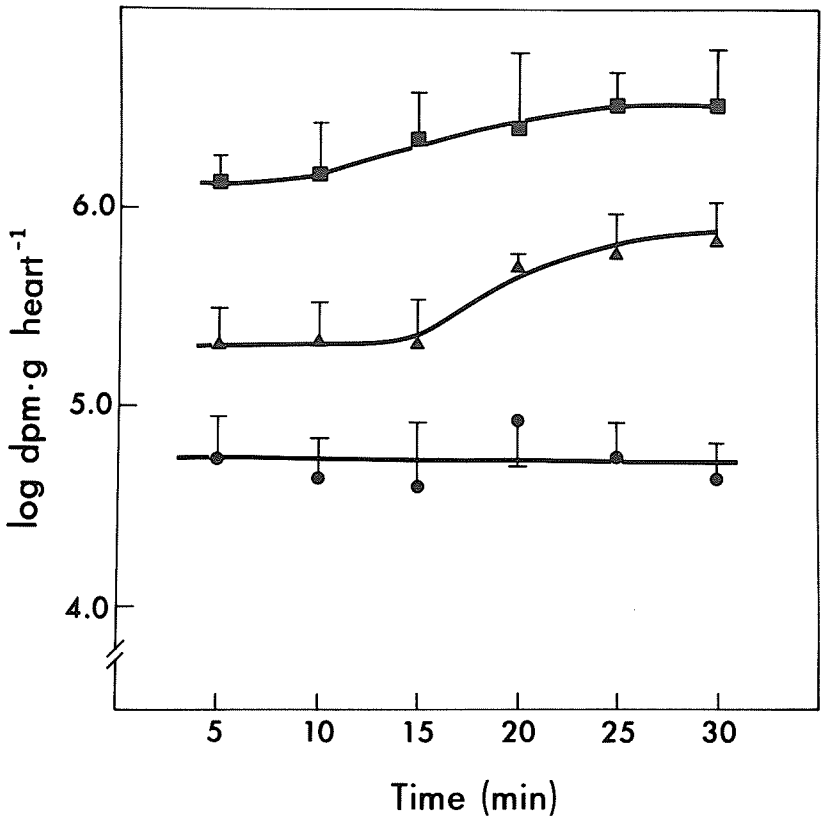


Fig. 10. Time course of [1-^{3}H] ethanolamine incorporation into the major ethanolamine-containing compounds. Hamster hearts were perfused with 0.1 μM [1-^{3}H] ethanolamine in Krebs-Henseleit buffer for 5 - 30 min. Subsequent to perfusion, hearts were homogenized in chloroform/methanol (2/1; v/v), to which an equal volume of 0.1 N KCl was added. The aqueous phase was analyzed by thin-layer chromatography for labeled metabolites: (●—●) ethanolamine; (■—■) phosphoethanolamine; (▲—▲) CDP-ethanolamine. Each point represents the mean of four separate experiments. The vertical bars are standard deviations.

