THE ROLE OF EXOGENOUS FREE FATTY ACIDS IN PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS IN ISOLATED HAMSTER HEART: INCORPORATION AND REGULATION

by

Thomas Mock

a thesis presented to the Faculty

of Graduate Studies

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FOR MY FAMILY AND FRIENDS

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ABSTRACT

The metabolism of exogenous fatty acids and their role in phospholipid biosynthesis in the isolated hamster heart was examined. The isolated hamster hearts were perfused in the Langendorff mode with Kreb's-Henseleit buffer containing labelled fatty acid and albumin. Linearity of uptake was observed with 20-320 μM stearic and oleic acids and 50-300 μM arachidonic acid at 15 minutes of perfusion. The uptake of both stearic and oleic acids was higher than the uptake of arachidonic acid by the isolated hamster heart. The majority (85%) of stearic acid after uptake was recovered as free stearic acid and only a small amount was associated with the triacylglycerol fraction. In contrast, when hearts were perfused with oleic and arachidonic acids only 40% of the fatty acids taken up were in the form of free fatty acid, and substantial amounts were recovered in the triacylglycerol fraction. A concentration-dependent and time-dependent incorporation of all these fatty acids into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was observed. A 3-fold difference between the labelling of PC and PE was observed with labelled stearic acid, but the difference was reduced to 2-fold with labelled arachidonic or oleic acids. These results indicate that fatty acid chain length and degree of unsaturation are important factors in determining the uptake by the hearts and the extent of incorporation into PC and PE.

The route(s) of fatty acid incorporation into PC and PE were determined by perfusing the heart with radioactive fatty acid for a

defined period (pulse-labelling) and subsequently perfusing with unlabelled fatty acid (chase) for different periods. A precursor-product relationship between 1:2-diacylglycerol and PC and PE was established when hamster hearts were perfused with stearic and oleic acids. It can be therefore concluded that the majority of these fatty acids are incorporated into PC and PE by the CDP-choline and CDP-ethanolamine pathways respectively. A similar precursor-product relationship between 1:2-diacylglycerol and PC and PE was not evident with arachidonic acid. In vitro studies on the formation of PC by reacylation of lysophosphatidylcholine (LPC) demonstrated that arachidonic acid was the preferred substrate. Our data indicate that arachidonic acid is incorporated into PC mainly via the reacylation process. These studies suggest that the route of fatty acid incorporation into PC and PE in the hamster heart is dependent on the fatty acid species.

The effect of free fatty acids on de novo synthesis of PC was studied. An increase in PC labelling was observed when hearts were perfused with labelled choline in the presence of 50 μM stearic acid; no increase was observed with either oleic or arachidonic acid. The choline-containing metabolites were analyzed for radioactivity and a significant decrease in the radioactivity associated with phosphocholine, with a corresponding increase in the labelling of PC, was observed upon perfusion with stearic acid. This indicates that the rate-limiting step catalyzed by CTP:phosphocholine cytidylyltransferase was stimulated by stearic acid. However, hamster heart cytidylyltransferase activity associated with the cytosolic and

microsomal fractions, was not stimulated when assayed in vitro in the presence of stearic acid. Nevertheless, a significant increase in microsomal-associated activity was detected when the microsomal fraction was prepared from hearts perfused with stearic acid. Therefore, we postulate that the increase in PC labelling observed was due to the stimulation of the microsome-associated cytidylyltransferase by stearic acid. We further postulate that the translocation of cytidylyltransferase from the cytosol to the microsomes was probably not responsible for the observed increase. It appears that stearic acid indirectly stimulated the microsome-associated enzyme activity in the perfused heart. The stimulation of CTP:phosphocholine cytidylyltransferase by free fatty acid thus represents an additional regulatory mechanism for PC biosynthesis in the hamster heart.

ABBREVIATIONS

CoA	Coenzyme A
CDP	Cytidine 5'-diphosphate
CTP	Cytidine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g	Gravitational force
hr	Hour
kg	Kilogram
LPC	Lysophosphatidylcholine
mg	Milligram
min	Minute
ml	Millilitre
nm	Nanometre
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
s	Second
TLC	Thin-layer chromatography
µl	Microlitre
v	Volume
w	Weight

INTRODUCTION

I THE BIOLOGICAL MEMBRANE

Biological membranes are cellular components whose structural integrity is imperative for normal cellular function and growth. The biological membrane has the capacity to perform and mediate many specialized functions because of the unique conformation adopted by the membrane components in an aqueous medium. Such specialization of function is typified by the plasma membrane, which acts as a barrier between cell constituents and the external environment. It effects a screening device that allows passage into the cell of some molecules but not others. In addition, the plasma membrane also contains receptors that enable a cell to communicate with the external medium and with other cells (Cantor and Schimmel, 1980).

The distinctive feature of membranes, from the standpoint of chemical composition, is the high concentration of phospholipids (Tanford, 1973). The major phospholipids in mammalian cells are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). These amphiphilic molecules provide the structural framework of membranes. In an aqueous medium the phospholipids tend to orient themselves in a thermodynamically favorable conformation by maximizing hydrophobic and hydrophilic interactions, thereby maintaining structural integrity. This conformation also provides an anchor for proteins which, because of the fluid nature of the membrane, are capable of considerable lateral movement as suggested in the Fluid Mosaic Model of membrane structure (Singer and Nicholson, 1972). Phospholipids have also been shown to modulate the activity of some membrane-bound enzymes, such as beef heart B-hydroxybutyrate dehydrogenase (Isaacson et al, 1977). The

absolute requirement for PC exhibited by this enzyme is attributed to specific lipid-protein interactions . It is believed that, as a boundary lipid, PC induces a kinetically active and thermodynamically favorable form of the enzyme.

The mammalian cell is a dynamic entity, it responds and adapts itself in such a manner to ensure its continued viability. The synthesis of membranes and their principal components, PC and PE, is an integral part of the dynamic process. An understanding of the biosynthesis of PC and PE and the regulation thereof, lends itself to a greater understanding of biological phenomena.

II PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

a) PHOSPHATIDYLCHOLINE BIOSYNTHESIS

Phosphatidylcholine (lecithin) was first isolated from egg yolk and brain (Gobley, 1850). The chemical composition, however, was first reported in 1868, which enabled a provisional structure of the phospholipid to be derived based on its glycerophosphoric acid, fatty acid and choline content. (Diakanow, 1868, Strecker, 1868).

In most animal tissues PC represents the major phospholipid, accounting for 25-60% of total lipid phosphorus. One feature of naturally occurring PC is the tendency for the 1-position to be esterified with a saturated fatty acid and the 2-position esterified with an unsaturated fatty acid (Ansell and Spanner, 1982). However, there are exceptions, most notably in the lung where a disaturated

species of PC is predominant (Farnell and Avery, 1975). PC and PE are non-randomly distributed in the plasma membrane thereby generating lipid asymmetry in the membrane. For the most part PE is confined to the inner leaflet of the bilayer while PC is found mainly in the outer leaflet of the bilayer (Rothman and Lenard, 1977, Chap et al., 1977).

Several pathways responsible for the synthesis of PC in mammalian tissues have been identified (Fig. 1). De novo synthesis of the phospholipid by the CDP-choline pathway (Kennedy, 1962) is responsible for the majority of PC synthesized in most tissues. Acylation of lysophosphatidylcholine (LPC) (Lands, 1960, Lands, 1965), transacylation of LPC (Erbland and Marinetti, 1965, van den Bosch et al., 1965), stepwise methylation of PE (Bremer and Greenberg, 1961) and base-exchange reactions (Dils and Hübscher, 1961) are also responsible for PC biosynthesis. However, the contribution of each pathway to total PC biosynthesis appears to be species and tissue-specific. These pathways will be discussed in more detail in the following section. Emphasis will be placed on de novo synthesis and the reacylation reactions as they are more germane to the present study.

i) CDP-choline pathway

The de novo pathway for PC biosynthesis was first elucidated by Kennedy and his co-workers and represents the major pathway for PC biosynthesis in the liver. Central to this pathway is the formation of the activated base, CDP-choline, and the lipid moiety, 1:2-diacylglycerol. These metabolites condense yielding PC in the

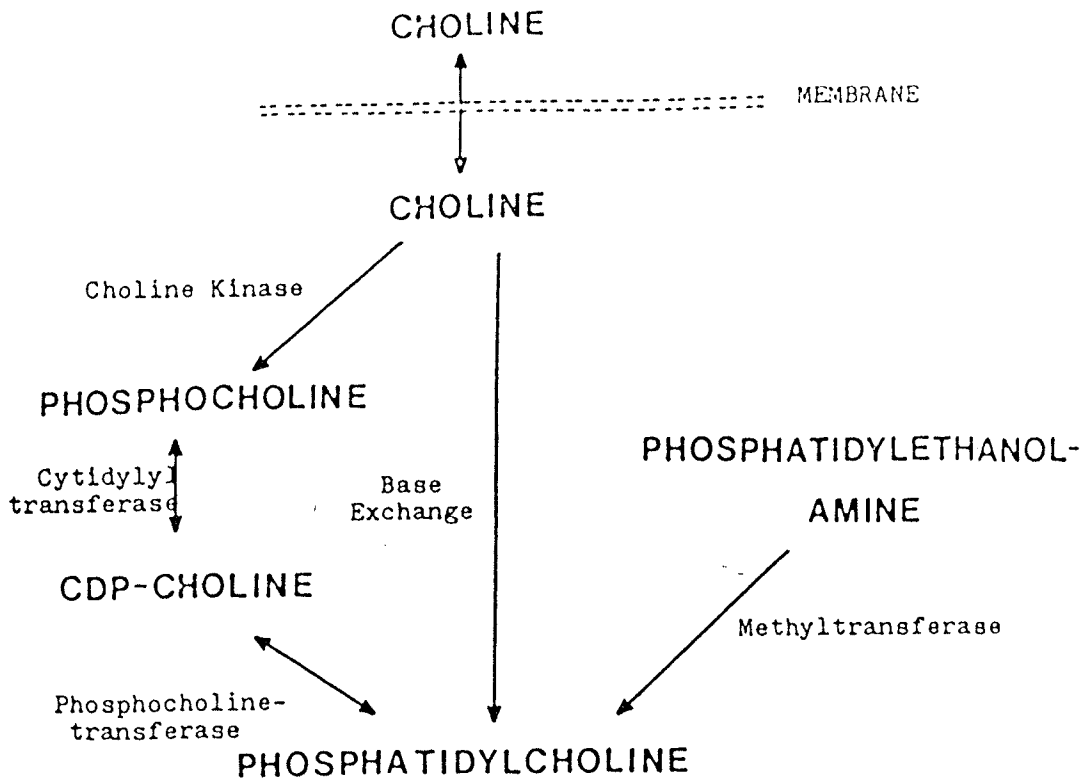

Fig.1. Major metabolic pathways for phosphatidylcholine biosynthesis.

final step of the reaction sequence. The synthesis of these intermediates will be discussed separately.

The first step in the production of CDP-choline is catalyzed by choline kinase (Wittenberg and Kornberg, 1953). Choline kinase is a Mg^{2+}-ATP dependent enzyme present exclusively in the cytosol of mammalian cells. Exogenous choline is transported into the cell and is rapidly phosphorylated by choline kinase yielding phosphocholine. Recently, it has been shown that exogenous ethanolamine inhibits the uptake of choline in the isolated perfused hamster heart (Zelinski and Choy, 1984). The regulation of the intracellular pool of choline may influence the amount of PC formed by this pathway.

The formation of the activated base, CDP-choline, by CTP: phosphocholine cytidylyltransferase results from the condensation of phosphocholine and CTP. This reaction is generally perceived to be the rate-limiting step in the de novo biosynthetic pathway (Zelinski et al., 1980). This enzyme displays selectivity for CTP and dCTP and requires Mg^{2+} or Mn^{2+} for activity (Borkenhagen and Kennedy, 1957). This enzyme has been found in both the cytosolic and microsomal fractions of the rat liver and the hamster heart (Choy et al., 1977, Choy, 1982).

The final step in the de novo pathway is catalyzed by CDP-choline:1,2-diacylglycerol phosphocholinetransferase. Phosphocholinetransferase activity resides in the microsomal fraction and has a requirement for Mg^{2+} or Mn^{2+}. Numerous studies have been undertaken

to determine the specificity, if any, of the enzyme for various diacylglycerol species which are required for condensation with CDP-choline to form PC. Disaturated substrates such as 1:2-dipalmitoylglycerol are poor substrates in vitro, except in the lung, while diacylglycerols which contain an unsaturated fatty acid at the C-2 position are preferred substrates (Holub, 1978, Arthur and Choy, 1984).

Most of the 1:2-diacylglycerols utilized for PC and PE biosynthesis are synthesized de novo from phosphatidic acid. Phosphatidic acid is produced through a series of acylation reactions of glycerol-3-phosphate or dihydroxyacetone phosphate (Fig.2). Phosphatidic acid phosphohydrolase then cleaves the phosphate group yielding 1:2-diacylglycerols.

Formation of dihydroxyacetone phosphate occurs during glycolysis and gluconeogenesis. Dihydroxyacetone phosphate acyltransferase activity has been reported in microsomes and peroxisomes (Jones and Hajra, 1977, Hajra et al., 1978). This enzyme is characterized by a preferential utilization of saturated acyl-CoA esters, producing 1-acyldihydroxyacetone phosphate. However, this compound has not been isolated from natural sources. The precursor-product relationship of 1-acyldihydroxyacetone phosphate and phosphatidic acid has not been detected in vivo. Reduction of 1-acyldihydroxyacetone phosphate to 1-acyl-glycerol-3-phosphate is catalyzed by acyldihydroxyacetone phosphate oxidoreductase. Enzyme activity has been detected in both the microsomal and peroxisomal fractions (Labelle and Hajra, 1972).

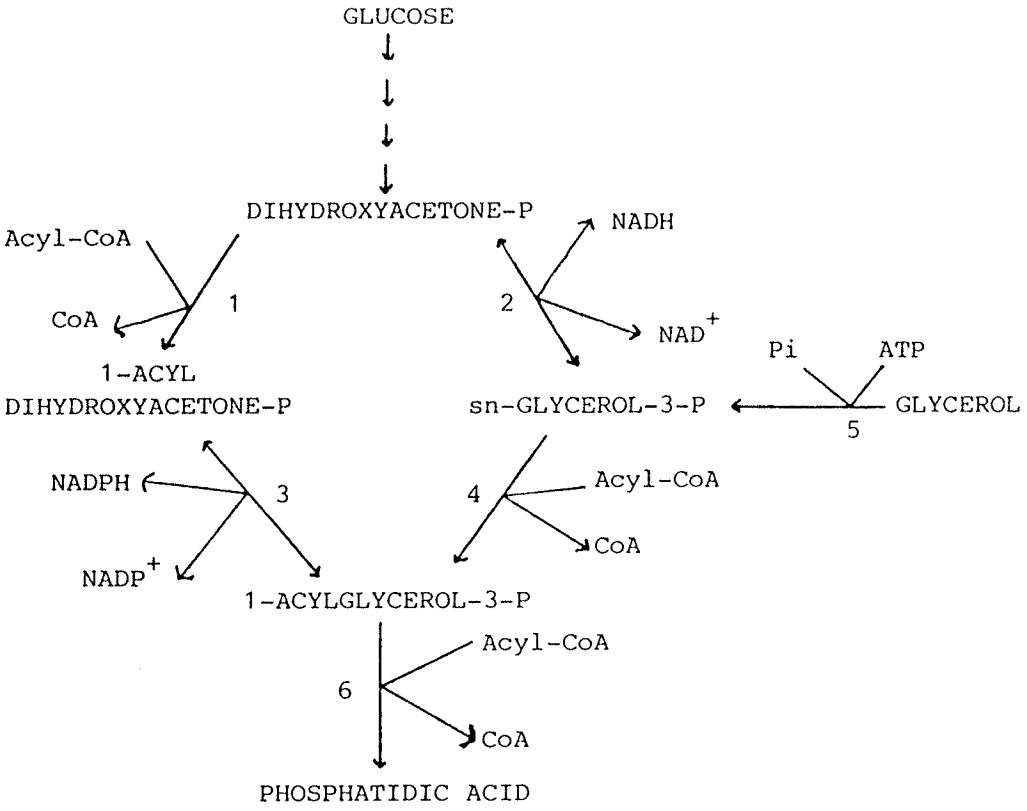

Fig. 2. Biosynthesis of phosphatidic acid

1. Dihydroxyacetone phosphate acyltransferase
2. sn-Glycerol-3-phosphate dehydrogenase
3. Acyl(alkyl)dihydroxyacetone phosphate oxidoreductase
4. sn-Glycerol-3-phosphate acyltransferase
5. Glycerol kinase
6. 1-Acyl-sn-glycerol-3-phosphate acyltransferase

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This enzyme differs from the biosynthetic glycerol-3-dehydrogenase as it utilizes NADPH rather than NADH for its source of reducing power. Peroxisomes, then, may contribute to cellular phosphatidic acid formation as they can generate the precursor 1-acyl-glcerol-3-phosphate by virtue of their intrinsic acyltransferases and oxidoreductase activities.

Glycerol-3-phosphate is generated from reduction of dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase, and to a lesser extent by phosphorylation of glycerol by glycerol kinase (Lin, 1977). Contribution to the steady state glycerol-3-phosphate pool is mainly by the dehydrogenase reaction as many tissues are not capable of utilizing exogenous glycerol.

Separate glycerol-3-phosphate acyltransferase and 1-acyl-glycerol-3-phosphate acyltransferase activites have been reported in rat liver microsomes (Yamashita and Numa, 1972, Yamashita et al., 1972,1973,1975). Preferential utilization of saturated CoA esters by glycerol-3-phosphate acyltransferase yielding 1-acyl-glycerol-3-phosphate was detected. However, the production of phosphatidic acid by 1-acyl-glycerol-3-phosphate acyltransferase preferentially utilized monoenoic and dienoic acyl-CoA esters. The existence of two distinct enzymes which sequentially esterify fatty acyl-CoA's with different specificities may account for the asymmetric distribution of fatty acids in liver phospholipids (Strickland, 1973).

Two different glycerol-3-phosphate acylating systems have been reported in rat liver; one residing in the microsomes and the other

mitochondrial in origin. Both possess fatty acyl-CoA selectivity. The importance of each system in terms of contribution to the cellular phosphatidic acid pool is subject to much debate (van den Bosch, 1974, van Golde and van den Bergh, 1977). The existence of two separate acylating systems raises an interesting question as to whether the systems contribute to a single pool of phosphatidic acid or whether they are regulated differently and thus contribute to separate pools of phosphatidic acid which in turn may be used for compartmentalized phospholipid biosynthesis.

Using different experimental approaches the relative importance of dihydroxyacetone phosphate and glycerol-3-phosphate to phosphatidic acid synthesis has been estimated. Based on pool size and kinetic paramaters, direct acylation of dihydroxyacetone phosphate account for 1% of total phosphatidic acid synthesis (Schlossman and Bell, 1976), while exploiting the reduction step using [^{3}H]NADH and [^{3}H]NADPH, it has been determined that 50% of phosphatidic acid is synthesized via dihydroxyacetone phosphate (Agranoff and Hajra, 1971). The activity of glycerol-3-phosphate dehydrogenase is severely depressed in tumor cells (Lin, 1977), alluding to the importance of the dihydroxyacetone phosphate route in phosphatidic acid biosynthesis in transformed cells. Irrespective of the relative contribution of these routes to phosphatidic acid synthesis the production of ether-linked phospholipid is initiated only by the acylation of dihydroxyacetone phosphate.

ii) Deacylation-reacylation of choline phospholipids

Lysophosphatidylcholine reacylation and lysophosphatidylcholine (LPC) transacylation are two modes of PC resynthesis that do not lead to net PC synthesis (Fig.3). Rather, they effect a "tailoring" process, wherein fatty acids of desired lengths and degree of unsaturation are esterified to the free hydroxyl group of existing LPC.

