Review-Hypothesis

Biosynthesis of acyl-specific glycerophospholipids in mammalian tissues

Postulation of new pathways

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A review of the literature concerning the synthesis of acyl-specific glycerophospholipids indicates that the known biosynthetic sequences cannot satisfactorily explain these specific acylations. New de novo and salvage pathways are proposed to account for the acyl composition of highly unsaturated and saturated glycerophospholipids. In these hypothetical pathways, de novo synthesized glycerophosphodiesters are postulated to be key intermediates to establish the specific acyl composition of the resulting glycerophospholipids, and to be integrated with the known cytidine pathways. A re-interpretation of the experimental literature in terms of these postulated pathways is provided, with some methods to test these proposed sequences.

Glycerophophodiester *Glycerophospholipid* *Retinal phospholipid* *Lung phosphatidylcholine* *Fatty acid* *Platelet-activating factor*

Abbreviations: P_i, orthophosphate; GP, glycerophosphate; P-choline, phosphorylcholine; P-ethanolamine, phosphorylethanolamine; CDP-choline, cytidinediphosphocholine; CDP-ethanolamine, cytidinediphosphoethanolamine; CDP-serine, cytidinediphosphoserine; CDP-glycerol, cytidinediphosphoglycerol; CDP-DG, cytidinediphosphodiglyceride; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; GPG, glycerophosphorylglycerol; GPS, glycerophosphorylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGGP, phosphatidylglyceroglycerophosphate; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; DG, *sn*-1,2-diglyceride; C18:0, octadecanoate; C18:2*n*–6, 9,12-octadecadienoate; C18:3*n*–3, 9,12,15-octadecatrienoate; C20:4*n*–6, 5,8,11,14-eicosatetraenoate; C22:5*n*–6, 4,7,10,13,16-docosapentaenoate; C22:6*n*–3, 4,7,10,13,16,19-docosahexanoate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine

1. INTRODUCTION

Glycerophospholipids are known to display a remarkable degree of molecular and metabolic heterogeneity in mammalian tissues. Collins [1] was the first to demonstrate the metabolic heterogeneity of glycerophospholipids more than 20 years ago. In studies with in vivo $^{32}P_i$ incorporation into rat liver phospholipids, he was able to show that the most unsaturated PC and PE species had the highest radiospecific activities. In accordance with these observations, later studies established non-random acyl incorporation into glycerophospholipids and the specific acyl composition of glycerophospholipids of various mammalian tissues and species [2–6]. Since that time, a great deal of research has been dedicated to understand the molecular mechanism whereby the specific acyl

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composition of glycerophospholipids is established. In spite of these efforts these mechanisms remain uncertain. This paper briefly reviews the present literature on the subject and proposes new pathways to explain the published data.

2. THE SEARCH FOR ACYL SPECIFICITY

2.1. *Highly unsaturated phospholipid species*

Phospholipids of nervous tissue, retina, testes, and muscle contain particularly high levels of C22:6*n*−3 or C22:5*n*−6 [7–9]. These observations motivated an active investigation to search for acyl transfer specificity in the known phospholipid biosynthetic pathways, namely the convergent PA [10–12] and cytidine pathways [12–15] which share the *sn*-1,2-diglycerides (DG) as intermediates for the synthesis of PC and PE; however, most of the research to answer this question has been done with liver tissue.

A search for similarities between PA, DG, PC and PE species in their fatty acid composition produced negative results [16,17], suggesting that the highly unsaturated acyl composition of PC and PE was not determined at the level of PA synthesis. Even in the retina where microsomal PA contains about 20% C22:6*n*−3 [18], the percent of this fatty acid in PC and PE is about twice as high [8,9,19], thus suggesting that there must be other pathways to account for the high levels of C22:6*n*−3 in PC and PE in this tissue. In addition, some of the molecular species of PC and PE contain C22:6*n*−3 in both positions of the GP backbone [20]. This unusual acyl composition has not been reported for any molecular species of PA. It is likely that the fairly high levels of C22:6*n*−3 found in retinal PA are a reflection of the acyl-CoA pool composition, which may be determined to a large extent by highly selective transport of C22:6*n*−3 from plasma lipoproteins [21]. This fatty acid is known to be exported from the liver in plasma lipoprotein phospholipids [22].