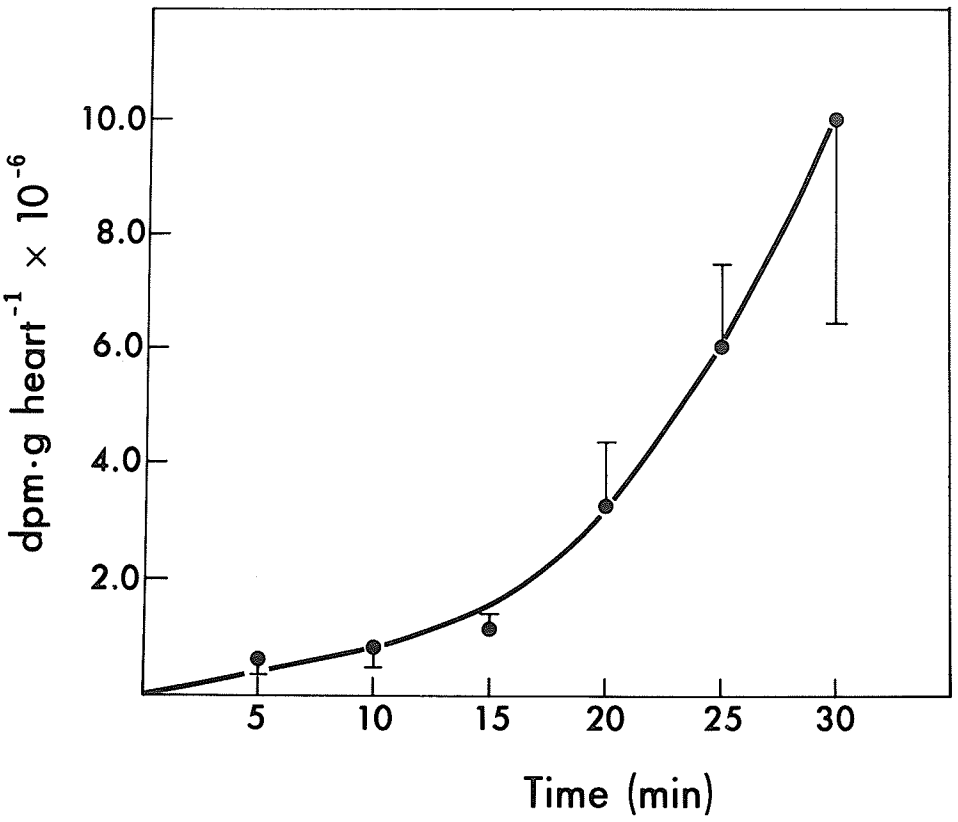


Fig. 11. Time course of [1-^{3}H] ethanolamine incorporation into phosphatidylethanolamine.

The lower phase from Fig. 10 was analyzed by thin-layer chromatography for radioactivity in phosphatidylethanolamine. Each point represents the mean of four separate experiments, the vertical bars indicate standard error of means.

activity of CDP-ethanolamine at 15 - 30 min of perfusion, the rate of PE formation via the CDP-ethanolamine pathway was estimated to be 290 nmol·min^{-1}· g heart^{-1}.

b) Pool Sizes of Precursors to Phosphatidyl-ethanolamine

In order to calculate the contribution of the CDP-ethanolamine pathway to the formation of PE in the heart, the pool size of CDP-ethanolamine was required. Hence, the amount of ethanolamine, phosphoethanolamine, and CDP-ethanolamine were determined in the hamster heart (Table IV). These concentrations were quite different from those obtained by Sundler, (1973), in rat liver. The concentration of ethanolamine in hamster plasma was initially determined by Dowex AG-1-X8 column chromatography, as described in "Materials and Methods". Since the value obtained via this procedure was quite different from the results obtained for ethanolamine concentration in rat plasma (Sundler and Åkesson, 1975), an alternative method was used. Utilizing the amino acid analyzer, the concentration of ethanolamine in hamster plasma was estimated to be 0.9 mM. This value was in good agreement with values obtained by column chromatography. Therefore it is likely that ethanolamine concentration in plasma, varies from species to species. The amount of PS in hamster heart was estimated to be 3.93 μmol· g wet weight^{-1}.

Table IV

Concentration of ethanolamine, phosphoethanolamine and CDP-ethanolamine in hamster heart.

The pool sizes of the ethanolamine-containing metabolites in hamster hearts were measured as described in "Materials and Methods". Each value represents the mean of three separate experiments.

	Pool Sizes
	μmol/g heart
Ethanolamine	5.25[a] ± 1.10[b]
Phosphoethanolamine	2.55 ± 0.52
CDP-ethanolamine	1.86 ± 0.66

[a] Mean

[b] Standard deviation

c) Phosphatidylethanolamine Biosynthesis from Phosphatidylserine

The contribution to PE formation by the decarboxylation of PS was also investigated in the hamster heart. The isolated hearts were perfused with 0.6 μM L-[3-^{3}H] serine (Specific radioactivity = 17.0 Ci/mmol) for 10 - 40 min, and the radioactivity incorporated into PS and PE was determined (Fig. 12). From the average specific radioactivity of PS at 10 - 40 min of perfusion, the rate of PE formed via decarboxylation of PS was estimated to be 9.0 $nmol \cdot min^{-1} \cdot g\ heart^{-1}$.

d) In vitro Enzyme Activities Involved in Phosphatidylethanolamine Biosynthesis

A 10% hamster heart homogenate was prepared in 0.25 M sucrose and subcellular fractions were obtained by differential centrifugation. The subcellular localization of these enzymes was determined, and the activities of these enzymes are shown in Table V. The presence of enzyme activities for PE formation via CDP-ethanolamine pathway, by decarboxylation of PS, and by base exchange of ethanolamine with other phospholipids indicated that all these pathways may contribute to PE formation in hamster heart.