LPC is formed intracellularly from PC by the action of phospholipase A1 and A2. These enzymes cleave ester linkages of PC yielding LPC and free fatty acid. The acylation of LPC to form PC was first demonstrated by Lands (1960,1965). Two acyltransferases were characterized each exhibiting different fatty acyl-CoA specificities. These acylating enzymes are different from the phosphatidic acid-generating acyltransferases (Lands and Hart, 1965). In general, lysolecithin acyltransferase esterifies an unsaturated fatty acid to the free hydroxyl group at the 2-position of LPC, whereas, 2-acylglycerophosphocholine acyltransferase esterifies a saturated fatty acid to the free hydroxyl group at the C-1 position of 2-acylglycero-3-phosphocholine (Lands and Merkl, 1963).

Uptake of LPC and subsequent acylation to form PC has been demonstrated in the isolated perfused hamster heart (Savard and Choy, 1982). LPC is normally present in the serum associated with albumin. The majority of serum LPC is generated by the action of lecithin:cholesterol acyltransferase, where the fatty acid at the C-2 position of lipoprotein PC is transferred to cholesterol yielding LPC

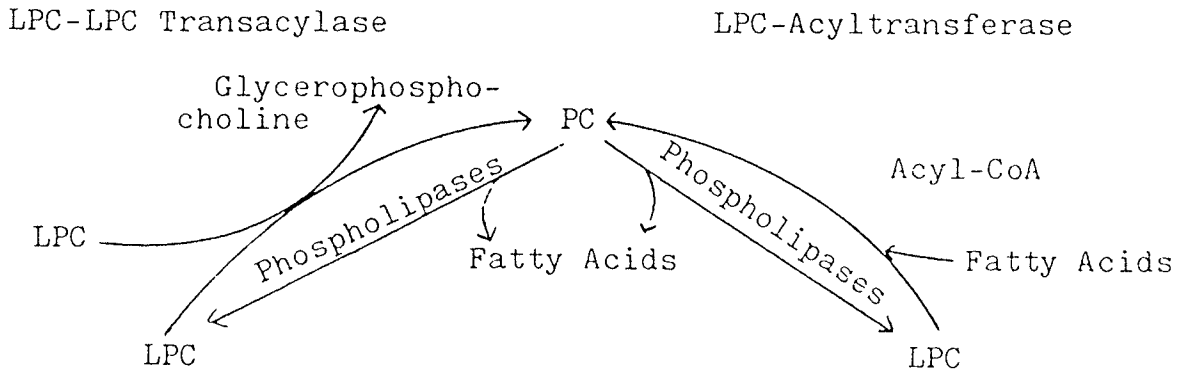


Fig. 3. Deacylation-reacylation reactions for the biosynthesis of phosphatidylcholine.

and cholesterol ester. A small amount of LPC may also be formed from lipoproteins and cell surface PC by lipoprotein and hepatic lipases (Nilsson-Ehle et al., 1980).

Studies with Chinese Hamster Ovary cells have revealed an interesting relationship between LPC uptake and metabolism and de novo synthesis of PC. It was found that a mutational block in the CDP-choline pathway led to an enhanced uptake of LPC (Esko et al., 1982). It has also been established that acylation of LPC is augmented when de novo PC synthesis is interfered with, which may indicate that the acyltransferases are regulated by intracellular PC levels or by one or a number of the choline metabolites.

Another reacylation type of reaction is transacylation of LPC, catalyzed by LPC-LPC acyltransferase. Two molecules of 1-acyl-3-phosphocholine are required which generate PC and glycerophosphocholine. The reaction is mainly cytosolic in nature (van den Bosch et al., 1965) and does not require ATP or any CoA derivates. No LPC-LPC acyltransferase activity has been detected in the hamster heart (Savard and Choy, 1982).

iii) Progressive methylation of phosphatidylethanolamine

Pioneering work by Bremer and Greenberg (1961), revealed the S-adenosyl methionine-dependent N-methylation of PE to form PC. In the adrenal medulla two methyltransferases have been localized in the microsomes with differing pH optima, cation requirements and Km values for S-adenosyl methionine (Hirata et al., 1978). The formation of PC

by progressive methylation of PE is of quantitative significance only in the liver (Sundler and Åkesson, 1975), where it accounts for 20% of PC synthesized.

Working with red cell ghosts, Hirata and associates have determined an asymmetric distribution of the two methyltransferases in the membrane, and have attributed a biochemical significance to this arrangement (Hirata and Axelrod, 1980). These authors believe that a signal-receptor interaction initiates methylation on one side of the membrane, followed by translocation of the monomethyl derivative to the other side of the membrane which then undergoes further methylation thereby markedly affecting regional membrane fluidity (Hirata and Axelrod, 1978). According to these same authors (1980), the decreased membrane viscosity that occurs upon signal-initiated PE methylation allows for increased coupling between receptors and effectors resulting in an enhanced transmission of signal across the membrane. This hypothesis remains a contentious issue, however, as Moore et al (1984), using rat basophil leukemic cells, mast cells and mouse thymocytes, have found that phospholipid methylation is not peremptory for the receptor-mediated transmission of the Ca^{2+}-signal. Furthermore, evidence provided by Vance and de Kruijff (1980) indicates that the low methyltransferase activity normally present in red blood cells could not effect the changes in membrane microviscosity required for the enhanced coupling between receptor and effector.

iv) Base-exchange

The base-exchange reactions for the formation of PC and other phospholipids are defined as those which result in direct incorporation of several amino alcohols into their corresponding phospholipid (Fig. 4). The reactions are characterized by being energy-independent and Ca^{2+}-requiring with maximum activity at alkaline pH (Kanfer, 1980). Serine, ethanolamine and choline and their corresponding phospholipids are known to participate in these reactions although net synthesis of these phospholipids does not occur merely alterations in the structure of pre-existing phospholipids. The alteration in the polar head group is expected to cause significant changes in the membrane by virtue of the asymmetric distribution of these phospholipids (Rothman and Lenard, 1977, Chap et al., 1977). Zelinski et al .(1980), using the isolated perfused hamster heart, noted a distinct lag in PC labelling upon perfusion with labelled choline, which suggests the base-exchange reaction is of minor importance in the production of PC in the hamster heart.

b) PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

PE accounts for 10-40% of total lipid phosphorus in most animal tissues, thus representing , along with PC, the major phospholipids in mammalian membrane systems. In sheep red blood cells, however, PE and sphingomyelin represent the major phospholipids as PC is conspicuously absent (White, 1973). PE is also characterized by having a saturated fatty acid at the C-1 position and an unsaturated fatty acid at the C-2 position in most instances.

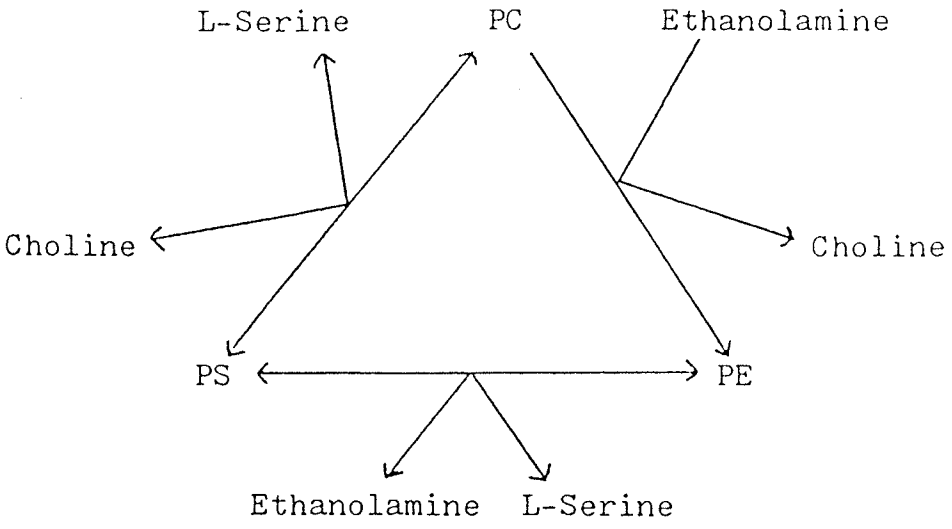


Fig. 4. Base-exchange reactions for interconversion of phospholipids.

PE and PC biosynthesis share similar pathways. PE may be synthesized de novo from ethanolamine (Kennedy, 1962), by Ca^{2+}-mediated base exchange (Dils and Hübscher, 1961) and by acylation of lysophosphatidylethanolamine (Merkl and Lands, 1963, Lands and Hart, 1965). These pathways parallel those for PC biosynthesis and will not be further elaborated on. A unique pathway for PE biosynthesis was described by Bremer et al. (1960), where PE was formed by decarboxylation of phosphatidylserine (PS).

i) Decarboxylation of phosphatidylserine

Bremer et al (1960), first demonstrated the in vivo production of PE by decarboxylation of PS, using labelled serine. Evidence provided by Wilson et al (1960), discounted the participation of decarboxylation of free serine to the ethanolamine pool, thereby clearly demonstrating that only lipid-bound serine was the precursor for PE via the decarboxyation reaction. Further work by Borkenhagen et al (1961), and Dennis and Kennedy (1972), established a mitochondrial source for this enzyme. The relatively minor importance of this pathway to PE biosynthesis in the hamster heart is evidenced by the work of Zelinski et al. (1982), who found that only 3% of PE synthesized in the perfused hamster heart arose from phosphatidylserine decarboxylation.

III INCORPORATION OF FATTY ACIDS INTO TISSUE LIPIDS

In the simplest sense, PC and PE may be visualized as being composed of three parts, the glycerol backbone, the head groups and

the fatty acyl groups. Each confers different structural and functional properties upon the particular phospholipid. These moieties are made available for PC and PE biosynthesis by different processes. The production of glycerol-3-phosphate may proceed through glycolysis, via dihydroxyacetone phosphate, or by phosphorylation of glycerol by glycerol kinase. The amino alcohol, choline, for the most part, is supplied in the diet and transported into the cell. The lipoidal moiety may originate from purely endogenous sources, however, it has been demonstrated that exogenous neutral lipids may also contribute to cellular PC and PE biosynthesis. In the serum, neutral lipids are often found associated with lipoproteins or bound to serum albumin thus facilitating transport within the body. The uptake and incorporation into phospholipids of non-esterified fatty acids will be discussed in the following section.

a) Uptake of fatty acid

Non-esterified fatty acids (free fatty acids) are usually found in the serum associated with albumin and as such must dissociate from albumin prior to uptake by a particular cell. The molar ratio of fatty acid to albumin has been determined to be a major factor in the rate of fatty acid uptake. Spector et al. (1965), using Ehrlich ascites tumor cells, and Evans (1964), using the isolated perfused rat heart have shown that higher fatty acid/albumin molar ratio (5:1) resulted in greater uptake when compared with a lower molar ratios (2:1). Spector and Steinberg (1965) contend that fatty acid uptake is dependent on cellular binding sites competing with binding sites on

plasma albumin for available fatty acid. As the molar ratio of fatty acid to albumin increases the lower affinity albumin-binding sites are occupied allowing the equilibrium to shift and favor cellular binding and consequently enhanced internalization of the fatty acid is achieved. The extent of myocardial extraction of free fatty acid is also dependent upon the molecular structure of the fatty acids (Evans et al., 1963, Evans, 1964). These authors have demonstrated the existence of an inverse relationship between chain length and myocardial extraction. Comparison studies with 12, 14, 16 and 18-carbon fatty acids showed uptake decreased as chain length increased, in addition, unsaturated fatty acids of a given length were preferentially extracted when compared with saturated fatty acids of the same chain length. Whether these uptake phenomena are due to differences in relative affinities of fatty acid species for albumin or their differences in solubilities in aqueous media remains unknown.

The majority of fatty acids taken up by the heart are oxidized as they represent the preferred substrate for energy metabolism (Neely et al., 1972). It has been shown that an increased cardiac work load necessitates increased oxidative activity and leads to an enhanced uptake of fatty acids. The availability of competing substrates for energy production, however, has a marked effect on myocardial fatty acid uptake and subsequent fatty acid metabolism. Olson (1962), using isolated rat heart has shown that both acetoacetate and pyruvate in the perfusion medium will inhibit fatty acid uptake. This inhibition has been attributed to energy-efficient activation of these substrates. Fatty acid activation requires ATP for thioester

formation, while both acetoacetate and pyruvate can enter the tricarboxylic acid cycle without resort to a kinase. These results suggest fatty acid uptake may be inexorably tied to utilization within the cell.

b) Incorporation into tissue lipid

The internalized fatty acids may be used in the synthesis of neutral glycerides and phospholipids. Stein and Stein (1963), found incorporation of labelled fatty acids into neutral lipids and phospholipids in the isolated perfused rat heart. This incorporation was, to some extent, species-specific, in as much as some fatty acids represented better substrates for esterification reactions. This specificity led to a varied intracellular distribution of isotopically labelled fatty acids. Linoleic and oleic acids were found to be incorporated into phospholipids, in particular PC, to a greater extent than palmitic acid; conversely incorporation of palmitic acid into triacylglycerols was greater than linoleic and oleic acids. Non-random incorporation of the fatty acids into phospholipids was evidenced in the asymmetric distribution of esterified labelled fatty acids found upon analysis of phospholipids. Of the fatty acids taken up by the rat heart, over 70% of the label was recovered in the triacylglycerol fraction and 30% in the phospholipid fraction with all fatty acids tested.

In contrast, Olson (1962) found an equal distribution of labelled fatty acid between phospholipids and neutral lipids.

However, addition of pyruvate to the perfusion medium elicited a "sparing" effect which, by becoming the preferred substrate for energy production, diverted the fatty acid into triacylglycerol synthesis rather than oxidation and resulted in a 7-fold increase incorporation of fatty acid into triacylglycerols.

Tamboli et al. (1983), studied both the intact rat heart and isolated myocytes and have found that upon uptake oleic acid is preferentially used in triacylglycerol synthesis while only a nominal amount (less than 10%) is used in phospholipid synthesis. In view of the apparent discrepancies in the literature, further clarification is required to determine the relationship between intracellular utilization of internalized fatty acids and its relation to molecular structure, under a defined set of experimental conditions.

Similar studies were conducted in rat liver (Åkesson, 1970), where labelled fatty acids were intraportally injected into rats and their incorporation into PC and PE monitored. Extraction and analysis of lipids at precise time points after injection of labelled fatty acids enabled this investigator to evaluate initial esterification rates. Considerable evidence was presented indicating participation of de novo synthesis and lysolipid acylation as principal routes for incorporation of fatty acid into phospholipids. The high degree of specificity exhibited by acyltransferases was also noted in these and other studies (Åkesson et al., 1970a, 1970b).

Exogenous free fatty acids have also been demonstrated to be

incorporated into phospholipids by cultured hamster fibroblasts (Maziere et al., 1982). These investigators found an increased incorporation of unsaturated fatty acids into phospholipids upon SV40 transformation compared to an established cell line, whereas no increase was observed with saturated fatty acids. This may lead to the observed changes in membrane fluidity that accompanies oncogenic transformation (Maziere et al., 1982). In vitro analysis showed enhanced acylation of both unsaturated and saturated fatty acids in transformed cells, suggesting that acyltransferase activity is not limiting fatty acid incorporation into phospholipids.

IV EFFECT OF FREE FATTY ACIDS ON DE NOVO SYNTHESIS OF PHOSPHATIDYLCHOLINE

Free fatty acids, in addition to their role as substrates for PC and PE synthesis, may alter the choline flux through the CDP-choline pathway and hence stimulate PC biosynthesis. This stimulation of PC biosynthesis observed in isolated hepatocytes (Pelech et al., 1983), is brought about as a direct result of fatty acids influencing enzyme activity.

a) CTP:phosphocholine cytidylyltransferase

The rate of [^{14}C]choline incorporation has been shown to be augmented in rat hepatocytes cultured in oleate-supplemented media (Pelech et al., 1983). This stimulation is mediated through activation of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase; such stimulation could also be produced in

vitro. The mechanism of action of fatty acid stimulation is believed to involve the translocation of the cytidylyltransferase from the cytosol to the microsomes. The addition of fatty acids in vitro results in a measurable loss of cytosolic activity and a corresponding increase in microsomal-associated activity which is manifested in the stimulation of the cytidylyltransferase. A current theory holds that cytidylyltransferase activity is enhanced upon translocation to the microsomes in part by activation by phospholipids in the microsomes (Sleight and Kent, 1983), and in addition, the reaction product CDP-choline, will then be more readily accessible to cholinephosphotransferase which is found in the microsomes.

b) CDP-choline:1,2-diacylglycerol cholinephosphotransferase

Free fatty acids have been shown to stimulate rat liver choline phosphotransferase in vitro when additional diacylglycerol was present in the reaction mixture (Sribney and Lyman, 1973). These authors feel that free fatty acids may effect a conformational change in the enzyme which makes it more apt to accept the diacylglycerol substrate thus enhancing catalysis.

V RESEARCH AIMS

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represent the major phospholipids in the mammalian heart (White, 1973). They function not only in a structural capacity by providing the framework of cardiac membranes but also may serve to modulate the activity of certain membrane-bound enzymes. Although the

pathways leading to PC formation (Zelinski et al., 1980) and PE formation (Zelinski and Choy, 1982) in the hamster heart have been well characterized the role of exogenous free fatty acids in PC and PE biosynthesis is at present unknown.

The importance of fatty acids in cardiac energy metabolism has been well established (Evans, 1964). Moreover, exogenous fatty acids have been shown to be incorporated into phospholipids in the isolated perfused rat heart (Stein and Stein, 1963). However, these studies did not address the problem of route(s) of incorporation of fatty acids into phospholipids. The effect of fatty acid chain length and degree of unsaturation on the incorporation characteristics remains largely unknown. One of the aims of the present research is to evaluate the incorporation of fatty acids into PC and PE with respect to these features.

Free fatty acids have also been shown to stimulate PC biosynthesis in isolated rat hepaocytes as a result of activation of CTP:phosphocholine cytidylyltransferase (Pelech et al., 1983). Another aim of this research is to assess the possible regulatory effects of free fatty acids in PC biosynthesis in the hamster heart.

This research may contribute to a more complete understanding of PC and PE biosynthesis in the mammalian heart. It is hoped that an investigation into regulation of PC biosynthesis by fatty acids will provide insight that will enhance our overall knowledge of this control mechanism and its relevance in normal and disease states.

MATERIALS AND METHODS

I MATERIALS

a) Chemicals

CDP-choline, CTP, ATP, choline chloride, Coenzyme A, fatty acid-free bovine serum albumin, Tween 20 and stearic, oleic and arachidonic acids were purchased from Sigma Chemical Company. 1,2-Diacylglycerol was obtained from Serdary Research Laboratories. Acetic anhydride was purchased from BDH Chemicals. Thin-layer chromatography plates (Sil-G25) were obtained from Brinkmann. [Me-^{3}H]Choline, [Me-^{14}C]CDP-choline and [^{3}H]acetic anhydride were purchased from New England Nuclear. [1-^{14}C]Stearic acid, [1-^{14}C]oleic acid, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, [1-^{14}C]palmitoyl LPC and aqueous counting scintillant (ACS) were obtained from Amersham Corporation. [Me-^{3}H]Phosphocholine was prepared enzymatically as described by Paddon and Vance, (1977). Gas-chromatography standards and diethylene glycol succinate columns (GC) were purchased from Supelco. All other chemicals were of reagent grade and were obtained from Fisher Chemical Company. All solution were prepared with double-distilled water and were adjusted to the required pH.

b) Experimental Animals

Syrian Golden Hamsters, 100-125 g were maintained on Purina Hamster Chow and tap water, ad libitum, in a light and temperature-controlled room.