In view of the above results, several investigations were initiated to determine whether the DG specificities of the phosphorylcholinetransferase (EC 2.7.8.2) and phosphorylethanolaminetransferase (EC 2.7.8.1) were enough to account for the acyl composition of the various molecular species of these phospholipids in vivo. Results obtained with rat liver PC and PE indicated that the phosphorylcholinetransferase had no specificity for the DG species [23–25] while the phosphorylethanolaminetransferase had a modest specificity towards the C22:6*n*−3-containing diglycerides [24,26,27], which only partially accounts for the high levels of C22:6*n*−3 in the *sn*-2 position of rat liver PE. Similar results were obtained with brain microsomes [28]. These findings led Hill and Lands [29] to suggest that the in vivo specific fatty acid acylations might occur by acyltransferase reactions with monoacyl derivatives [30,31]. However, experiments to test this hypothesis failed to show the putative specificities in rat liver PC [29, 32,33] and PS [34]. In view of the above negative results some investigators tested the hypothesis of catabolic specificity, i.e., that phospholipases A_1 (EC 3.1.1.32) and A_2 (EC 3.1.1.4) would show high selectivity toward phospholipid species with the least unsaturated acyl moieties in the *sn*-2 position. This would therefore lead to a relative abundance of highly unsaturated species. Somewhat consistent with this hypothesis, Waite and Sisson [35] showed a higher rate of hydrolysis of C18:2*n*−6 over C20:4*n*−6 by a purified rat liver mitochondrial phospholipase A_2. However, the hydrolysis of C20:4*n*−6 was also higher than that of C18:3*n*−3. Other reports failed to show any acyl specificity for the phospholipase A_2 hydrolysis of PC and PE [36,37], therefore the biological role of this phospholipase in terms of the above hypothesis is doubtful.

2.2. *Highly saturated phosphatidylcholine species*

In lung alveolar tissue, more than 30% of the phosphatidylcholines are of the dipalmitoyl class [38]. This disaturated PC has unique surface tension-lowering properties and is the major functional component of the alveolar surfactant [39]. Evidence suggests that this PC is synthesized in the type II alveolar cells, and is stored in lamellar bodies which are secreted into the alveolar cells. There it is assembled with other components into a pulmonary surfactant which is then secreted onto the alveolar surface. A major role of this surfactant is to inhibit alveolar collapse at low transpulmonary pressures (review [40,41]).

As in the case of the highly unsaturated PC and PE species, studies with the phosphorylcholinetransferase from type II pneumocytes indicated that the cytidine pathway lacked the necessary spe-

cificity to form the dipalmitoyl-PC species [42,43]. A search for specificity in remodeling pathways has suggested that acylation of the lyso derivatives can partially account for some of the biosynthesis of dipalmitoyl-PC species, together with a preponderance of palmitoyl-CoA in the acyl-CoA pool [44–46]. The lysophospholipase transacylase (EC 3.1.1.5), on the other hand, shows no specificity [47,48]. However, incubations with type II pneumocyte sonicates with CDP-[^{14}C]choline resulted in more than half of the label being incorporated into disaturated PC species [49]. So it seems that the choline moiety of the CDP-choline may participate in an acyl-specific pathway for the synthesis of PC. Thus, a de novo pathway for the synthesis of the dipalmitoyl-PC must exist in addition to the remodelling pathway.

2.3. *Platelet-activating factor*

Another unusual PC species for which the biosynthetic pathway is also unknown is 1-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine, also known as platelet-activating factor (PAF). This alkyl phopholipid has been reported to exhibit powerful anti-hypertensive activity [50], and appears to be a potent platelet-activating factor [51]. A phosphorylcholinetransferase from various tissues has been reported to use the 1-alkyl-2-acetyl-*sn*-glycerol as the phosphorylcholine acceptor from CDP-choline [52]. However, the reaction is inhibited by DG and the activity in the spleen seems too high for its physiological needs for PAF synthesis, and is similar to that of other tissues [9]. Thus it is unlikely that the cytidine pathway plays any role in the determination of specificity for the synthesis of PAF.

As in the case for other phosphatidylcholine species, the search for acyl specificity in the synthesis of PAF has also included the deacylation-reacylation pathway. Phospholipase A_2 activity on PAF and acetylation of the lyso-PAF residue has been reported [53,54]. However, this acetylation reaction is totally inhibited by even less than equimolar concentration of oleyl-CoA, indicating that this acyltransferase lacks the necessary specificity to synthesize PAF.

2.4. *Other glycerophospholipids*

Phosphatidylinositol (PI), CL, and PS also have specific fatty acid compositions which cannot be accounted for by the known pathways. The role of CDP-DG as a precursor of PI and CL is well accepted [55–57]. This liponucleotide can be formed from PA via the enzyme CTP:phosphatidate cytidylyltransferase (EC 2.7.7.41). The PA moiety is then esterified to inositol by CDP-diglyceride:inositol phosphatidyltransferase (EC 2.7.8.11). This metabolic sequence predicts a similar acyl composition of PA, CDP-diglyceride, and PI. In vivo studies reported by Thompson and MacDonald [58], however, have shown that the high level of arachidonate found in CDP-DG and PI is not found in the total pool of PA. These authors have also indicated that the fatty acid profile of CL is very different from that of CDP-diglyceride and PA.