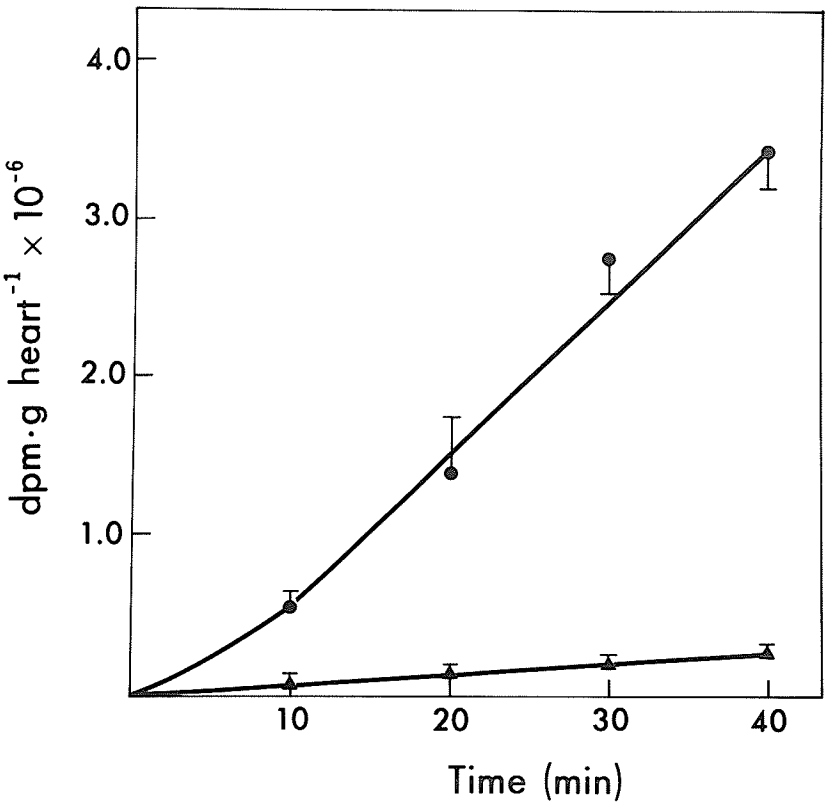


Fig. 12. The incorporation of L-[^{3}H] serine into phosphatidylethanolamine.

Isolated hamster hearts were perfused with L-[^{3}H] serine as described in Fig. 4. Phosphatidylethanolamine (▲—▲) and phosphatidylserine (●—●) were separated by thin-layer chromatography. Each point represents the mean of three separate experiments. The vertical bars are standard deviations.

Table V

The activities of phosphatidylethanolamine biosynthetic enzymes from hamster heart.

Enzyme activities were assayed as described in "Materials and Methods".

Enzyme Activities	
	($nmol \cdot min^{-1} \cdot mg^{-1}$)
Ethanolamine kinase (Cytosolic)	.134[a] ± .001[b] (4)[c]
Phosphoethanolamine cytidylyltransferase (Cytosolic)	.383 ± .054 (4)
Phosphoethanolaminetransferase (Microsomal)	.019 ± .002 (4)
	($nmol \cdot 30\ min^{-1} \cdot mg^{-1}$)
Base exchange of phospholipids with ethanolamine (Microsomal)	.132 ± .033 (3)
Phosphatidylserine decarboxylase (Mitochondrial)	.085 ± .048 (3)