II METHODS

a) Extraction of lipids and storage of lipid extracts

A prerequisite for this study is the development of methodology for the efficient and effective extraction of cardiac lipids. Subsequent manipulation and storage must be conducted so as to minimize any alteration in the lipid levels.

i) Extraction and quantitation of cardiac phospholipids

Mongrel dogs were anesthesized by intravenous injection of sodium pentobarbitol (30 mg/kg), and the hearts rapidly excised and placed on ice. The tissue was cut into small pieces and the lipids were extracted by homogenization in either chloroform/methanol (2/1; v/v), chloroform/methanol (1/1; v/v) or chloroform/methanol (1/2; v/v) with a Polytron Homogenizer (Brinkmann PT10/35). The tissue was placed in 10 vol. of solvent (w/v) and homogenized twice for 30 s each at a setting of 6. Depending on the homogenizing solvent, the homogenate was either filtered or centrifuged and the insoluble material was reextracted with the same solvent for the required number of times. Addition of an equal volume of 0.1 N KCl to the homogenate resulted in phase separation. The upper phase was drawn off and discarded and the lower phase was evaporated under nitrogen. The lipid residue was dissolved in chloroform/methanol (2/1; v/v) to a final volume of 1 ml/g wet wt of the tissue. The lipid extract was then spotted on to thin-layer chromatography plates with a Camag Linomat III TLC spotter. The plates were developed in chloroform/methanol/acetic acid/water (75/60/10/8; v/v) which resulted

in good separation of lysophosphatidylcholine (Rf 0.11), sphingomyelin (Rf 0.16), phosphatidylcholine (Rf 0.36), phosphatidylethanolamine (Rf 0.66), cardiolipin (Rf 0.80) and neutral lipids.

The LPC bands were analyzed for lipid phosphorus content by the procedure of Bartlett (1959). The bands corresponding to LPC were scraped into test tubes to which 0.5 ml of sulfuric acid was added. The tubes were incubated overnight at 160° and then allowed to cool to room temperature. Upon cooling 1.5 ml of hydrogen peroxide was added to each tube and the mixtures returned to the heating block for an additional 3 hr at 160°. The tubes were again allowed to cool upon which 9.1 ml of 0.26% ammonium molybdate and 400 µl 1-amino-2-naphthol-4-sulfonic acid reagent (1.537 g in 10 ml water) were added and the tubes vortexed. The tubes were then placed in a boiling water bath for 10 minutes after which the tubes were vortexed and centrifuged at 1,000 x g for 10 min. Absorbance at 820 nm was measured against a silica gel blank.

ii) Storage of lipid extracts

Lipids were extracted from the fresh canine heart by chloroform/methanol (1/2; v/v) and identical amounts stored at -20° in chloroform/methanol (2/1;v/v), chloroform/methanol/water (86/14/1; v/v) or under nitrogen without solvent for 14 days. One microcurie of [1-^{14}C]palmitoyl LPC was added to each lipid extract in order to monitor LPC degradation. Aliquots were taken at various time intervals and separation and quantitation of LPC was performed as described in the previous section.

b) Perfusion of isolated hamster heart

i) General

To effectively study the contribution of exogenous fatty acids to PC and PE biosynthesis a suitable model was required. The isolated hamster heart perfused with Kreb's Henseleit buffer was employed in this capacity.

Preparation of Kreb's-Henseleit buffer (Kreb's and Henseleit, 1932), was as follows: 70.1 g sodium chloride, 21 g sodium bicarbonate and 9.91 g anhydrous dextrose were combined and dissolved in 1 litre of distilled water to give Solution A. Solution B was a mixture of 3.55 g potassium chloride 2.94 g magnesium sulfate and 1.63 g sodium phosphate (monobasic) in 100 ml distilled water. Solution C contained 3.734 g calcium chloride in 100 ml distilled water. These solutions were stored separately at 4°. Fresh buffer was used in all perfusion studies and was prepared by combining 100 ml Solution A, 10 ml Solution B and 5 ml of Solution C and distilled water to a volume of 1 litre. All Kreb's-Henseleit solutions were maintained at 37° and aerated with 95% oxygen-5% carbon dioxide during perfusion.

Hamsters were decapitated and the hearts quickly excised and placed in Kreb's-Henseleit buffer saturated with 95% oxygen-5% carbon dioxide at 37°. The heart was cannulated via the aorta in the Langendorff mode (1895), and the pulmonary artery was incised to ensure adequate coronary drainage. A small amount of buffer was

forced through the heart to expel any remaining blood. All hearts were then pre-perfused with Kreb's-Henseleit buffer for a minumum of 5 minutes to stabilize the heart.

The viability of the heart was assessed by electrocardiac recording. The negative lead was attached to the cannula thereby monitoring atrial activity, while the positive lead was placed at the apex of the heart and monitored ventricular activity. This arrangement allowed the atrial and ventricular activities to be monitored simultaneously. The signals were amplified and recorded by a Sanborn paper recorder. No significant alterations in electrical activity was observed throughout the perfusion period.

ii) Time-course studies

a) Fatty acid uptake by the isolated heart

In this study a perfusate concentration of 50 μM fatty acid and 10 μM fatty acid-free albumin was used. Fatty acids were prepared in chloroform solution and the required amounts placed in Erlenmeyer flasks. [1-^{14}C]Stearic acid and [1-^{14}C]oleic acid were added to the flasks to give a specific activity of 4 μCi/μmol. The solvents were evaporated to dryness under a stream of nitrogen. Freshly prepared Kreb's-Henseleit buffer and fatty acid-free albumin were subsequently added and the mixtures sonicated well. The resultant fatty acid suspension was used as soon as possible.

The isolated hamster heart was perfused with Kreb's-Henseleit

buffer saturated with 95% oxygen-5% carbon dioxide for at least 5 minutes to restore the beat. The heart was then perfused with the fatty acid suspension for 3-30 minutes. After perfusion the heart was purged with 10 ml of Kreb's-Henseleit buffer to remove labelled compounds trapped in the vasculature of the heart. An additional 20 ml of air was forced through the heart to remove the buffer. The heart was cut open, blotted dry and weighed. The tissue was homogenized in 4 ml of chloroform/methanol (1/2; v/v) with a Polytron Homogenizer. An aliquot of the homogenate was drawn and the radioactivity determined by scintillation counting. The total uptake of labelled fatty acid by the perfused heart was calculated from the specific activity of the fatty acid in the perfusate.

b) Analysis of phosphatidylcholine and phosphatidylethanolamine

Extraction of phospholipids was performed by a slight modification of the procedure of Bligh and Dyer (1959). Phase separation in the homogenates was effected by the addition of 4 ml of chloroform and 4 ml of water. The tubes were vortexed and spun at 1,000 x g for 10 minutes. The aqueous upper phase was drawn off and 4 ml of theoretical lower phase chloroform/methanol/water was added (86/14/1; v/v). The mixture was again vortexed and spun at 1,000 x g for 10 minutes. The upper phase was discarded and the organic lower phase pooled with the previous lower phase. The solvent was then removed under nitrogen and the lipid residue dissolved in a small amount of chloroform/methanol (2/1; v/v). Aliquots of the extracts were spotted onto thin-layer chromatography plates which were then

developed in chloroform/methanol/water/acetic acid (70/30/4/2; v/v). This solvent system gave excellent separation of PC and PE and the minor phospholipids. The bands corresponding to PC and PE were scraped into scintillation vials and 1 ml water, 200 µl acetic acid and 10 ml aqueous counting scintillant were added to each vial. The vials were thoroughly mixed and sonicated and then subjected to scintillation counting.

iii) Concentration-dependence studies

a) Fatty acid uptake by the isolated heart

Preparation of the perfusate in this study differed from the previous section only in the fatty acid concentration. The desired concentration was achieved through addition of unlabelled fatty acid. The amount of radioactive fatty acid was the same in all cases.

The isolated hamster heart was perfused with Kreb's-Henseleit buffer, containing the desired amount of fatty acid, for 15 min. The tissue was homogenized after perfusion as described in the previous section. Aliquots of the homogenate were taken and the amount of associated radioactivity determined by liquid scintillation counting. The uptake of fatty acid was calculated from the known specific activity of the fatty acid.

b) Analysis of phosphatidylcholine and phosphatidylethanolamine

Phospholipids were extracted and separated as described previously. The amount of radioactivity associated with PC and PE was

determined. Corrections for differences in specific activity were required to standardize incorporation data.

iv) Pulse-chase studies on the metabolism of fatty acids

Isolated hamster hearts were either pulsed for 10 minutes with 50 μM [1-^{14}C]stearic acid or 50 μM [1-^{14}C]oleic acid or for 15 minutes with 50 μM [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid. The hearts were subsequently chased for 10-60 minutes with unlabelled fatty acid (all perfusates contained 10 μM bovine serum albumin). The hearts were homogenized as previously detailed and aliquots taken of the homogenates to determine amount of radioactivity associated with the hearts.

a) Analysis of tissue lipid

Phospholipids were extracted and separated as mentioned earlier. The fractions on the thin-layer chromatography plate corresponding to standard PC and PE were scraped into scintillation vials and the amount of radioactivity determined.

Neutral lipids in the extracts were separated by the procedure of Manners et al. (1969). TLC plates were washed twice with diethyl ether and then dried thoroughly before use. Aliquots of the lipid extracts were spotted on to the plates and where necessary, 1:2-diacylglycerol standard was also applied. The plates were developed in ether/petroleum ether/acetic acid (65/35/0.5; v/v) to about 6 cm from the top of the plate. The plates were then removed from the developing chamber and dried in an oven for 15 min at 105°. The

plates were then further developed in the same direction in ether/petroleum ether (6/94; v/v) to the top of the plate. The lipids were then visualized by iodine staining. Complete separation of 1:3-diacylglycerol, 1:2-diacylglycerol, fatty acid and triacylglycerol was achieved (when compared with authentic lipid standards). The bands corresponding to 1:2-diacylglycerol, fatty acid and triacylglycerol were scraped into vials and counted. No artifactual production of 1:2-diacylglycerol via hydrolysis of triacylglycerol was found. Isomerization of 1:2-diacylglycerol to 1:3-diacylglycerol was determined to be insignificant.

b) Determination of 1:2-diacylglycerol, phosphatidylcholine, and phosphatidylethanolamine

Isolated hamster hearts were perfused with unlabelled fatty acid for 10 minutes (representing the initial pulse period) and for 70 minutes (pulse period + 60 minute chase period). The hearts were homogenized and the lipids extracted as before.

PC and PE in the lipid extracts were separated in a solvent containing chloroform/methanol/water/acetic acid (70/30/4/2; v/v) and the bands visualized by iodine staining. The bands corresponding to PC and PE were scraped into test tubes and analyzed for lipid phosphorus content as described earlier for LPC.

Neutral lipids were separated as described in the previous section, however, no 1:2-diacylglycerol carrier was used. 1:2-Diacylglycerols were visualized by iodine staining and the bands

scraped into test tubes. The lipids were eluted from the silica gel with 3 x 2 ml washes of chloroform/methanol (2/1; v/v). The supernatants were pooled in test tube and the solvent removed under a stream of nitrogen. 1:2-Diacylglycerol levels were estimated by the acetic anhydride procedure of Ishidate and Weinhold (1981). To each sample of 1:2-diacylglycerol, 125 µl pyridine, 35 µl unlabelled acetic anhydride, 15 µl [^{3}H]acetic anhydride (150 µCi) and 1.75 µl perchloric acid were added and the tubes vortexed. The tubes were allowed to stand at room temperature for 60 minutes and then incubated at 37° for an additional 30 minutes. The reaction mixtures were then cooled on ice and 1 ml of water was added. The tubes were further incubated at 37° for 20 minutes. After completion, 1.5 ml of hexane was added and the tubes vortexed. The upper hexane layer was drawn off and saved. The reaction mixture was further subjected to 2 x 1.5 ml washes of hexane. All three hexane phases were pooled and 1.5 ml of 50% methanol in water was added and the tubes vortexed. The lower layer was removed and discarded; the remaining solvent was evaporated under a stream of nitrogen. Separation of 1:2-diacylglycerol acetates was performed as described by Banschbach et al. (1974). The residue was dissolved in a small amount of chloroform/methanol (2/1; v/v) and spotted onto Silica Gel G plates. Carrier diacyglycerol acetates was added to each lane to aid in separation and visualization. The plates were developed in heptane/ether/acetic acid (80/20/2; v/v) and the bands visualized by iodine staining. The bands corresponding to 1:2-diacyglycerol acetates were scraped into scintillation vials and counted. Acetylation of 1:2-diacylglycerol standards were performed

simulataneously for calibration purposes and for the production of 1:2-diacylglycerol acetate carriers. The production of 1:2-diacylglycerol acetate was linear from 10 nmol-100 nmol diacylglycerol using this method.

v) Analysis of free fatty acids by gas-liquid chromatography

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50 μM fatty acid for 15 minutes. The hearts were homogenized in chloroform/methanol (1/2; v/v) and the lipids extracted as described previously. A small amount of butylated-hydroxytoluene (5 mg/ml) was added as an anti-oxidant.

Aliquots of the lipid extracts were applied to TLC plates which were then developed in petroleum ether/ether/acetic acid (80/20/1; v/v) (Kates, 1972). The plates were sprayed with 0.025% 2,7 dichlorofluoroscein (in ethanol) and viewed under ultraviolet light. The fatty acid bands were scraped into test tubes and the lipid eluted with 3 x 3 ml washes of chlorform/methanol/water/acetic acid (50/39/10/1; v/v). The dichlorofluoroscein dye was removed after addition of 3 ml of 4 M ammonium hydroxide. The top layer, which contained the dye, was removed and discarded, the remaining solvent was evaporated under a stream of nitrogen. The residue was then dissolved in a small amount of chloroform/methanol (2/1; v/v). To ensure the fatty acid fraction was free of any lipid contaminants, the fatty acid extract was re-applied to a TLC plate and developed in chloroform/methanol/acetic acid (98/2/1; v/v) (Snyder, 1973). Fatty

acids were eluted as described above. In a separate experiment, [1-^{14}C]oleic acid was added to the homogenate and the lipids extracted and separated as before. Analysis of the fatty acid fraction by scintillation counting revealed a recovery of >95%.

Fatty acid methyl esters were prepared by addition of 2 ml of 0.5 N HCl in methanol to the fatty acid fractions and incubated overnight at 80°. The solvent was then removed by heat under a stream of nitrogen. The residue, containing fatty acid methyl esters, was dissolved in a small amount of hexane.

The gas chromatograph used in this study was a Shimadzu GC Mini 2 equipped with a temperature programmer. Before each run the machine was calibrated by injection of commercially available fatty acid methyl ester standards (GLC-20, GLC-30). All runs were performed using the identical parameters. A small amount of heptadecanoic acid methyl ester standard was added to each sample as an internal standard. Samples were then injected into the gas-chromatograph and the resultant methyl ester profile recorded on a Wescan Paper Recorder. Fatty acid methyl ester peaks were identified by comparison with standards and their areas measured. The area of each peak was divided by the total area of all peaks to calculate the percent distribution of free fatty acids.

vi) Pulse-chase studies on the metabolism of choline

Isolated hamster hearts were perfused with 10 μM [Me-^{3}H]choline for 30 minutes and subsequently chased with 10 μM choline and 0-50 μM

fatty acid for an additional 30 minutes. The hearts were then purged with 10 ml Kreb's-Henseleit buffer and 20 ml air as previously outlined. The tissue was homogenized in 20 ml chloroform/methanol (2/1; v/v).

a) Analysis of phosphatidylcholine

The homogenate was filtered and a 400 µl aliquot taken and applied to a TLC plate. The plate was developed in chloroform/methanol/water/acetic acid (70/30/4/2; v/v) and the PC fraction scraped into a vial and the radioactivity associated with it determined by liquid scintillation counting.

b) Analysis of choline-containing metabolites

Addition of 10 ml 0.1 N KCl to the homogenate resulted in phase separation. The upper aqueous phase was removed and evaporated to dryness by flash evaporation. The residue was dissolved in a small volume of water and applied along with appropriate carriers to a TLC plate. The plate was then developed in methanol/0.6% sodium chloride/ammonium hydroxide (50/50/5; v/v) which resulted in separation of choline, phosphocholine and CDP-choline. The areas corresponding to the choline metabolites were scraped into vials and analyzed for radioactivity by scintillation counting.

vii) Determination of enzyme activities for phosphatidylcholine biosynthesis

a) CTP:phosphocholine cytidylyltransferase

This enzyme is located in both the cytosolic and microsomal fractions (Choy et al., 1977). These fractions were prepared from hamster heart as follows. Hamsters were sacrificed by decapitiation and the hearts quickly removed and placed in ice-cold 0.145 M sodium chloride. The hearts were weighed and homogenized in a Polytron Homogenizer (10% homogenate). The homogenate was centrifuged at 12,000 x g for 15 min. The resulting supernatant was then centrifuged at 100,000 x g for 60 min. The pelleted material was designated the microsomal fraction and the supernatant was designated the cytosolic fraction. The microsomal pellet was resuspended in 0.145 M NaCl - 5 mM Tris-HCl (pH 7.4) and used immmediately in the assay for enzyme activity.

The reaction mixture contained 100 mM Tris-succinate (pH 6.0), 12 mM magnesium acetate, 2 mM cytidine triphosphate, 1 mM [Me-^{3}H]phosphocholine and microsomal or cytosolic protein in a total volume of 100 µl. The reaction mixture was incubated at 37° for 30 minutes. The reaction was subsequently stopped by placing the tubes in a boiling water bath for 2 min. An aliquot of the reaction mixture was applied to a TLC plate, along with carrier CDP-choline, and the plate developed in methanol/0.6% sodium chloride/ammonium hydroxide (50/50/5; v/v). The plate was then viewed under ultraviolet light and the CDP-choline spot identified. The corresponding fraction was then scraped into a scintillation vial and analyzed for radioactivity.

CTP:phosphocholine cytidylyltransferase was also assayed in vitro in the presence of 25 µM and 50 µM stearic acid. Stearic acid

was added in chloroform solution to each test tube and the solvent removed under nitrogen prior to addition of the other reactants. The enzyme assay was performed as described above.

The subcellular distribution of enzyme activity after perfusion with stearic acid was also determined. Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50 μM stearic acid for 30 min. The hearts were homogenized and subcellular fractions prepared as described earlier. The resultant microsomal and cytosolic fractions were then assayed for CTP:phosphocholine cytidylyltransferase activity as detailed above.

b) CDP-choline:1,2-diacylglycerol cholinephosphotransferase

This enzyme is associated with the microsomal fraction and was assayed according to the procedure of Arthur et al. (1984). Hamster heart microsomes were prepared by homogenization of the tissue in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.3 M Sucrose and 2 mM EDTA (10% homogenate). The homogenate was centrifuged at 12,000 x g for 10 min, and the resulting supernatant then centrifuged at 100,000 x g for 60 min. The crude microsomal pellet was then resuspended in a buffer containing 0.15 M Tris-HCl pH 8.0, and centrifuged again at 100,000 x g for 60 min. The resulting washed microsomal pellet was resuspended in the initial buffer and centrifuged again for 60 min. The microsomal pellet was then suspended in a buffer containing 10 mM Tris-HCl pH 7.4 and 0.25 M sucrose. The microsmal preparations were assayed for cholinephosphotransferase activity within 24 hr.

A typical reaction mixture contained 100 mM Tris-HCl (pH 8.5), 10 mM magnesium chloride, 5 mM EDTA, 0.4 mM [Me-^{14}C] CDP-choline (10 µCi/µmol), 0.4 mM 1:2-diacyglycerol solution, 10 µM fatty acid (none for control) and 100 µg of microsomal protein in a total volume of 1 ml. A working solution of 1:2-diacylglycerol was prepared by sonication in the presence of Tween-20. The final concentration of Tween-20 in the reaction mixture was 60 µg/ml.

The reaction was initiated by the addition of microsomal protein and was incubated at 37° for 15 min. No microsomal protein was added in the reaction blank. The reaction was stopped by the addition of 1 ml chloroform + 0.5 ml of 1 M HCl. The resulting lower phase was washed three times with 40% methanol in water. An aliquot of the washed lower phase was analyzed for associated radioactivity by liquid scintillation counting. This procedure measured the formation of radioactive PC from the labelled precursor CDP-choline.

c) Acylation of lysophosphatidylcholine

Fatty acid Coenzyme A ligase and acyl CoA: 1-acyl-glycerophosphocholine acyltransferase activities are both microsomal in origin (Ballas and Bell, 1980, Polokoff and Bell, 1978). Heart microsomes were assayed for the incorporation of fatty acids into LPC forming PC by a slight modification of the procedure of Connor et al. (1981). The microsomes were prepared as described in the previous section.

A typical reaction mixture contained 150 nmoles [1-^{14}C]LPC (2.67

μCi/μmol), 100 nmoles fatty acid, 100 μmoles potassium phosphate buffer (pH 7.4), 25 μmoles adenosine triphosphate, 10 μmoles magnesium chloride, 100 μmoles sodium fluoride and 0.2 μmoles Coenzyme A in a total volume of 1 ml. The reaction was initiated by the addition of 100 μg microsomal protein and was incubated at 25° for 10-30 min. At each 10 min interval, a 250 μl aliquot was removed and to it was added 3.75 ml of chloroform/methanol (1/2; v/v) to stop the reaction. The tubes were vortexed and centrifuged at 1,000 x g for 10 min. The supernatant was decanted and 1 ml of chloroform/methanol/water (1/2/0.8; v/v) was added to the pellet. The tubes were vortexed and centrifuged at 1,000 x g for 10 min. The supernatants were pooled and 3.75 ml chlorform and 2.75 ml water was added to cause phase separation. The upper phase was removed and discarded and the lower phase was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in chloroform/methanol (2/1; v/v) and applied to a TLC plate. The plate was developed in chloroform/methanol/water/acetic acid (70/30/4/2; v/v) and the PC fraction scraped and analyzed for radioactivity.

d) Protein determinations

Protein concentrations of the subcellular fractions were determined by the method of Lowry et al., (1951). Aliquots of the subcellular fractions were incubated overnight in 0.5 ml of 0.66 N sodium hydroxide. A mixture of 13% sodium carbonate - 2% cupric sulfate - 4% sodium potassium tartrate, (1.5 ml), was added to each tube and the mixture vortexed well. The mixture was allowed to sit at

room temperature for 10 min after which 0.5 ml of 2 N Folin-Ciocalteau Reagent was added. The mixture was again vortexed and allowed to sit at room temperature for 30 min. Absorbance was measured at 625 nm against albumin standards.

EXPERIMENTAL RESULTS

I EXTRACTION AND STORAGE OF CARDIAC LIPIDS

It has been previously shown that extraction of cardiac lipids with acidified butanol resulted in a marked elevation in LPC levels (Mock et al., 1984). This elevation was attributed to the hydrolysis of acid-labile PC. In order to prevent any intrapreparative loss in PC, only neutral solvents containing chloroform and methanol were evaluated with respect to efficiency in lipid extraction. To assess the extent of lipid extraction after each solvent wash, the amount of LPC extracted was quantitated by lipid-phosphorus determination (Bartlett, 1959).

The three solvents used to extract the tissue lipids were, chloroform/methanol (2/1; v/v), chloroform/methanol (1/1; v/v) and chloroform/methanol (1/2; v/v). Although all three solvents used effectively extracted LPC after 5 extractions, the solvent containing chloroform/methanol (1/2; v/v) yielded maximum recovery of LPC after only 3 extractions (Table 1). The values obtained for cardiac LPC content were comparable with those from a previous report (Man et al., 1983). The lower specific gravity of chloroform/methanol (1/2; v/v) also allowed for the separation of the insoluble pellet from the solvent by low-speed centrifugation. By virtue of the efficient and effective extraction of cardiac lipids by chloroform/methanol (1/2; v/v), all subsequent extractions were conducted using this solvent. The use of this solvent also eliminated the artifactual production of lysophospholipids and the corresponding loss of parent phospholipid.

As depicted in Figure 5, storage of the lipid extracts in

TABLE 1

Extraction of lysophosphatidylcholine from canine heart by various solvents

Fresh canine cardiac tissue was homogenized and tissue lipid extracted with the three solvents listed below. The lysophosphatidylcholine levels after each extraction were determined as described in "Materials and Methods"

	Solvent (nmol lipid-P/g wet wt)		
Number of extractions	$CHCL_3/CH_3OH$ (2/1; v/v)	$CHCL_3/CH_3OH$ (1/1; v/v)	$CHCL_3/CH_3OH$ (1/2; v/v)
1	76.8 ± 9.3	79.8 ± 6.1	94.5 ± 9.8
2	101.8 ± 4.3	104.9 ± 7.4	117.0 ± 8.0
3	109.2 ± 7.4	113.2 ± 6.1	123.6 ± 4.3
5	111.3 ± 5.8	122.3 ± 7.2	118.8 ± 4.6

Note: The values listed are the means ± standard deviation of three separate experiments

$CHCL_3$ - chloroform

CH_3OH - methanol

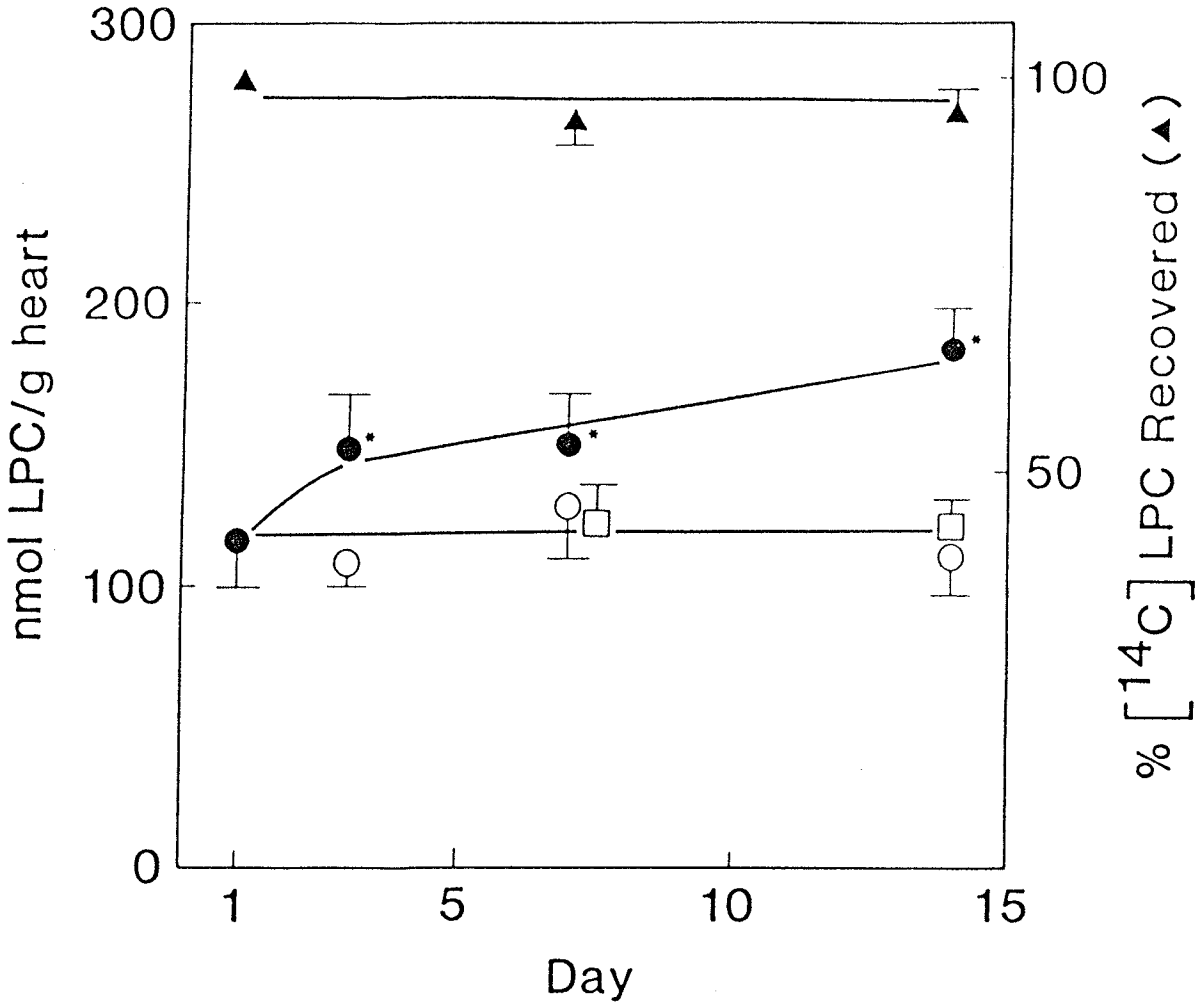


Fig. 5. Stability of lysophosphatidylcholine in lipid extract stored in various media

Lipids extracted from canine heart left ventricle were divided into three aliquots and these aliquots were stored at -20°C in either chloroform/methanol (2/1) (○), chloroform/methanol/water (86/14/1) (●) or without solvent under nitrogen (□) for the periods indicated. One microcurie of [1-^{14}C]palmitoyl lysophosphatidylcholine was added to each of the aliquot preparations prior to storage. The lysophosphatidylcholine in the lipid extracts was isolated by thin-layer chromatography and assayed as described at the prescribed intervals. Each value represents the mean of six different experiments. The vertical bars are standard deviation. *$P < 0.05$ when compared with the sample from day 1.

chloroform/methanol/water (86/14/1; v/v) resulted in the elevation of LPC levels when compared with extracts stored in chloroform/methanol (2/1; v/v) or without solvent under nitrogen. The artifactual production of LPC probably occurred via hydrolysis of PC during storage. No change was seen in the amount of radioactivity associated with LPC under all modes of storage. In order to prevent any loss of PC during storage, all lipid extracts in the following studies were stored in chloroform/methanol (2/1; v/v) under nitrogen.

II PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS: INCORPORATION OF FREE FATTY ACIDS

a) Time-course studies

The initial approach in this study was to demonstrate the uptake and incorporation into PC and PE of exogenous fatty acids in the isolated perfused hamster heart. Isolated hamster hearts were perfused with [1-^{14}C]stearic or [1-^{14}C]oleic acid as described in "Materials and Methods". The uptake of radioactive fatty acids by the hearts was found to be hyperbolic in nature from 0-30 min with 50 μM stearic and 50 μM oleic acid (Figures 6 and 7). Maximum uptake occurred by 15 min with both fatty acids and remained constant thereafter. The uptake of fatty acid by the isolated hamster heart observed in this study (approx. 700 nmoles/g heart/15 min) is in close agreement with that obtained by Stein and Stein (1963), using the isolated perfused rat heart.

The heart homogenates after perfusion were separated into two

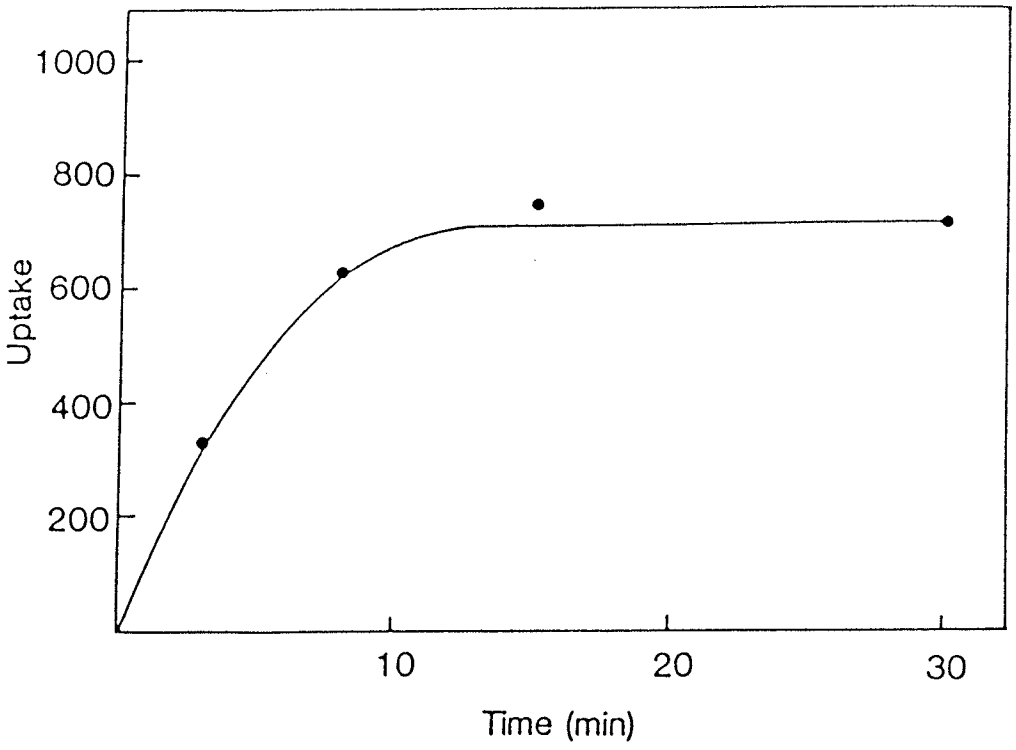


Fig. 6. Time course of stearic acid uptake in isolated hamster hearts. Isolated hamster hearts were cannulated via the aorta in the Langendorff Mode (1895), with Kreb's-Henseleit buffer containing 50 µM [1-^{14}C]stearic acid (4 µCi/µmol) and 10 µM albumin for 3-30 minutes. After perfusion the hearts were homogenized in chloroform/methanol (1/2; v/v) and the total radioactivity in the homogenates determined. The total stearic acid uptake by the isolated hearts was calculated from the specific radioactivity of stearic acid in the perfusate. Uptake is expressed as nmol/g heart. Each point represents the mean of three separate experiments.

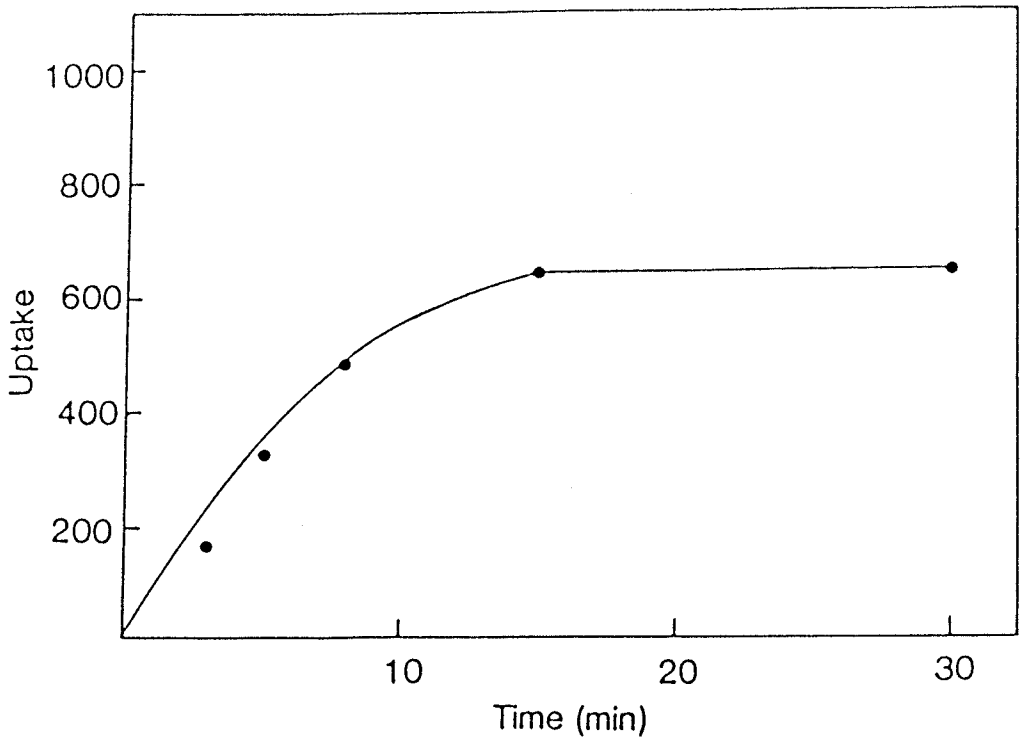


Fig. 7. Time course of oleic acid uptake in isolated hamster hearts. Isolated hamster hearts were perfused with 50 μM [1-^{14}C]oleic acid (4 μCi/μmol) and 10 μM albumin for 3-30 minutes. Uptake of oleic acid was calculated as described in Figure 6, and is expressed as nmol/g heart. Each point represents the mean of three separate experiments.

phases and PC and PE in the organic phase were separated by thin-layer chromatography with a solvent system containing chloroform/methanol/water/acetic acid (70/30/4/2; v/v). Incorporation of [1-^{14}C]stearic and [1-^{14}C]oleic acid into PC and PE was found to be linear between 0-30 min (Figures 8 and 9). The incorporation of [1-^{14}C]stearic acid was greater into PC than into PE at all periods of perfusion. Similarly, incorporation of [1-^{14}C]oleic acid into PC exceeded incorporation into PE. Most noteworthy, however, was that the difference in labelling between PC and PE was 3-fold with stearic acid but only a 2-fold difference in labelling was observed with oleic acid.

b) Concentration-dependence studies

The uptake of fatty acid by the isolated hamster heart and incorporation into PC and PE was studied as a function of fatty acid concentration. Hearts were homogenized after perfusion and aliquots of the homogenate taken to determine uptake of fatty acid. The uptake of [1-^{14}C]stearic acid and [1-^{14}C]oleic acid was linear between 20 μM-320 μM (Figures 10 and 11). Linearity of uptake was also observed with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid at perfusate concentrations between 50 μM-300 μM (Figure 12). However, the total uptake of fatty acids by the isolated hearts differed. At all concentrations tested, stearic acid and oleic acid were taken up to a greater extent than arachidonic acid.