[a] Mean

[b] Standard deviation

[c] Number of experiments

DISCUSSION

I PHOSPHATIDYLCHOLINE BIOSYNTHESIS

The purpose of this study was to identify the contribution of each known pathway for the synthesis of PC in hamster heart. In order to mimic the *in vivo* conditions of the beating heart, initial experiments were performed by direct infusion of [Me-^{3}H] choline into the posterior *vena cava* of the hamster under light ether anesthesia. Incorporation of total radioactivity in the hamster heart after 15 min of infusion was less than 1% and 12% of the radioactivity in plasma was converted to other choline metabolites. This is not surprising since a rapid oxidation of choline was demonstrated in rat liver during perfusion (Sundler *et al.*, 1972). In order to increase the efficiency of [Me-^{3}H] choline uptake by the heart, and also to eliminate the uptake of other labeled metabolites, the hamster heart was isolated and perfused with Krebs-Henseleit buffer (Krebs and Henseleit, 1932), by the procedure of Langendorff (Langendorff, 1895). As shown in Fig. 3, a significant amount of radioactivity was incorporated into the heart after 30 min of perfusion. Moreover, this mode of perfusion provided a facile mechanism for monitoring the electrocardiac activity of the beating heart. No change in electrocardiac function was observed during perfusion, which suggested that the viability of the heart was maintained throughout the perfusion period.

The apparent substrate concentration for half saturation velocity (Km) of choline uptake in isolated, perfused heart

was estimated to be 0.1 mM. This result was quite different from that obtained with brain cell culture (Yavin, 1976), where 2 separate Km values were identified (Km = 0.016 mM and 0.95 mM). Since the choline concentration in hamster plasma is 0.18 mM, it is quite possible that plasma choline concentration may provide a mechanism for the regulation of choline uptake in the hamster heart.

The ability of hamster hearts to metabolize choline is clearly demonstrated. Unlike the papillary and trabecular muscles of the cat heart, (Bosteels *et al.*, 1970), the majority of choline taken up by the hamster heart was rapidly phosphorylated to phosphocholine. The results indicate that more than 90% of PC in hamster heart was synthesized *de novo* from choline via the CDP-choline pathway. Although the exact contribution of base exchange to PC biosynthesis was not determined, the distinct lag of radioactivity incorporation into the phospholipid during the first 15 min of perfusion was similar to the results of Yavin, (1976), with brain cell culture. This lag in label incorporation into the phospholipid is indicative of the minor contribution of base exchange reaction. A small amount (2.5%) of PC in the heart was also formed by progressive methylation of PE. The low activities of PE *S*-adenosylmethionine methyltransferase and base exchange enzyme (Table I) when assayed *in vitro*, under optimum conditions, also reflect the minor contribution of these two pathways.

One perplexing finding is the low concentration of phosphocholine in the hamster heart (Table II) as compared to rat liver (Sundler et al., 1972, Choy et al., 1978) and HeLa cells (Vance et al., 1980). Consequently, the specific radioactivity of phosphocholine from 30 - 60 min of perfusion with 0.1 or 50 μM [Me-^{3}H] choline was several times higher than its precursor. Although preferential phosphorylation of labeled choline might cause a high specific radioactivity in phosphocholine, this does not seem to be the case, since similar results were obtained with [Me-^{14}C] choline. Also, the relatively small fluctuation in choline and phosphocholine concentrations before and after perfusion (Table II) did not account for this increase in specific radioactivity. A possible explanation for this apparent discrepancy is that labeled choline taken up by the heart did not equilibrate with the total pool of intracellular choline prior to phosphorylation. Similar interpretations of results were suggested in the in vivo study of PC biosynthesis in rat liver (Sundler et al., 1972).

The rate-limiting step in the CDP-choline pathway was investigated by pulse-chase experiments with [Me-^{3}H] choline (Fig. 7). From pool size determinations, the specific radioactivity of phosphocholine was over 10-fold higher than that of CDP-choline at all times of perfusion. This clearly indicates that the rate-limiting step for PC biosynthesis is at the conversion of phosphocholine to

CDP-choline. These results further confirm the regulatory role of phosphocholine cytidylyltransferase (Vance and Choy, 1979). A rapid decrease in specific radioactivity was observed in phosphocholine during the chase period. The specific radioactivity of CDP-choline remained constant, possibly due to a rapid equilibrium between CDP-choline and PC. This hypothesis is supported by the fact that phosphocholinetransferase mediates a freely reversible reaction (Kanoh and Ohno, 1973).