Analysis of PC and PE by thin-layer chromatography demonstrated

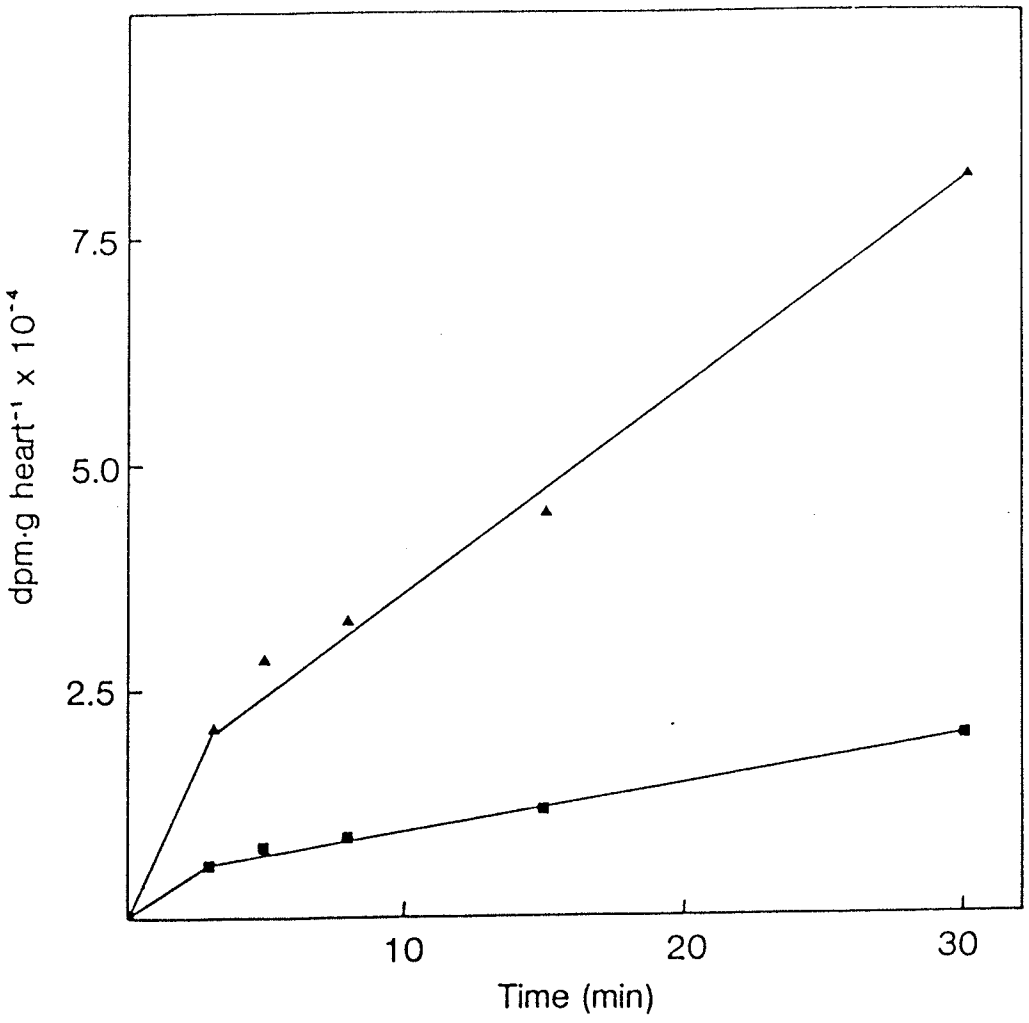


Fig. 8. Time course of [1-^{14}C]stearic acid incorporation into phosphatidylcholine and phosphatidylethanolamine

Phase separation was effected in the homogenates from Fig. 6. The lower (chloroform) phase was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine (▲) and phosphatidylethanolamine (■). Each point represents the mean of three separate experiments.

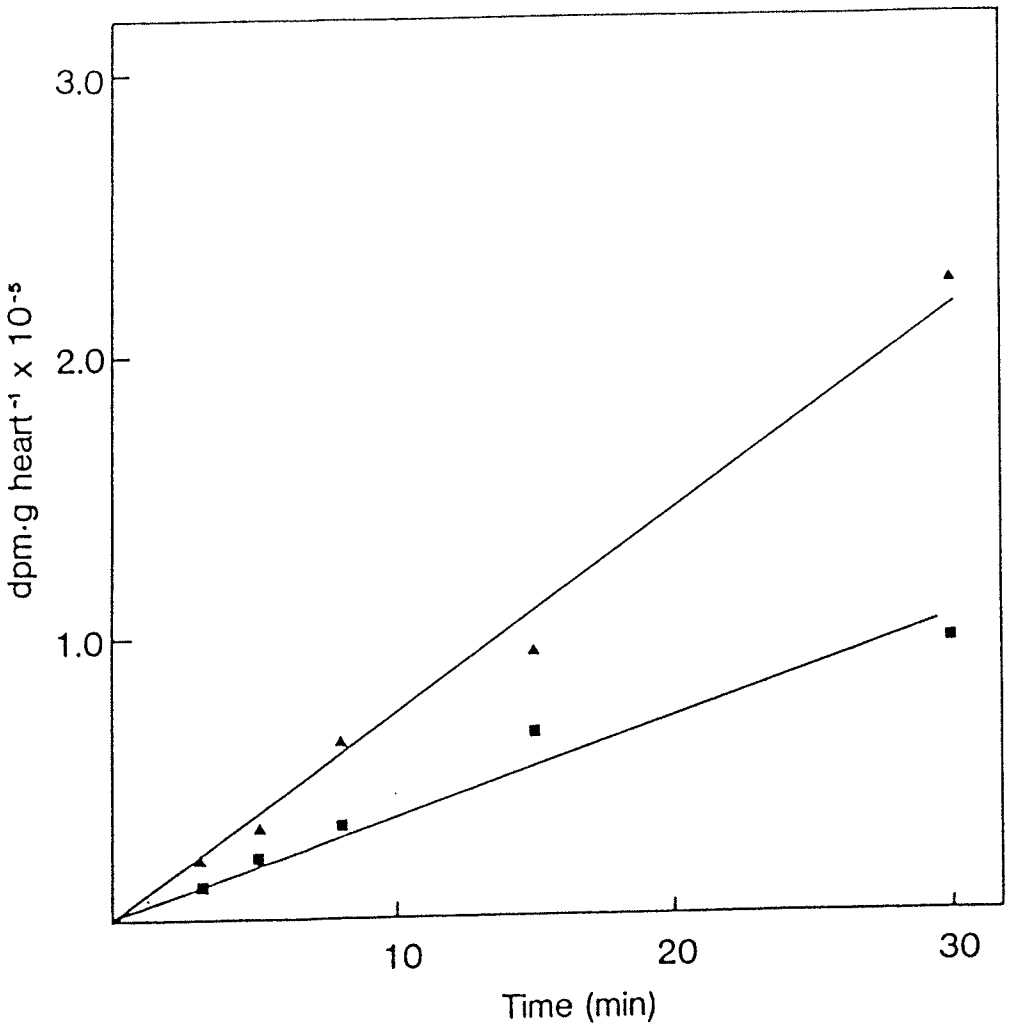


Fig. 9. Time course of [1-^{14}C]oleic acid incorporation into phosphatidylcholine and phosphatidylethanolamine

The homogenates from Fig. 7 were separated into two phases. The lower (chloroform) phase was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine (▲) and phosphatidyl-ethanolamine (■). Each point represents the mean of three separate experiments.

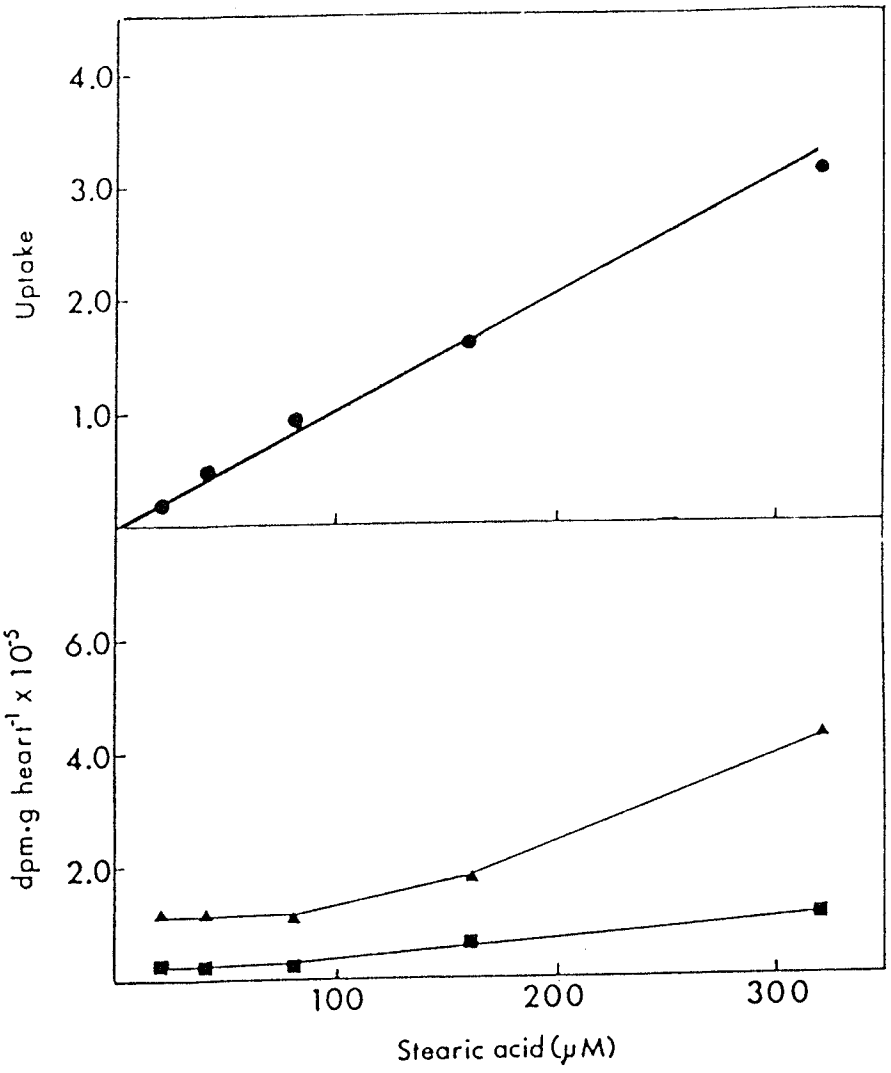


Fig. 10. Total uptake and incorporation of [1-^{14}C]stearic acid in isolated hamster hearts.

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 20-320 μM [1-^{14}C]stearic acid and 10 μM albumin for 15 minutes. After perfusion the hearts were homogenized in chloroform/methanol (1/2; v/v) and the total radioactivity in the homogenate determined. Uptake of radioactivity (●) is expressed as μmol/g heart/15 minutes. Phase separation was then effected in the homogenates and the organic phase was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine (▲) and phosphatidylethanolamine (■). Each point represents the mean of three separate experiments.

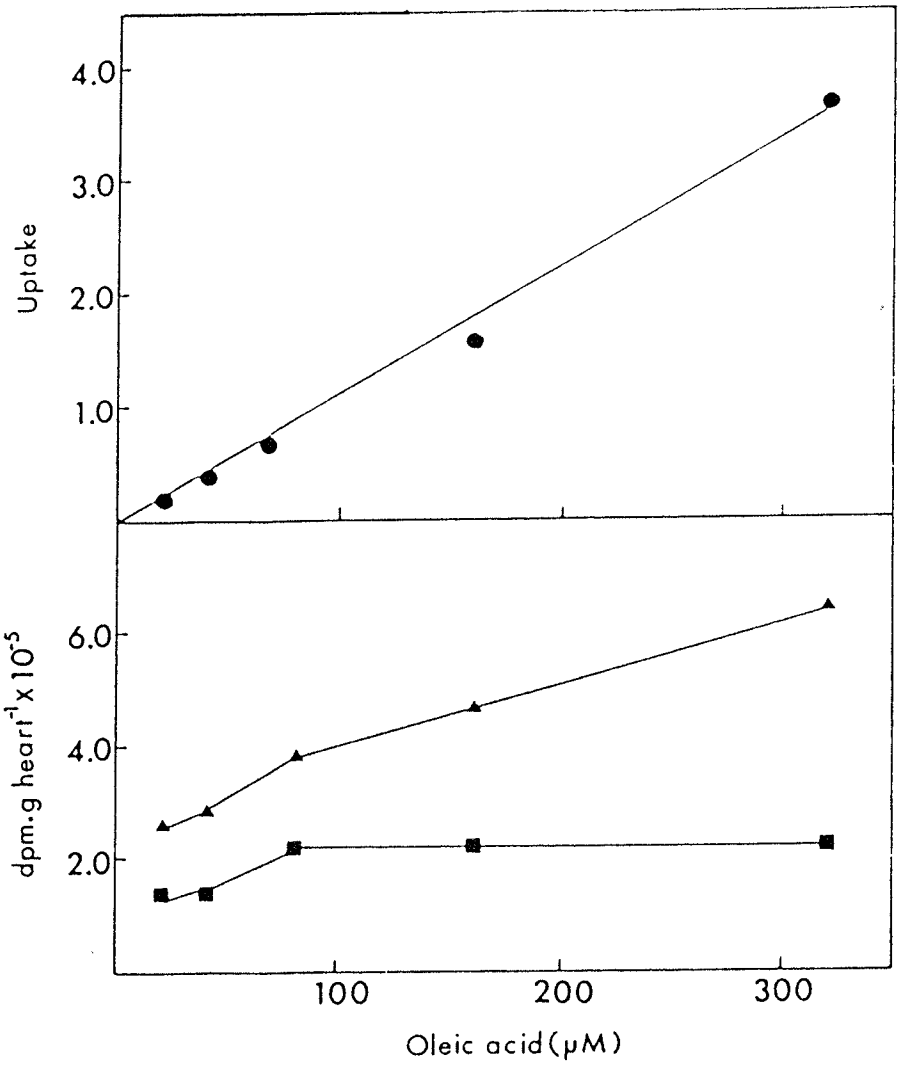


Fig. 11. Total uptake and incorporation of [1-^{14}C]oleic acid in isolated hamster hearts

The experimental conditions were identical to those described in Fig. 10. The total uptake (●) and incorporation of [1-^{14}C]oleic acid into phosphatidylcholine (▲) and phosphatidylethanolamine (■) was determined. Each point represents the mean of three separate experiments.

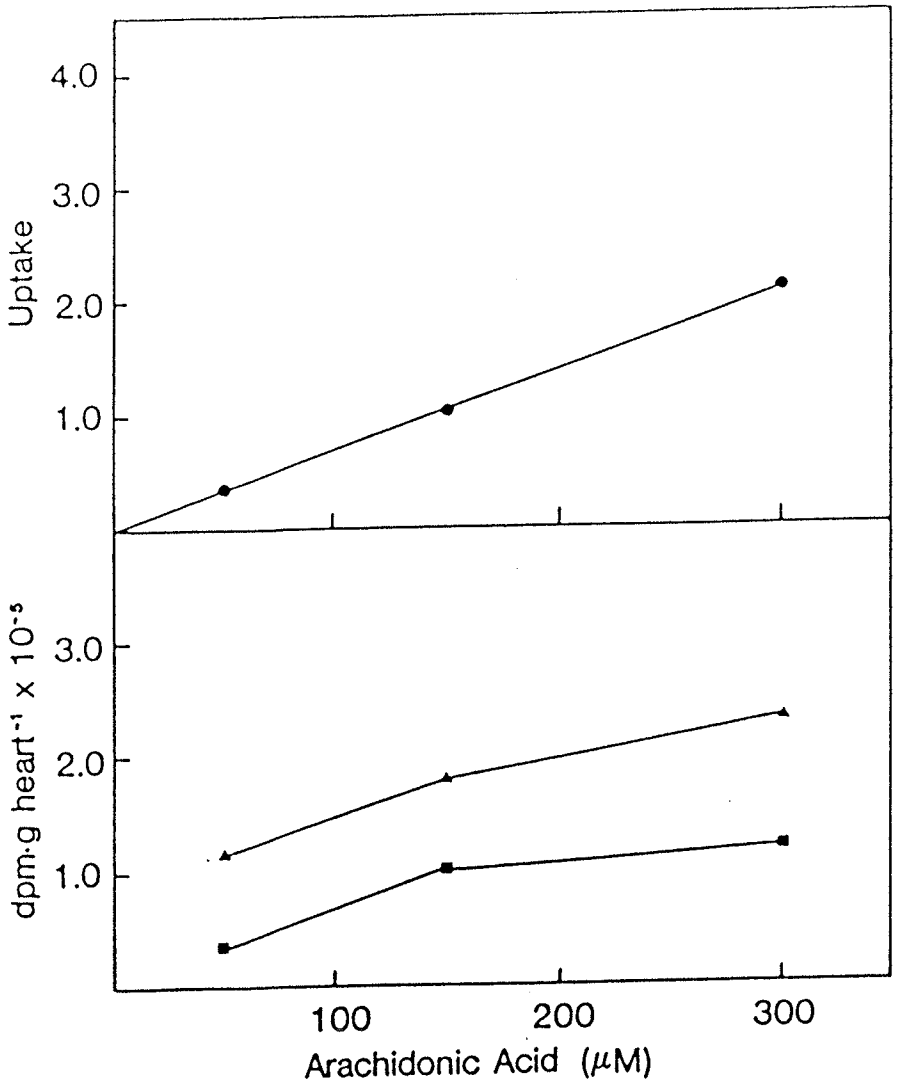


Fig. 12. Total uptake and incorporation of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid in isolated hamster hearts.

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50-300 μM [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid and 10 μM albumin for 15 minutes. The total uptake (●), expressed as μmol/g heart/15 minutes, and the amount of radioactivity incorporated into phosphatidylcholine (▲) and phosphatidylethanolamine (■) was determined as described in Fig. 10. Each point represents the mean of five separate experiments.

a concentration-dependent incorporation of fatty acid into these phospholipids (Figures 10, 11 and 12). Incorporation of [1-^{14}C]stearic acid, [1-^{14}C]oleic acid and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid into PC and PE increased as the fatty acid concentration increased. Incorporation of [1-^{14}C]oleic acid into the PC and PE fractions exceeded that of stearic and arachidonic acid at all concentrations. Stearic acid and arachidonic acid were incorporated to a similar extent except at higher fatty acid concentrations where incorporation of stearic acid into PC was greater than arachidonic acid incorporation. A 3-fold difference in PC and PE labelling was observed with [1-^{14}C]stearic acid. In contrast, a 2-fold difference in incorporation into PC and PE occurred with [1-^{14}C]oleic acid and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid. These data suggest that uptake of fatty acid by the hearts and the rate of incorporation of fatty acids into PC and PE is dependent on the fatty acid species.

c) Pulse-chase studies

In order to study the metabolism and incorporation of fatty acids into PC and PE, pulse-chase studies were conducted. Isolated hamster hearts were pulse-labelled for 10 min with 50 μM [1-^{14}C]stearic acid or 50 μM [1-^{14}C]oleic acid or for 15 min with 50 μM [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid. The hearts were subsequently chased in a non-recirculating fashion for 0-60 min with the respective unlabelled fatty acids at the same perfusate concentration. The hearts were homogenized after the chase period and

aliquots taken of the homogenate to determine associated radioactivity.

A decrease in the radioactivity associated with the homogenate was observed throughout the chase period with each fatty acid (Figures 13, 14 and 15). A similar decrease in the radioactivity associated with the fatty acid fraction was also observed. Such a decrease may be attributed to oxidation of the fatty acids or passage of the label out of the cell and into the effluent. To test the latter hypothesis aliquots of the effluent were taken throughout the chase period and the associated radioactivity determined. With all three fatty acids approximately 10%-20% of the original amount of radioactive label was recovered in the chase buffer. This observation suggested that the fatty acid or a metabolic product, was extruded from the cell during the chase period.

The heart homogenates obtained after perfusion were separated into two phases and 1:2-diacylglycerol, triacylglycerol, free fatty acid, PC and PE in the organic phase were analyzed by thin-layer chromatography as outlined in "Materials and Methods". The majority of [1-^{14}C]stearic acid taken up (85%), remained as free fatty acid. In contrast only 20%-30% of [1-^{14}C]oleic acid and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid was recovered in the fatty acid fraction (Figures 13, 14 and 15). A substantial amount of [1-^{14}C]oleic acid and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid was incorporated into triacylglycerols. Incorporation of [1-^{14}C]stearic acid into triacylglycerols represented a relatively minor fate of the

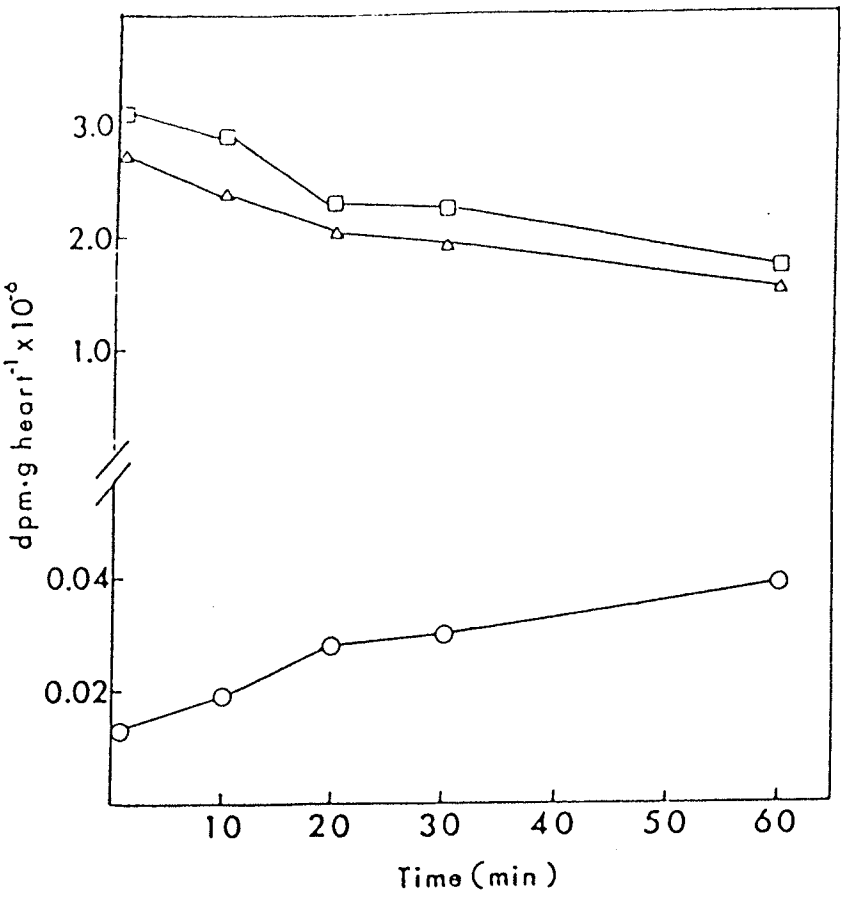


Fig. 13. Metabolism of labelled fatty acids and triacylglycerols in hamster heart after pulse-labelling with [1-^{14}C]stearic acid.

Isolated hamster hearts were pulse-labelled with 50 µM [1-^{14}C]stearic acid and 10 µM albumin in Kreb's-Henseleit buffer for 10 minutes and subsequently chased with 50 µM stearic acid for 0-60 minutes. After perfusion the hearts were homogenized in chloroform/methanol (1/2; v/v) and the associated radioactivity determined (□). The homogenate was separated into two phases and the organic phase was analyzed by thin-layer chromatography for radioactivity in the free fatty acid (△) and triacylglycerol fractions (○). Each point represents the mean of three separate experiments.

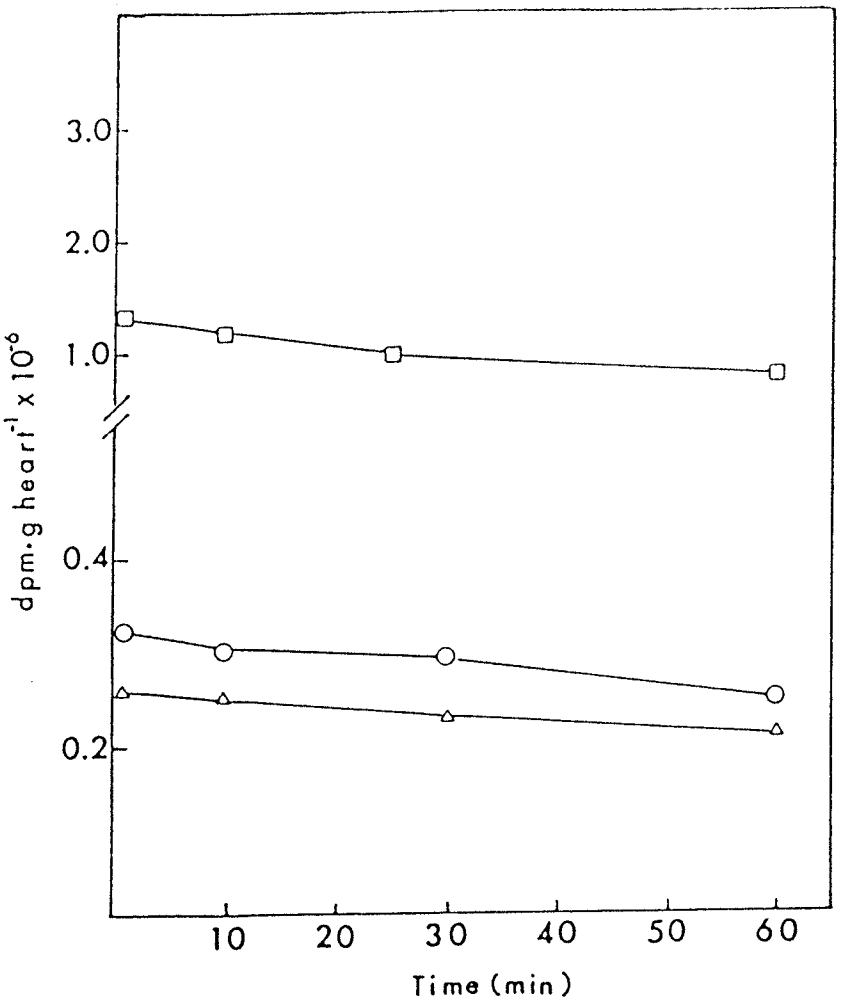


Fig. 14. Metabolism of labelled fatty acids and triacylglycerols in hamster heart after pulse-labelling with [1-^{14}C]oleic acid.

Isolated hamster hearts were pulse-labelled with 50 µM [1-^{14}C]oleic acid and subsequently chased with 50 µM oleic acid as described in Figure 13. Radioactivity in the homogenate (□), fatty acid fraction (△) and in the triacylglycerol fraction (○) was determined. Each point represents the mean of five separate experiments.

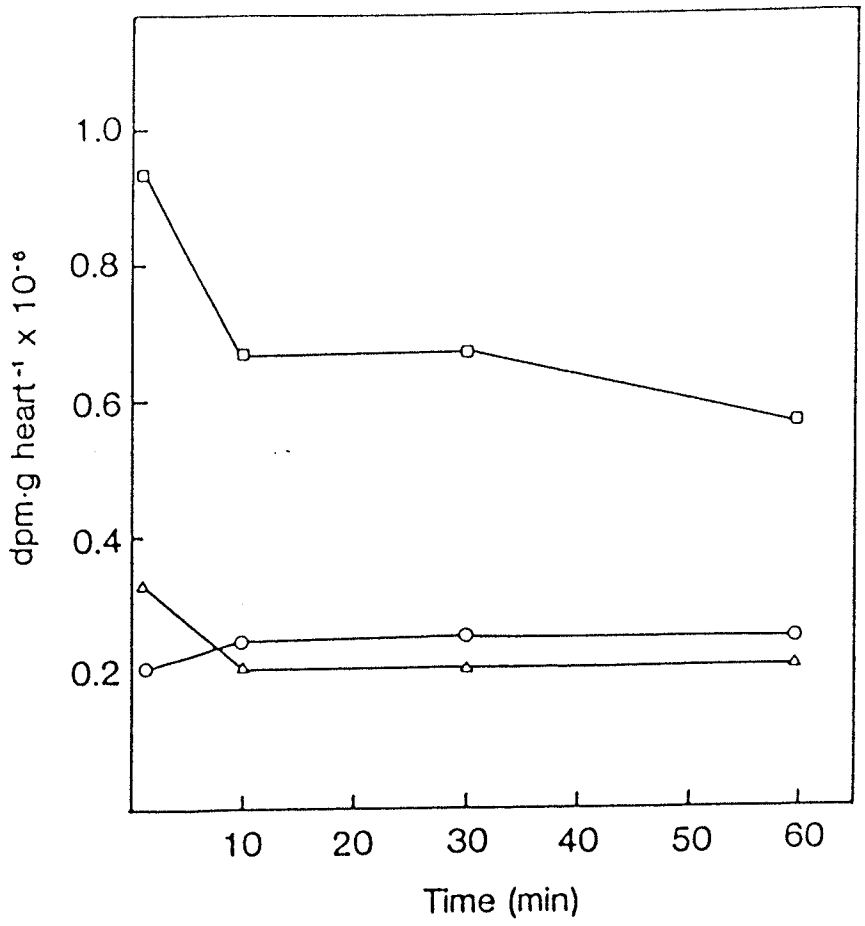


Fig. 15. Metabolism of labelled fatty acids and triacylglycerols in hamster hearts after pulse-labelling with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid.

Isolated hamster hearts were pulse-labelled with 50 µM [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid and 10 µM albumin in Kreb's-Henseleit buffer for 15 minutes and subsequently chased with 50 µM arachidonic acid for 0-60 minutes. Radioactivity in the homogenate (□), fatty acid fraction (△) and in the triacylglycerol fraction (○) was determined as described in Figure 13. Each point represents the mean of five separate experiments.

fatty acid taken up. No significant changes in the radioactivity associated with the triacylglycerol fractions were observed throughout the chase periods with oleic and arachidonic acids. However, a slight increase in the labelling of triacylglycerols during the chase period was evident with stearic acid. The remainder of the radioactivity associated with the homogenates after perfusion was recovered in the aqueous phase.

Pulse-chase studies conducted with stearic acid revealed a decrease in the radioactivity associated with 1:2-diacylglycerol with a concomitant increase in PC and PE labelling (Figure 16). The decrease in 1:2-diacylglycerol labelling and the increase in PC and PE labelling observed was temporally related. A similar pattern of labelling was also observed with oleic acid (Figure 17). These data suggest that labelled 1:2-diacylglycerol may serve as the immediate precursor for PC and PE biosynthesis. A similar decrease in 1:2-diacylglycerol labelling was also observed with arachidonic acid (Figure 18). However, this decrease was not coupled with an increase in PC or PE labelling. Since a precursor-product relationship was not apparent this suggested that arachidonic acid was incorporated into PC and PE by a route(s) other than the de novo synthetic pathway.

Since any alterations in the levels of PC, PE and 1:2-diacylglycerol will inevitably alter the labelling patterns, the cardiac levels of these metabolites were quantitated. No changes in 1:2-diacylglycerol content were observed during the chase period (Table 2). The 1:2-diacylglycerol contents of the hearts are in

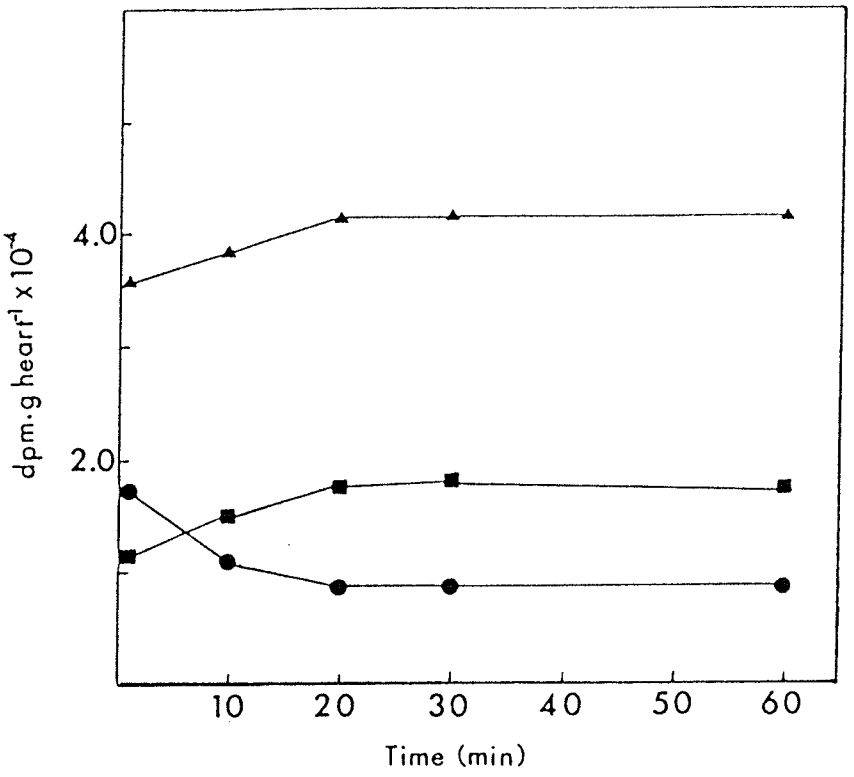


Fig. 16. Metabolism of phosphatidylcholine, phosphatidylethanolamine and 1:2-diacylglycerol in hamster hearts after pulse-labelling with [1-^{14}C]stearic acid.

Phase separation was effected in the homogenates from Fig. 13. The lower organic phase was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine (▲), phosphatidylethanolamine (■) and 1:2-diacylglycerol (●). Each point represents the mean of three separate experiments.

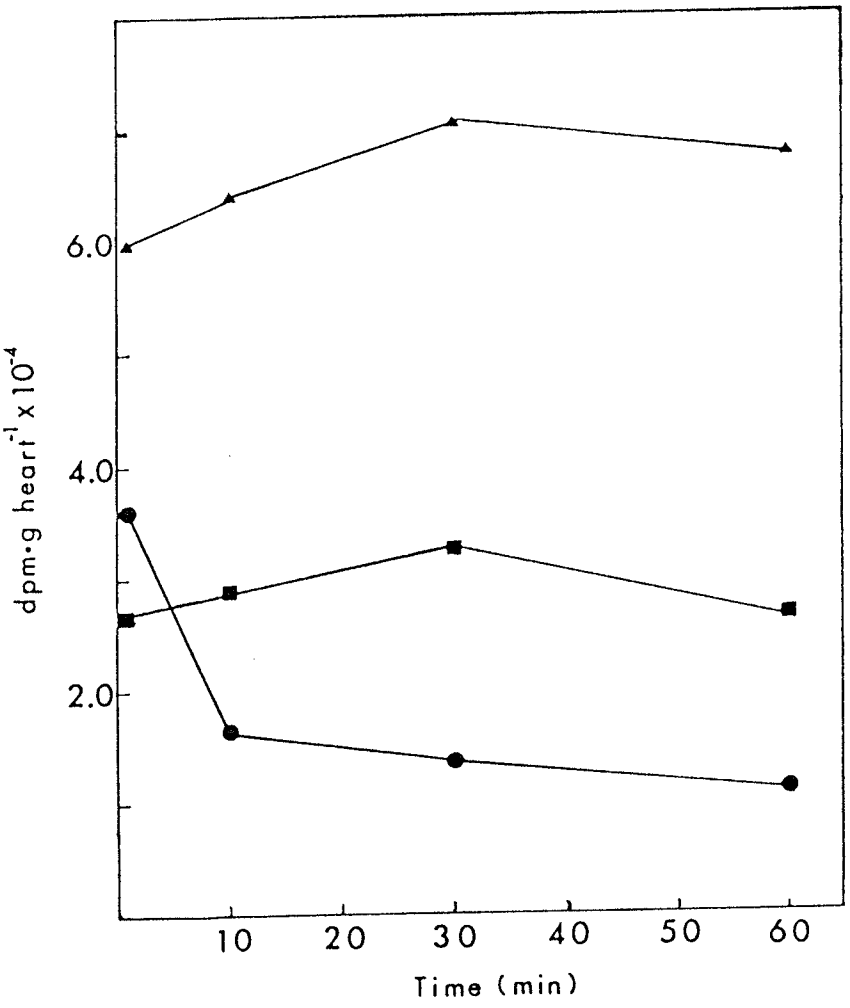


Fig. 17. Metabolism of phosphatidylcholine, phosphatidylethanolamine and 1:2-diacylglycerol in hamster hearts after pulse-labelling with [1-^{14}C]oleic acid.

The homogenates from Fig. 14 were separated into two phases. The lower organic phase was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine (▲), phosphatidylethanolamine (■) and 1:2-diacylglycerol (●). Each point represents the mean of three separate experiments.

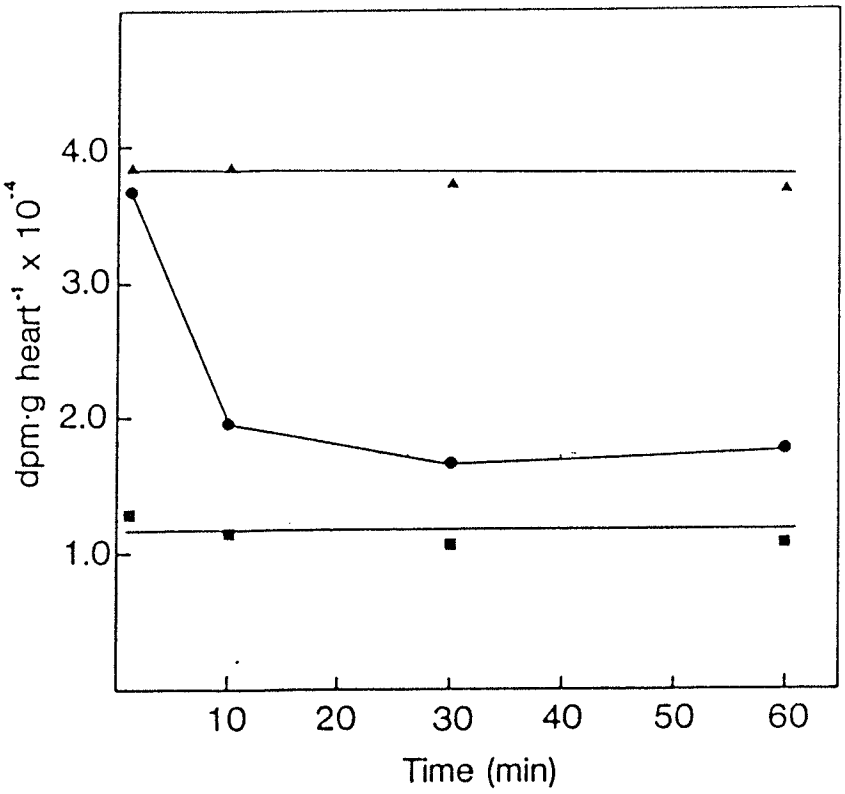


Fig. 18. Metabolism of phosphatidylcholine, phosphatidylethanolamine and 1:2-diacylglycerol in hamster hearts after pulse-labelling with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid

The homogenates from Fig. 15 were separated into two phases. The chloroform phase was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine (▲), phosphatidylethanolamine(■) and 1:2-diacylglycerol (●). Each point represents the mean of five separate experiments.

TABLE 2

Concentration of 1:2-diacylglycerol, phosphatidylcholine and phosphatidylethanolamine in hamster heart.

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50μM fatty acid and 10μM albumin for 10 minutes (initial pulse period) and for 70 minutes (10 minute pulse + 60 minute chase). The hearts were homogenized after perfusion and separated into two phases. Phosphatidylcholine, phosphatidylethanolamine and 1:2-diacylglycerol in the organic phase were separated by thin-layer chromatography and quantitated as described in "Materials and Methods".