One interesting aspect pertaining to the pulse-chase experiments is the metabolic fate of labeled phosphocholine in the heart. Throughout the chase period, less than half of the radioactivity lost from phosphocholine was incorporated into PC, and the majority of the radioactivity was recovered in the effluent as labeled choline (Table III). Although the origin of the labeled choline is not known, it was demonstrated in HeLa cells that alkaline phosphatase and phosphocholine phosphatase are the same enzyme (Paddon and Vance, 1977). The presence of phosphocholine phosphatase and alkaline phosphatase in the heart (Li et al., 1979) makes it feasible to postulate that a portion of the labeled phosphocholine is dephosphorylated into choline and subsequently eluted into the effluent. Another possible pathway for the regeneration of labeled choline is from the hydrolysis of PC by the action of phospholipase D. This does not seem to be the case since the amount of labeled choline formed should

increase with increasing amounts of radioactivity incorporated into PC during the chase period. Moreover, phospholipase D activity has not been reported in the heart. Hence, in the hamster heart, CDP-choline may not be an obligatory product for phosphocholine, and some phosphocholine may be hydrolyzed to choline by alkaline phosphatase. The significance of this alternate pathway for phosphocholine metabolism is not known at the present time.

II PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

The purpose of this study was to evaluate the contribution of the CDP-ethanolamine, base exchange and the PS decarboxylation pathways to the formation of PE in the isolated hamster heart. The CDP-ethanolamine and base exchange pathways were evaluated simultaneously by perfusion with [1-^{3}H] ethanolamine in Krebs-Henseleit buffer (Krebs and Henseleit, 1932), as described by Langendorff, (1895). As shown in Fig. 8, a significant amount of radioactivity was incorporated into the heart, up to 30 min of perfusion. The viability of the heart was maintained throughout the perfusion period, as assessed by electrocardiac recordings of the beating heart as previously described.

The apparent substrate concentration for half saturation velocity (Km) of ethanolamine in isolated, perfused heart was estimated to be 0.17 mM, (Fig. 9, inset). The ethanolamine concentration in hamster plasma is 0.9 mM

and it is therefore unlikely that the plasma ethanolamine concentration regulates ethanolamine uptake by the hamster heart.

PE biosynthesis in the heart was clearly demonstrated by perfusion studies, where 96% of PE was synthesized *de novo* from ethanolamine via the CDP-ethanolamine pathway. Although the exact contribution of the base exchange pathway to PE biosynthesis was not determined, the lag of radioactivity incorporated into the phospholipid during the first 15 min of perfusion was reminiscent of the results obtained for PC biosynthesis in hamster heart (Zelinski *et al.*, 1980) and in rat brain cell culture (Yavin, 1976). This lag of label incorporation into PE is indicative of the relatively minor contribution of the base exchange reaction. In addition to these two pathways, it was found that approximately 3% of PE in the heart was formed by decarboxylation of PS. The low activities of PS decarboxylase and base exchange enzyme (Table V) when assayed *in vitro* also reflect the relatively minor contribution of these two pathways.

From pool size determinations, the specific radioactivity of phosphoethanolamine is higher than that of its precursor. Preferential phosphorylation of [1-^{3}H] ethanolamine could be one explanation for high specific radioactivity of phosphoethanolamine, but this does not seem to be the case, since similar results were obtained with [2-^{14}C] ethanolamine. Since this is incompatible

with a precursor-product relationship between total heart pools of two compounds, one possible explanation for this apparent discrepancy is that labeled ethanolamine taken up by the heart did not equilibrate with the total pool of intracellular ethanolamine prior to phosphorylation. A similar interpretation of results was suggested by Sundler, (1973).

Although the rate-limiting step in the CDP-ethanolamine pathway is not entirely clear, it appears that this step is catalyzed by phosphoethanolamine cytidylyltransferase. Results from perfusion experiments with [1-^{3}H] ethanolamine indicate that the majority of the radioactivity taken up by the heart was associated with phosphoethanolamine, and the amount of radioactivity in ethanolamine or CDP-ethanolamine was several times lower. This suggests that the ability to incorporate labeled ethanolamine into PE is dependent on the rate of conversion of phosphoethanolamine into CDP-ethanolamine thus identifying the step catalyzed by phosphoethanolamine cytidylyltransferase as rate-controlling. Similar results and interpretations were reported for rat liver (Sundler and Åkesson, 1975).