Metabolite	Time period of chase	
	0 minutes	60 minutes
	(μmol/g heart)	
1:2-Diacylglycerol	0.13 ± 0.03	0.14 ± 0.03
Phosphatidylcholine	12.7 ± 0.2	12.3 ± 0.6
Phosphatidylethanolamine	9.9 ± 0.2	10.3 ± 0.3

Each value represents the mean ± standard deviation of four separate experiments.

close agreement with previously reported values (Choy, 1982). Similarly, no alteration in cardiac PC or PE levels were noted (Table 2).

d) Free fatty acid profile

Pelech et al. (1983) have reported a stimulatory effect of relatively high concentrations of certain fatty acids on the CDP-choline pathway in isolated rat hepatocytes. The initial approach to determine if a similar mechanism exists in the isolated hamster heart was to evaluate the effects of perfusion with 50 µM stearic acid, 50 µM oleic acid and 50 µM arachidonic acid on the free fatty acid profile of the hearts. Lipids were extracted from the hearts after perfusion and free fatty acids were separated from other neutral lipids by thin-layer chromatography. Free fatty acid methyl esters were prepared by methanolysis and were analyzed by gas-liquid chromatography. As seen in Table 3, only myristic, lauric, palmitic and stearic acids were found to be of quantitative importance in the free fatty acid fraction. A small amount of oleic, linoleic, linolenic and arachidonic acids was also detected. Two unidentified peaks, suggestive of long-chain fatty acids, were also seen in some chromatograms. A typical gas-liquid chromatogram is shown in Figure 19. The only change in the free fatty acid profile which was found to be quantitatively significant was an increase in stearic acid content upon perfusion with stearic acid. Such a result was expected as a large proportion of stearic acid taken up remained as free fatty acid as reported earlier (Pulse-chase studies). Total free fatty acid

TABLE 3

Free fatty acid profile of hamster heart

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50μM fatty acid and 10μM albumin for 15 minutes. After perfusion the hearts were homogenized in chloroform/methanol (1/2; v/v) and the homogenates were subjected to phase extraction. Free fatty acids in the organic phase were separated, converted into methyl esters and the fatty acid methyl esters were analyzed by gas-chromatography as outlined in "Materials and Methods".

		Fatty acid methyl ester (nmoles/g heart)		
Perfusate fatty acid	lauric	myristic	palmitic	stearic
Control	-	13.9 ± 6.4 (6)	103.9 ± 37.9 (56)	64.1 ± 14.2 (35)
Oleic	2.0 (2)	11.6 ± 4.6 (6)	114.0 ± 34.6 (57)	64.9 ± 9.1 (33)
Arachidonic	-	18.8 ± 5.9 (5)	112.6 ± 26.8 (55)	77.6 ± 27.8 (38)
Stearic	2.0 (1)	20.0 ± 14.5 (4)	125.2 ± 14.3 (24)	362 ± 218* (69)

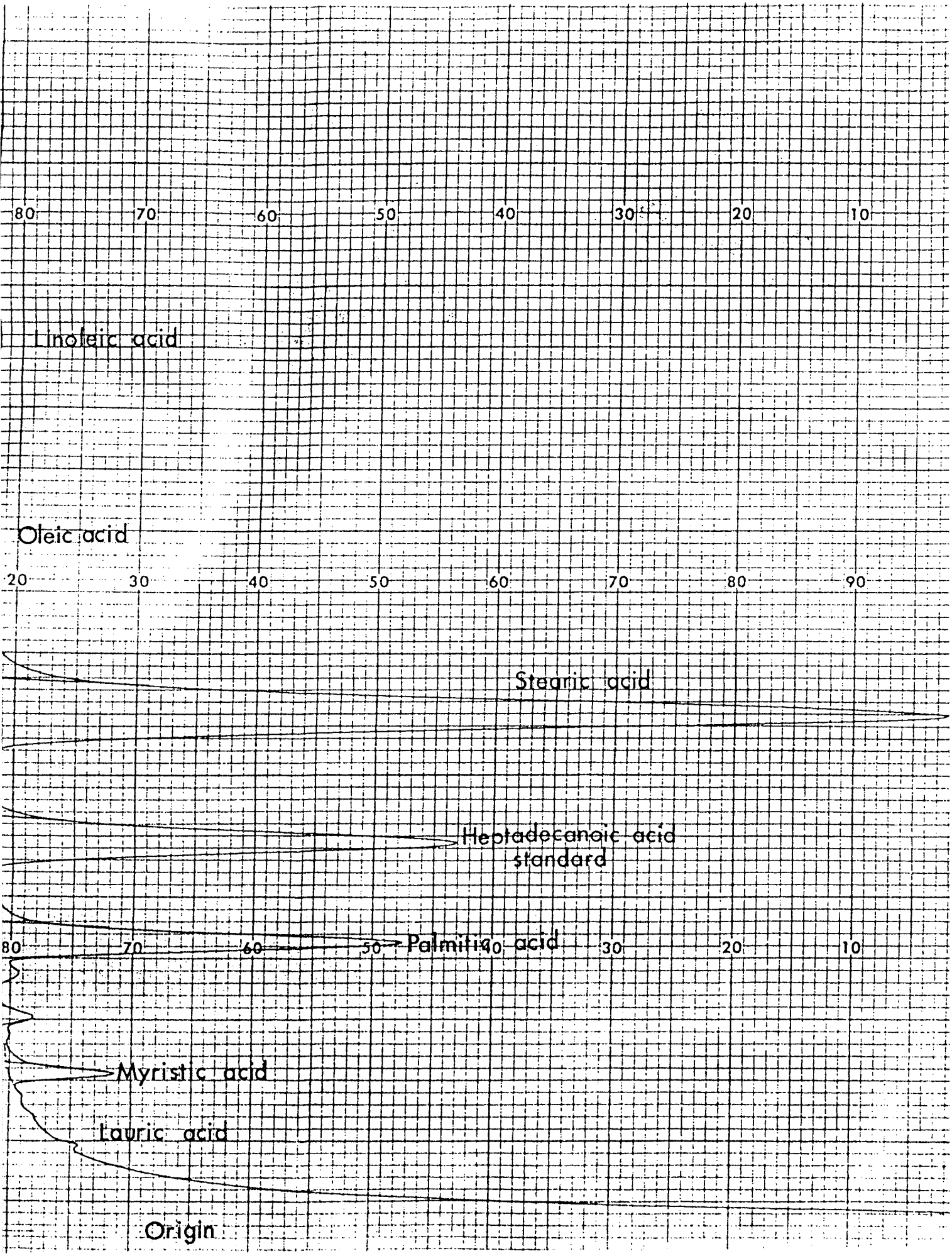
Each value represents the mean ± standard deviation of three separate experiments
Note: Numbers in parantheses denotes % contribution of that fatty acid to total fatty acid content.

*$P<0.05$ when compared to control

Fig. 19. A typical gas-chromatogram of the fatty acid methyl esters prepared from the free fatty acid fraction of hamster hearts

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50µM fatty acid and 10µM albumin for 15 minutes. After perfusion the hearts were homogenized in chloroform/methanol (1/2; v/v) and the homogenates separated into two phases. Free fatty acids in the organic phase were separated by thin-layer chromatography. Fatty acid methyl esters were prepared and subjected to gas-chromatography as described in "Materials and Methods". The program utilized for the separation of the fatty acid methyl esters had the following parameters:

Injection temperature:	230°C
Temperature range:	180°C-200°C at 1°C/min.
Range:	10^2
Attenuation:	8
Chart Speed:	2 cm/min.


80
70
60
50
40
30
20
10
Linoleic acid
Oleic acid
20
30
40
50
60
70
80
90
Stearic acid
Heptadecanoic acid
standard
80
70
60
50
Palmitic acid
40
30
20
10
Myristic acid
Lauric acid
Origin

content (45 ± 5 μg/g heart) was similar to that reported by Kramer and Hulan (1978) in the rat heart.

III PHOSPHATIDYLCHOLINE BIOSYNTHESIS: EFFECT OF FREE FATTY ACIDS

a) Pulse-chase studies on choline incorporation

In order to investigate the possible regulatory effects of free fatty acids on the CDP-choline pathway, isolated hamster hearts were pulse-labelled with 10 μM [Me-^{3}H]choline for 30 min and subsequently chased with 10 μM choline and 0-50 μM fatty acid for an additional 30 min. Control hearts were designated as those which did not contain fatty acid in the chase buffer. After perfusion the hearts were homogenized and PC was analyzed by thin-layer chromatography. No increase in choline incorporation into PC was observed when hearts were chased with 25 μM fatty acid, however, a significant increase in PC labelling occurred when hearts were chased with 50 μM stearic acid (Table 4). A possible explanation for the increase in PC labelling observed is that stearic acid stimulated the activity of one or more of the enzymes in the CDP-choline pathway. Choline flux through this pathway would therefore be enhanced. To investigate further, the choline metabolites in homogenates of hearts chased with stearic acid were analyzed by thin-layer chromatography. A significant decrease in labelled phosphocholine was observed in hearts chased with 50 μM stearic acid (Table 5). No significant difference in the radioactivity associated with choline or CDP-choline was detected. These data suggest the activity of the rate-controlling enzyme CTP:phosphocholine cytidylyltransferase was increased in the presence

TABLE 4

Effect of fatty acids on phosphatidylcholine biosynthesis

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 10μM [Me-^{3}H]choline (10μCi/ml) for 30 minutes and subsequently chased for 30 minutes with 10μM choline and 0-50μM fatty acid. The hearts were homogenized after perfusion and the homogenates separated into two phases. The lower organic phases was analyzed by thin-layer chromatography for radioactivity in phosphatidylcholine.

	stearic	oleic	arachidonic
Concentration of fatty acid	dpm phosphatidylcholine x 10^{-7}/g heart		
0	0.82 ± 0.12	0.82 ± 0.12	0.82 ± 0.12
25μM	1.04 ± 0.18	0.93 ± 0.16	0.87 ± 0.13
50μM	1.08 ± 0.11*	0.95 ± 0.21	0.94 ± 0.20

Each value represents the mean ± standard deviation of three separate experiments

*$P<0.05$ when compared with value obtained from pefusion with no fatty acid.

TABLE 5

Effect of stearic acid on labelling of choline and choline metabolites.

The aqueous upper phase from Table 4 was analyzed by thin-layer chromatography for radioactivity in choline, phosphocholine and CDP-choline.

Concentration of stearic acid	choline (dpm x 10^{-6}/g heart)	phosphocholine (dpm x 10^{-7}/g heart)	CDP-choline (dpm x 10^{-6}/g heart)
0	0.73 ± 0.07	1.67 ± 0.21	0.48 ± 0.05
25μM	0.72 ± 0.14	1.45 ± 0.38	0.44 ± 0.05
50μM	0.67 ± 0.10	1.36 ± 0.11*	0.41 ± 0.07

Each value represents the mean ± standard deviation of three separate experiments.

*$P<0.05$ when compared with value obtained from perfusion with no fatty acid.

of 50 μM stearic acid. The increase in enzyme activity would result in a faster turnover of the phosphocholine pool and consequently a decrease in the amount of radioactivity associated with it.

b) *In vitro enzyme activities involved in phosphatidylcholine biosynthesis*

i) *CTP:phosphocholine cytidylyltransferase*

The cytosolic and microsomal fractions were prepared and assayed for CTP:phosphocholine cytidylyltransferase activity as described in "Materials and Methods". Enzyme activity was measured in the absence and presence of 25 μM and 50 μM stearic acid. Both the cytosolic and microsomal fractions exhibited CTP:phosphocholine cytidylyltransferase activity with 60% of cellular activity residing in the microsomal fraction and 40% of cellular activity residing in the cytosolic fraction. No difference in enzyme activity was detected in either the microsomal or cytosolic fraction when 25 μM and 50 μM stearic acid was added *in vitro* to the reaction mixture (Table 6).

Hamster hearts were perfused in the absence and in the presence of 50 μM stearic acid for 30 min. The subcellular fractions were prepared and assayed for CTP:phosphocholine cytidylyltransferase activity as before. The assay of cytosolic and microsomal fractions prepared from hearts perfused without stearic acid revealed a 60% microsomal-40% cytosolic distribution of enzyme activity. However, an enhancement of microsomal activity was observed when fractions prepared from hearts perfused with 50 μM stearic acid were assayed.

TABLE 6

In vitro CTP:phosphocholine cytidylyltransferase activity

Hamster hearts were homogenized in 0.145M NaCl and subcellular fractions prepared by differential centrifugation. Microsomal and cytosolic CTP: phosphocholine cytidylyltransferase activity was assayed *in vitro* in the presence of 0-50μM stearic acid as described in "Materials and Methods".

Concentration of stearic acid	cytosolic activity (nmol/min/mg protein)	microsomal activity
0	0.54 ± 0.12	0.73 ± 0.08
25μM	0.51 ± 0.04	0.82 ± 0.11
50μM	0.55 ± 0.10	0.77 ± 0.14

Each value represents the mean ± standard deviation of three separate experiments.

No change in cytosolic activity was detected (Table 7).

ii) CDP-choline:1,2-diacylglycerol cholinephosphotransferase

Sribney and Lyman (1973) reported that liver cholinephosphotransferase activity was stimulated in the presence of fatty acids *in vitro*. In order to ascertain whether a similar phenomenon exists with the hamster heart enzyme, hamster heart microsomes were prepared and cholinephophotransferase activity was assayed as detailed in "Materials and Methods". The addition of 10 μM fatty acid to the assay medium did not significantly affect the activity of the enzyme (Table 8). Cholinephosphotransferase activity was not assayed at higher fatty acid concentrations because of the limited solubility of the 1:2-diacylglycerol substrate. An increase in fatty acid concentration would effectively compete with 1:2-diacylglycerol for the solubilizing action of the detergent Tween 20.

iii) Acylation of lysophosphatidylcholine

Another route of incorporation of fatty acids into PC is through direct acylation of existing LPC. The deacylation-reacylation reactions first described by Lands (1960), were found to exhibit a certain degree of acyl specificity. Hence, the acyl specificity of this PC synthesizing system was investigated in hamster heart microsomes. The *in vitro* production of labelled PC from free fatty acids and [1-^{14}C]palmitoyl LPC was used to monitor the acylation process. After incubation the reaction mixture was subjected to a two-phase extraction and PC in the organic phase was separated by

TABLE 7

In vitro CTP:phosphocholine cytidylyltransferase from hamster heart perfused with stearic acid.

Isolated hamster hearts were perfused with Kreb's-Henseleit buffer containing 50μM stearic acid for 30 minutes. The hearts were then homogenized and sub-cellular fractions prepared and assayed for activity as detailed in "Materials and Methods".

Concentration of stearic acid	cytosolic activity (nmol/min/mg protein)	microsomal activity (nmol/min/mg protein)
0	0.61 ± 0.18	0.93 ± 0.24
50μM	0.57 ± 0.21	1.33 ± 0.16*

Each value represents the means ± standard deviation of four separate experiments.

*$P<0.05$

TABLE 8

Effect of fatty acids on cholinephosphotransferase activity.
Hamster heart microsomes were prepared and assayed for cholinephosphotransferase activity *in vitro* in the presence of 10μM fatty acid as described in "Materials and Methods".

Fatty acid	Enzyme activity (nmole/min/mg protein)
Control (no fatty acid)	1.43 ± 0.26
Stearic	1.69 ± 0.14
Oleic	1.60 ± 0.21
Arachidonic	1.66 ± 0.27

Each point represents the mean ± standard deviation of three separate experiments.

thin-layer chromatography using a solvent system containing chloroform/methanol/water/acetic acid (70/30/4/2; v/v). The PC fraction was scraped into a scintillation vial and analyzed for associated radioactivity. As depicted in Figure 20, stearic acid represented a relatively poor substrate for LPC reacyltion. Arachidonic acid, and to a lesser extent, oleic acid, were relatively good substrates for PC formation via reacylation of LPC. These results indicate that unsaturated fatty acids are preferred substrates for LPC reacylation in the hamster heart. The importance of phospholipid deacylation-reacylation in the remodelling of fatty acyl groups in newly synthesized PC in hamster heart (Arthur and Choy, 1984), suggests that this mechanism represents a significant route of incorporation of some fatty acids into PC.

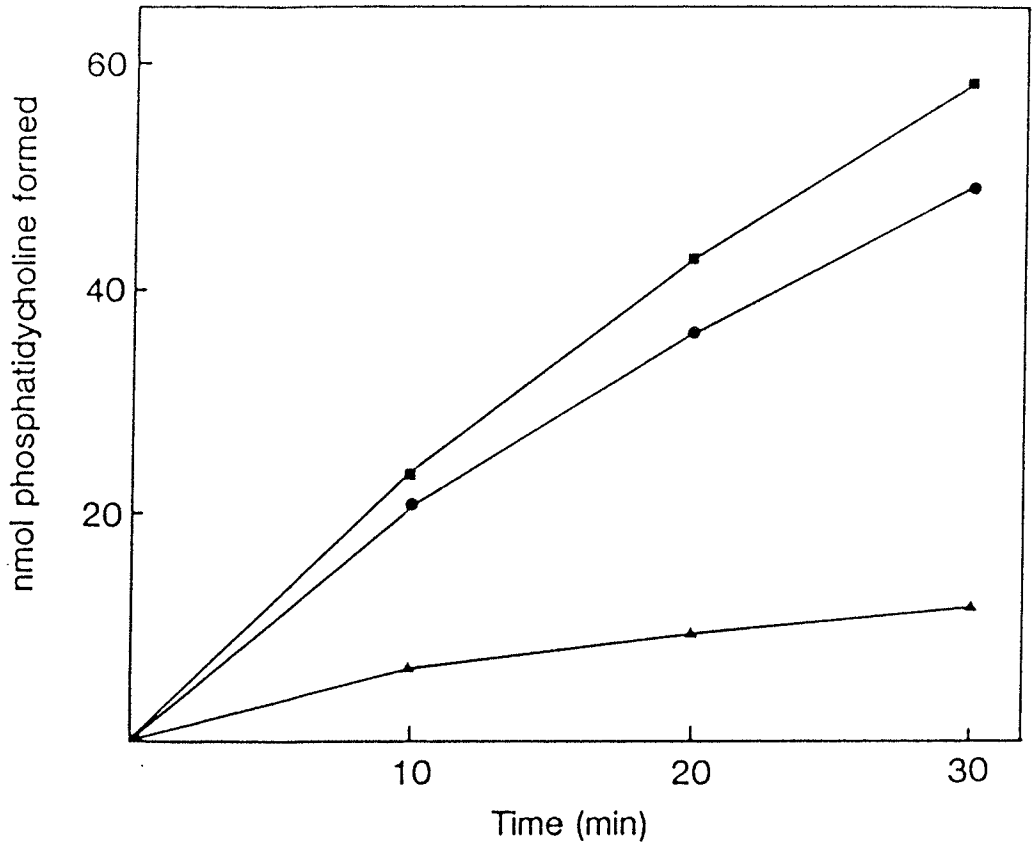


Fig. 20. The in vitro production of phosphatidylcholine from [1-^{14}C]palmitoyl lysophosphatidylcholine and free fatty acids.

Hamster heart microsomes were prepared and assayed for the incorporation of stearic acid (▲), oleic acid (●) and arachidonic acid (■) into [1-^{14}C]palmitoyl lysophosphatidylcholine forming phosphatidylcholine as described in "Materials and Methods". Each point represents the mean of two separate experiments.

DISCUSSION

I EXTRACTION AND STORAGE OF CARDIAC LIPIDS

Recently, considerable attention has been focussed on the role of LPC in the pathogenesis of cardiac arrhythmias (Sobel et al., 1979, Katz and Messineo, 1981, Man and Choy, 1982). These authors propose that myocardial ischemia leads to an accumulation of cardiac LPC which produces the electrophysiological changes associated with ischemia. These electrophysiological changes seen in the ischemic myocardium have been attributed to the cytolytic and membrane-perturbing properties of LPC described by Weltzien (1978). A positive correlation between LPC levels and the pathogenesis of cardiac arrhythmias is therefore dependent upon the accurate quantitation of LPC in the tissue before and after the onset of ischemia. Sobel et al. (1978), using the acid-butanol extraction scheme of Bjerve et al (1974), reported ischemic rabbit myocardium LPC levels which were 20-fold higher than those reported by Shaikh and Downar (1981). The elevated levels of LPC reported by Sobel et al was attributed to the artifactual production of LPC by the acid-catalyzed hydrolysis of vinyl ether linked phospholipids (plasmalogens). In order to evaluate the efficiency and accuracy in cardiac lipid extraction only neutral solvents were used in this study so as to eliminate artifactual production of LPC. The solvent mixture containing chloroform/methanol (1/2; v/v) was found to the most effective and efficient solvent in the extraction of cardiac LPC (Table 1). In addition, no intrapreparative loss of PC was detected when lipid extraction was conducted with this solvent.

Storage of lipid extracts in chloroform/methanol/water (86/14/1; v/v) resulted in an elevation in LPC levels, while storage in chloroform/methanol (2/1; v/v) or without solvent under nitrogen did not result in alterations in LPC levels (Figure 5). The increase in LPC levels observed upon storage of the lipid extract in chloroform/methanol/water (86/14/1; v/v) was attributed to hydrolysis of parent PC during storage. Dodge and Phillips (1965), found elevations in lipid-phosphorus levels associated with the LPC fraction when dry lipid extracts were stored at -25° for 20 days. Upon further analysis they found that auto-oxidation of PC and PE accounted for the increase in lipid-phosphorus levels found in the LPC fraction. It was found that auto-oxidation of PC and PE generated phospholipid species whose Rf values corrresponded to those of LPC when subjected to thin-layer chromatography, and hence and increase in lipid-phosphorus levels in the LPC fraction was produced. In view of this observation all storage vessels were thoroughly flushed with nitrogen prior to the addition of the lipid samples in this study. As storage of lipid extracts prior to analysis was often required in the present study, lipid extracts were stored in chloroform/methanol (2/1; v/v) so as to minimize any alterations in phospholipid levels and allow for accurate analysis of the lipid extract.

II THE UPTAKE AND INCORPORATION OF EXOGENOUS FREE FATTY ACIDS INTO PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE

Although the oxidation of fatty acids in mammalian hearts has been extensively studied, the incorporation of fatty acids into the

cardiac phospholipids has not received the same attention. Whereas phospholipids provide the structural framework of the cardiac membrane, the incorporation of fatty acids with the appropriate acyl group into the cardiac phospholipids is of extreme importance in the maintenance of membrane integrity and proper function. Hence, this study was directed to assess the relationship between fatty acid structure and its uptake and metabolism in the mammalian heart. Stearic, oleic and arachidonic acid were used in this study because of their widespread occurrence in cardiac phospholipids (White, 1973), and because they are representative of saturated, monounsaturated and polyunsaturated fatty acids respectively. However, the relative content and distribution among phospholipids in the heart varies with each fatty acid. It is therefore highly conceivable that the chain length and the degree of unsaturation exhibited by these fatty acids are of major importance in the incorporation characteristics.

A prerequisite for this study is the development of a model where conditions that most closely resemble those in vivo can be produced. Normal cardiac metabolism can only be realized in an intact tissue because of the requirement for propagation of the electrical signal to produce the characteristic beating of the heart. The isolated hamster heart perfused with Kreb's-Henseleit buffer (Kreb's and Henseleit, 1932) in the Langendorff Mode (Langendorff, 1895) provided a suitable model for this study. The viability of the hearts were maintained throughout all perfusion periods as suggested by electrocardiac recordings.

Uptake of fatty acid by the isolated perfused hamster heart was clearly demonstrated by perfusion with 50 μM [1-^{14}C]stearic acid and 50 μM [1-^{14}C]oleic acid (Figures 6 and 7). Maximum uptake occurred within 15 minutes with both stearic and oleic acid. This suggested that either the uptake mechanism had become saturated and could not accomodate additional fatty acid or the uptake was limited by intracellular utilization of the fatty acid. However, analysis of the perfusate before and after perfusion revealed that relatively little fatty acid remained in the perfusate after 15 minutes. It was therefore concluded at that perfusate fatty acid concentration the isolated hamster heart effectively extracted the majority of fatty acid supplied to it in the perfusate. These data indicate that the hamster heart possesses an highly efficient mechanism for fatty acid uptake.

Analysis of phosphatidylcholine and phosphatidylethanolamine revealed active incorporation of [1-^{14}C]stearic and [1-^{14}C]oleic acid which was found to be time-dependent (Figures 8 and 9). A substantial amount of radioactivity was incorporated into these phospholipids after only 3 minutes. On the other hand Zelinski *et al.* (1980), found a lag period of approximately 15 minutes for incorporation of [Me-^{3}H]choline into PC in the isolated hamster heart. Although both compounds are precursors for PC, their different rates of incorporation into this phospholipid suggests that their incorporation is subject to different modes of regulation. It can also be argued that exogenous stearic and oleic acid are more readily available for PC synthesis because they enter the required fatty acid pool quicker

than choline enters its respective pool. Incorporation of oleic acid into both PC and PE exceeded that of stearic acid indicating that oleic acid represented a better substrate for acylation reactons than did stearic acid. Also of interest was the observation that the difference between PC and PE labelling was 3-fold with stearic acid but only 2-fold with oleic acid. This enhanced incorporation of oleic acid into PE may account for the higher relative content of oleic acid in PE reported in the mammalian heart (White, 1973).

Linearity of uptake was observed with 20-320 μM [1-^{14}C]stearic acid and [1-^{14}C]oleic acid and with 50-300 μM [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid at 15 minutes of perfusion (Figures 10, 11 and 12). Most noteworthy, however, was that arachidonic acid was not taken up by the isolated hamster heart to the same extent as either stearic or oleic acid. Similar observations were made in the rat heart (Evans et al., 1963, Evans, 1964) where a decrease in fatty acid uptake as chain length increased was reported. It is not known whether this present observation is due to an increased affinity of arachidonic acid for albumin or whether the uptake is limited by the solubility of arachidonic acid in the aqueous medium. A concentration-dependent incorporation of all three fatty acid was demonstrated where [1-^{14}C]oleic acid incorporation into PC and PE exceeded that of either [1-^{14}C]stearic acid or [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid. A 3-fold difference in labelling of PC and PE was observed with [1-^{14}C]stearic acid but only a 2-fold difference was detected with [1-^{14}C]oleic acid or [5,6,8,9,11,12,14,15-^{3}H]arachidonic

acid. These results indicate that fatty acid incorporation into PC and PE in the hamster heart is not a random event but rather regulated to some extent by the fatty acid species.

Pulse-chase analysis has proved to be a powerful biochemical tool in the study of metabolic phenomena. Determination of the rate and extent of radioactive flux amongst metabolites provides useful information concerning the fate of labelled compounds. Hence, pulse-chase studies on the metabolism of fatty acids in the isolated hamster heart were conducted. The majority of stearic acid taken up (85%) remained as free fatty acids; relatively little stearic acid was incorporated into triacylglycerols. In contrast, incorporation of oleic and arachidonic acids into triacylglycerols represented a major fate of the fatty acid taken up. Clearly, the hamster heart preferentially utilizes unsaturated fatty acids for triacylglycerol synthesis. Most likely, the discrimination against exogenous stearic acid in triacylglycerol synthesis occurs at the level of the acyl-transferases. These findings are in contradiction to those of Stein and Stein (1963), where equal incorporation of stearic and oleic acids in triacylglycerols in the isolated perfused rat heart was reported. The existence of different acyltransferases for triacylglycerol synthesis with different acyl specificities in the rat heart and the hamster heart is therefore a distinct possibility. Although oxidation of fatty acid is generally considered to be the major fate of fatty acid taken up by the rat heart under most conditions (Neely et al., 1972), this was not demonstrated in the hamster heart in the present study. This apparent discrepancy could probably best be explained by

the different experimental conditions under which the perfusions were carried out. In this study a fatty acid concentration of 50 μM was used. On the other hand, perfuson of rat hearts were routinely conducted at fatty acid concentrations 10 times this amount, while dextrose concentrations were the same (5 mM). It could be argued, then, that the hamster heart preferentially utilized carbohydrate for energy metabolism at the fatty acid concentration used. The decrease in radioactivity associated with the heart homogenates and free fatty acid fractions was found to be a result of passage of the labelled fatty acid or a labelled metabolite out of the cell and into the effluent. It is conceivable then, the the hamster heart actively exports compounds as a consequence of fatty acid metabolism.

A precursor-product relationship was established between 1:2-diacylglycerol and PC and PE by pulse-chase analysis with [1-^{14}C]stearic acid and [1-^{14}C]oleic acid (Figures 16 and 17). The disappearance of radioactivity in 1:2-diacylglycerol was temporally related to the appearance of radioactivity in PC and PE. Moreover, no alterations in the levels of these metabolites was detected (Table 2). These data indicate quite conclusively that exogenous stearic and oleic acid are incorporated into PC and PE via the CDP-choline and CDP-ethanolamine pathway respectively. These results are in agreement with those reported by Åkesson (1970) and Åkesson et al. (1970a, 1970b), who monitored the fate of intraportally injected fatty acids in the rat liver. These investigators contend that the asymmetric distribution of fatty acids found in phospholipids

is generated during de novo synthesis by selective utilization of fatty acyl CoA's. The difference in [1-^{14}C]stearic acid and [1-^{14}C]oleic acid incorporation into PC and PE observed in this study suggests a similar mechanism operates in the hamster heart. Arthur and Choy (1984) have recently shown that hamster heart CDP-choline:1,2-diacylglycerol cholinephosphotransferase exhibits only a slight preference for 1:2-diacylglycerols containing an unsaturated fatty acid at the C-2 postion in vivo. Because of the lack of specificity for 1:2-diacylglycerols exhibited by hamster heart cholinephosphotransferase, it can be concluded that the relative rates of stearic and oleic acid incorporation into PC and PE are, for the most part, dictated by glycerol-3-phosphate acyltransferase specificity.

A similar precursor-product relationship was not demonstrated upon pulse-chase analysis with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, which suggested that arachidonic acid may have been incorporated into PC and PE by a route other than the de novo synthetic pathway. Indeed, in vitro analysis of hamster heart microsomes demonstrated that arachidonic acid was the preferred substrate for LPC-acylation when compared with stearic acid and , to a lesser extent, oleic acid (Figure 20). Similar studies were conducted with arachidonyl CoA in canine heart microsomes (Chien et al., 1984) and in rat lung microsomes (Holub et al., 1980) with comparable results. Deacylation-reacylation of phospholipids plays a major role in the remodelling of phospholipids (Arthur and Choy, 1984) and thus represent a facile mechanism for the incorporation of arachidonic acid into PC. Although

the participation of the de novo synthetic pathway for incorporation of arachidonic acid into PC and PE cannot be ruled out conclusively, the present data suggest that lysolipid acylation is responsible for the majority of arachidonic acid incorporated into PC and PE in the hamster heart. It is conceivable that oleic acid incorporation into PC and PE exceeded that of either stearic acid and arachidonic acid by virtue of the participation of the de novo synthetic pathway and also lysolipid acylation in the incorporation.

The foregoing study is the first systematic investigation into fatty acid incorporation into cardiac phospholipids. The results obtained indicate quite clearly that incorporation of exogenous fatty acids into phospholipids is not a random event but rather regulated to some extent by the fatty acid species. The different incorporation characteristics exhibited by the fatty acids studied signifies the importance of regulation of phospholipid biosynthesis in maintaining cardiac membrane integrity. It seems that the phospholipid biosynthetic machinery in the heart is controlled so as to ensure the appropriate fatty acyl content in membrane phospholipids.

III REGULATION OF DE NOVO PHOSPHATIDYLCHOLINE BIOSYNTHESIS BY FREE FATTY ACIDS

Free fatty acids have been shown to stimulate PC biosynthesis in isolated rat hepatocytes (Pelech et al., 1983). These authors postulate that the activity of CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in the CDP-choline

pathway, is enhanced as a result of translocation from the cytosol to the microsomes. The purpose of this study was to determine if a similar regulatory mechanism is present in the hamster heart. In view of the possible physiological and biochemical implications of this regulatory mechanism the use of *in situ* methodology would be the preferrred approach for this study. However, because of inherent technical difficulties with whole body experiments, the isolated perfused heart was chosen as the model for this investigation. Since the isolated heart lacks the innervation, hormonal control and the homeostatic regulation characteristic of the intact heart a postive correlation between the biochemistry of isolated and intact hearts cannot be drawn. The isolated perfused heart was nevertheless used because conditions which most closely resemble those *in vivo* may be obtained.

Isolated hamster hearts were pulse-labelled with 10 μM [Me-^{3}H]choline for 10 minutes and subsequently chased with unlabelled choline and 0 - 50 μM fatty acid for 30 minutes. Analysis of PC revealed a modest but significant increase in PC labelling when hearts were chased in the presence of 50 μM stearic acid (Table 4). Since CTP:phosphocholine cytidylyltransferase is the rate-controlling enzyme in the CDP-choline pathway in the hamster heart (Zelinski *et al.*, 1980), the increased choline flux through this pathway observed in this study was most likely due to activation of the cytidylyltransferase. Indeed, upon further analysis of choline-containing metabolites a significant decrease in radioactivity associated with phosphocholine was found (Table 5). Hence, the target

site for stearic acid activation was the rate-controlling enzyme CTP:phosphocholine cytidylyltransferase. Pelech et al. demonstrated a similar phenomenon in isolated rat hepatocytes cultured in 1 mM oleic acid. These investigators propose that fatty acids do not activate the enzyme directly but rather promote translocation of the enzyme from the cytosol to the microsomes thereby enhancing catalysis. However, assay of hamster heart CTP:phosphocholine cytidylyltransferase in the presence of 0-50 μM stearic acid in vitro did not result in an increase of microsome-associated activity (Table 6). On the other hand, an increase in microsomal activity was found when microsomes prepared from hamster hearts perfused with 50 μM stearic acid were assayed (Table 7). In contrast with observations made by Pelech et al. (1983) using isolated rat hepatocytes, no concomitant decrease in cytosolic activity was noted. The mechanism underlying the stimulation of activity is not known. These data suggest that under these experimental conditions, translocation of the cytidylyltransferase from the cytosol to the microsomes was likely not responsible for the increase in enzyme activity associated with the microsomes. Since fatty acid stimulation of CTP:phosphocholine cytidylyltransferase in the hamster heart is exclusively an in vivo phenomenon, it is highly possible that fatty acids will promote some other factor(s) present in intact cardiac cell which will cause the stimulation of microsome-associated enzyme activity.

Although in this study only stearic acid stimulated choline incorporation into PC, it would be erroneous to suggest that oleic

acid and arachidonic acid do not share similar properties. Perfusion with stearic acid did result in a marked increase in the free stearic acid content in the hamster heart, but no corresponding increase was observed with either oleic acid or arachidonic acid (Table 3). An increase in the free stearic acid content would explain the stimulation of [Me-^{3}H]choline incorporation into PC reported earlier. However, all these studies were conducted at a perfusate concentration of 50 μM fatty acid. It is not known if the free oleic and arachidonic acid content of the hamster heart would increase at a higher perfusate fatty acid concentration and correspondingly it is not known if stimulation of choline incorporation into PC would be manifested. Higher concentrations of fatty acid were not used in this study because of the deleterious effects of excess fatty acids on metabolic and mechanical function in the heart (Liedtke et al., 1978). A study with isolated cardiac myocytes in this respect would allow investigators to probe this problem more fully.

Sribney and Lyman (1973) reported activation of CDP-choline;1,2-diacylglycerol cholinephosphotransferase when assayed in the presence of fatty acids in vitro. These authors claim that fatty acids promote a conformational change in the enzyme which makes it more apt to accept the 1:2-diacylglycerol substrate thereby enhancing catalysis. It was not known, however, if hamster heart cholinephosphotransferase is also subject to fatty acid activation. In this study a fatty acid concentration of 10 μM did not cause a significant increase in cholinephosphotransferase activity when assayed in vitro (Table 8). However, the fatty acid concentration

used in this study was much lower than Sribney and Lyman used in their study (0.8 mM). It is therefore possible that fatty acid activation of the enzyme activity occurs only at higher fatty acid concentrations. This study was not pursued further because of the peculiar characteristics of detergent sensitivity and substrate specificity displayed by hamster heart cholinephosphotransferase (Arthur et al., 1984). Even if hamster heart choline phosphotransferase is shown to be activated by higher concentrations of fatty acids, such activation is expected to be of minor importance in regulating choline flux through the CDP-choline pathway. CTP:phosphocholine cytidylyltransferase, rather than cholinephosphotransferase, represents the rate-limiting enzyme in the CDP-choline pathway and therefore mitigates any biochemical or physiological significance of cholinephosphotransferase regulation.

IV CONCLUSION

From the foregoing study it is obvious that exogenous fatty acids are actively taken up and incorporated into PC and PE in the isolated hamster heart. However, the route of incorporation of exogenous fatty acids into PC and PE is dependent upon the fatty acid species. A precursor-product relationship between 1:2-diacylglycerol and PC and PE was demonstrated upon perfusion with stearic acid and oleic acid. This suggests that incorporation of stearic acid and oleic acid by the de novo synthetic pathway represents a major route of incorporation. A similar precursor-product relationship was not demonstrated between 1:2-diacylglycerol and PC and PE when isolated

hamster hearts were perfused with arachidonic acid. In vitro analysis did show that arachidonic acid was a preferred substrate for LPC acylation suggesting that the deacylation-reacylation pathway represented a major route for arachidonic acid incorporation.

It was also demonstated that stearic acid activated CTP:phosphocholine cytidylyltransferase activity in the hamster heart, and as a consequence, stimulated PC biosynthesis by the CDP-choline pathway. It is highly conceivable that free fatty acid-activation of the CDP-choline pathway represents a regulatory mechanism for PC synthesis in the hamster heart. A similar mechanism has been reported in isolated rat hepatocytes (Pelech et al., 1983). However, cellular PC levels may be more sensitive to fatty acid stimulation of the CDP-choline pathway in the liver than in the heart. It can be argued that because the liver actively exports PC as a component of lipoproteins, that measurable increases in PC levels can be realized as a result of fatty acid stimulation. The heart, on the other hand, synthesizes PC only for cardiac membrane synthesis, and therefore, actual increases in PC levels may not normally occur (Table 2). It can be argued that turnover of PC may serve to counter the increase in PC biosynthesis brought about by fatty acid stimulation of the CDP-choline pathway, and thereby maintain constant levels of PC. Nevertheless, if the phospholipid biosynthetic machinery in the hamster heart is sensitive to the free fatty acid levels, it would be expected that phospholipid biosynthesis can be regulated to some extent by serum levels of free fatty acids or free fatty acid precursors. A logical extension of

this research would involve studies directed towards characterization of abnormalities in phospholipid biosynthesis in a disease state such as cardiomyopathy. The possible regulatory function of free fatty acids in the disease state may then be defined.

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