A small amount of PS decarboxylase activity was detected in the microsomal fraction although this enzyme is thought to be exclusively mitochondrial (Borkenhagen et al., 1961). This activity was attributed to mitochondrial contamination of the microsomal fraction. Another interesting aspect of the measured enzyme activities in

the hamster heart, was the base exchange enzyme activity. This activity was 6 times greater than that reported for base exchange of choline with other phospholipids (Zelinski et al., 1980). At the present time the reason for such high enzyme activity for ethanolamine is unknown. One possible explanation is that ethanolamine is a preferred substrate for the Ca^{++}-stimulated base exchange reaction in hamster heart for specific purposes, e.g. for maintenance of phospholipid asymmetry in the biological membrane.

III COMPARISON OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS IN HAMSTER HEART

PC and PE are structurally related compounds. Their structure consists of a glycerol backbone with fatty acid chains attached in the 1 and 2 positions, a phosphate group, and a polar head group, in this case, choline or ethanolamine. Because of their structural similarities, it is not surprising that many similarities are found in the biosynthesis of these two compounds. Conversely, differences in the synthesis of PC and PE would also be expected, thereby contributing uniqueness to the individual phospholipid. It is the purpose of this section to discuss the similarities and differences in the biosynthesis of PC and PE in the hamster heart.

There are three known pathways leading to the formation of PC and PE. Both phospholipids are produced

by the base exchange pathway and the CDP-base (either choline or ethanolamine) pathway. In addition to these two common pathways, a unique mode of synthesis is available for each of these phospholipids, namely, progressive methylation of PE, in the case of PC biosynthesis and decarboxylation of PS, for PE biosynthesis. All of the fore-mentioned pathways were evaluated in the isolated perfused heart. It was concluded that all pathways were present in the heart, and that all pathways contributed in varying degrees, to the biosynthesis of PC or PE. The CDP-choline or CDP-ethanolamine pathways were found to be the major routes for PC and PE biosynthesis respectively. Although the rate of synthesis of the two phospholipids differs by a factor of approximately 8, (PC = 39 $nmol \cdot min^{-1} \cdot$ g $heart^{-1}$ and PE = 290 $nmol \cdot min^{-1} \cdot$ g $heart^{-1}$), the amounts of PC and PE found in the hamster heart are almost equal. A possible explanation for this phenomenon is that, as is the case in rat liver (Waite and Sisson, 1974), PE is a preferential substrate of phospholipase A_2. The turnover rates of PC and PE may therefore be quite different, which may account for observed differences in the rate of their biosynthesis.

The contribution of the base exchange pathway to biosynthesis of PC and PE was relatively minor. These results were consistant with the observations of Bjerve, (1973), who postulated that the base exchange pathway was not likely to be involved in total net synthesis of phospholipids.

It is possible that this reaction is involved only in alteration of the phospholipid microenvironments in certain subcellular organelles. Another interesting observation was that progressive methylation of PE and decarboxylation of PS accounted for approximately 3% of total PC or PE biosynthesis. The relative contribution to net phospholipid biosynthesis, in this case, was identical for these two independent pathways. Possibly the significance of these pathways is in regulation of cellular responses, as proposed by Hirata and Axelrod, (1980).

The concentrations of choline (0.18 mM) and ethanolamine (0.9 mM) in hamster plasma were found to be strikingly different. A possible explanation for the relatively low level of choline in plasma is that choline is a precursor for acetylcholine. The formation of acetylcholine is dependent on extracellular choline concentration (Haubrich et al., 1979). Since acetylcholine is a potent neurotransmitter it is essential to regulate its biosynthesis. Hence it is not surprising that choline is maintained in a relatively low level in plasma. On the other hand, it is not essential to have a highly regulated ethanolamine level in plasma because none of the immediate metabolites of ethanolamine have been identified as biological effectors. Although the concentrations of choline and ethanolamine are quite different in plasma, the Km's for uptake of these two compounds are relatively similar (Km choline = 0.1 mM and Km ethanolamine = 0.17 mM). These Km

values suggest that the membrane transport mechanisms for choline and ethanolamine may be similar.

There was an approximate 10-fold difference in the concentrations of ethanolamine-containing metabolites, as compared to choline-containing metabolites (Tables II and IV). The high level of water soluble ethanolamine-containing metabolites probably reflects the faster rate of PE biosynthesis in the hamster heart, as compared to PC biosynthesis.

Because the in vitro assays for the biosynthetic enzymes were under optimized conditions, it is not possible to make any unambiguous comparisons between the enzyme activities for PC and PE biosynthesis. Nevertheless, it is interesting to note that the activities of phosphocholinetransferase and phosphoethanolaminetransferase are similar. An additional point of interest is the rate of base exchange of choline or ethanolamine with phospholipids. According to Bjerve, (1973), ethanolamine may exchange with PC or PS, but choline is only able to exchange with PS. The ability of ethanolamine to freely exchange with two phospholipids as compared to choline which can only exchange with PS, may explain these differences in enzymatic activity.

The regulation of the CDP-choline and CDP-ethanolamine biosynthetic pathways was also investigated. The results suggest that both pathways are regulated by the second enzyme in the pathway, namely phosphocholine or phospho-

ethanolamine cytidylyltransferase. These findings further reinforce the regulatory roles of these enzymes, as proposed by Vance and Choy, (1979), for PC biosynthesis, and Sundler and Åkesson, (1975), for PE biosynthesis.

At present, it is not clear if a universal signal exists for the modulation of both cytidylyltransferases in mammalian tissues. Although both cytidylyltransferases were purified from rat liver, modulation of enzyme activity was studied only with phosphocholine cytidylyltransferase. Various modulators that might effect the activity of this enzyme *in vivo* and *in vitro* have been identified (Vance and Choy, 1979, Feldman *et al.*, 1978, Weinhold, P.A., personal communication). It is not known whether these compounds have the same effect on phosphoethanolamine cytidylyltransferase. Therefore, future efforts should be directed towards the positive identification of various modulators for this enzyme. The identification of common modulators for both cytidylyltransferases may allow investigators to identify the existence of universal signals, which may regulate both enzymes *in vivo*. Subsequently, the significance of such signals in the regulation of PC and PE biosynthesis could be investigated.

REFERENCES

Abe, M., Akino, T., and Ohno, K. (1972) Biochim. Biophys. Acta 280, 275-280.

Akino, T., Abe, M., and Arai, T. (1971) Biochim. Biophys. Acta 248, 274-281.

Akino, T., Yamazaki, I., and Abe, M. (1972) Tohoku J. Exp. Med. 108, 133-139.

Bajouz, B. (1971) Recent Adv. in Studies on Cardiac Struct. Metab. 2, 291-292.

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

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

Borkenhagen, L.F., and Kennedy, E.P. (1957) J. Biol. Chem. 227, 951-962.

Borkenhagen, L.F., Kennedy, E.P., and Fielding, L. (1961) J. Biol. Chem. 236, PC28-30.

Bosteels, S., Vleugels, A., and Carmeliet, E. (1970) J. Gen. Physiol. 55, 602-619.

Bremer, J., Figard, P.H., and Greenberg, D.M. (1960) Biochim. Biophys. Acta 43, 477-488.

Bremer, J., and Greenberg, D.M. (1960) Biochim. Biophys. Acta 37, 173-175.

Bremer, J., and Greenberg, D.M. (1961) Biochim. Biophys. Acta 46, 205-224.

Brophy, P.J., Choy, P.C., Toone, J.R., and Vance, D.E. (1977) Eur. J. Biochem. 78, 491-495.

Chap, H.J., Zwaal, R.F.A., and Van Deenan, L.L.M. (1977) Biochim. Biophys. Acta 467, 146-164.

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

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

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

Choy, P.C., Whitehead, F.W., and Vance, D.E. (1978) Can. J. Biochem. 56, 831-835.

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

Crews, F.T., Hirata, F., and Axelrod, J. (1980) J. Neurochem. 34, 1491-1498.

Dils, R.R., and Hübscher, G. (1961) Biochim. Biophys. Acta 46, 505-513.

Eibl, H. (1980) Chem. Phys. Lipids 26, 405-429.

Epstein, M.F., and Farrell, P.M. (1975) Pediat. Res. 9, 658-665.

Feldman, D.A., Kovac, C.R., Dranginis, P.L., and Weinhold, P.A. (1978) J. Biol. Chem. 253, 4980-4986.

Frosolono, M.F., Slivka, S., and Charms, B.L. (1971) J. Lipid Res. 12, 96-103.

Gibson, K.D., Wilson, J.D., and Udenfriend, S. (1961) J. Biol. Chem. 236, 673-679.

Haubrich, D.R., Gerber, N.H., and Plueger, A.B. (1979) in Nutrition and the Brain, (Barbeau, A., Growden, J.H., and Wurtman, R.J. eds.), vol. 5, pp. 57-73, Raven Press, New York.

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

Hirata, F., and Axelrod, J. (1978a) Proc. Natl. Acad. Sci. U.S.A. 75, 2348-2352.

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

Hirata, F., and Axelrod, J. (1980) Science 209, 1082-1090.

Hirata, F., Axelrod, J., and Crews, F.T. (1979a) Proc. Natl. Acad. Sci. U.S.A. 76, 4813-4816.

Hirata, F., Corcoran, B.A., Venkatasubramanian, K., Schiffmann, E., and Axelrod, J. (1979b) Proc. Natl. Acad. Sci. U.S.A. 76, 2640-2643.

Hirata, F., Toyoshima, S., Axelrod, J., and Waxdal, M.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 862-865.

Hirata, F., Viveros, O.H., Diliberto Jr., E.J., and Axelrod, J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1718-1721.

Hirs, C.H.W., Moore, S., and Stein, W.H. (1956) J. Biol. Chem. 219, 623-642.

Infante, J.P. (1977) Biochem. J. 167, 847-849.

Kahlenberg, A., Walker, C., and Rothrlick, R. (1974) Can. J. Biochem. 52, 803-806.

Kanoh, M., and Ohno, K. (1973) Biochim. Biophys. Acta 306, 203-217.

Kennedy, E.P. (1962) The Harvey Lectures 57, 143-171.

Kennedy, E.P., and Weiss, S.B. (1956) J. Biol. Chem. 222, 193-214.

Krebs, H.A., and Henseleit, K. (1932) Hoppe-Seylers Z. Physiol. Chem. 210, 33-36.

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

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

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

Manning, A.S., Hearse, D.J., Dennis, S.C., Bullock, G.R., and Coltart, D.J. (1980) Eur. J. Cardiol. 11, 1-21.

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

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

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

Polimeni, P.I. (1974) Am. J. Physiol. 277, 676-683.

Raheja, R.K., Kaur, C., Singh, A., and Bhatia, I.S. (1973) J. Lipid Res. 14, 695-697.

Rehbinder, D., and Greenberg, D.M. (1965) Arch. Biochem. Biophys. 108, 110-115.

Rothman, J.E., and Kennedy, E.P. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1821-1825.

Rothman, J.E., and Lenard, J. (1977) Science 195, 743-753.

Saito, M., Bourque, E., and Kanfer, J. (1975) Arch. Biochem. Biophys. 169, 304-317.

Scarpelli, E.M. (1968a) in The Surfactant System of the Lung, pp. 1-7, Lea and Febiger, Philadelphia.

Scarpelli, E.M. (1968b) in The Surfactant System of the Lung, pp. 9-29, Lea and Febiger, Philadelphia.

Schneider, W.J., and Vance, D.E. (1978) Eur. J. Biochem. 85, 181-187.

Singer, S.J., and Nicolson, G.L. (1972) Science 175, 720-731.

Steck, T.L., and Kant, J.A. (1974) Methods Enzymol. 31, 172-180.

Sundler, R. (1973) Biochim. Biophys. Acta 306, 218-226.

Sundler, R. (1975) J. Biol. Chem. 250, 8585-8590.

Sundler, R., and Åkesson, B. (1975) J. Biol. Chem. 250, 3359-3367.

Sundler, R., Arvidson, G., and Åkesson, B. (1972) Biochim. Biophys. Acta 280, 559-568.

Vance, D.E., and Burke, D. (1974) Eur. J. Biochem. 43, 327-336.

Vance, D.E., and Choy, P.C. (1979) Trends in Biochemical Sciences 4, 145-148.

Vance, D.E., and Dahlke, R.M. (1975) Can. J. Biochem. 53, 950-957.

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

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

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

Van Sande, M., and Van Camp, K. (1979) Investigative Urology 16, 445-446.

Waite, M., and Sisson, P. (1974) J. Biol. Chem. 249, 6401-6405.

Weinhold, P.A. (1968) J. Lipid Res. 9, 262-266.

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

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

Wilson, J.D., Gibson, K.D., and Udenfriend, S. (1960) J. Biol. Chem. 235, 3539-3543.

Yavin, E. (1976) J. Biol. Chem. 251, 1392-1397.

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