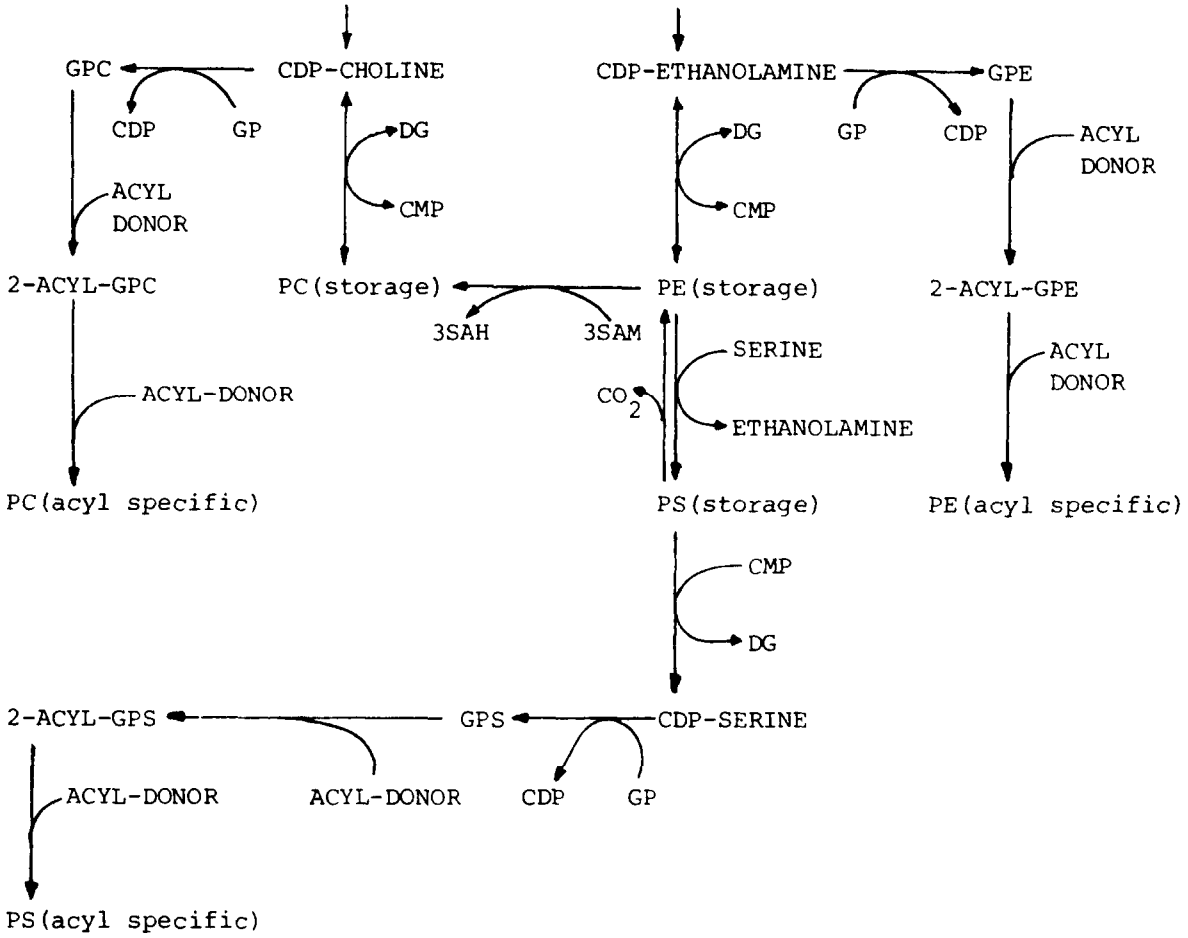
Among several hypotheses proposed to explain these discrepancies in precursor-product acyl composition [58], in vivo and in vitro searches for acyl selectivity of the phosphatidate cytidylyltransferase have produced disappointing results [59,60]. Although initial studies to search for a deacylation-reacylation cycle of CDP-diglyceride were encouraging and showed the presence of an enzyme catalyzing the acylation of CDP-monoacylglycerol by arachidonyl-CoA [61], the identity of the CDP-diglyceride product seems uncertain (Thompson, W., personal communication). Therefore, the reactions responsible for the specific acyl composition of PI remain unknown. A similar case is that for the acyl specificity of CL [58]. In respect to the specific fatty acid composition of PS, I have been unable to find any hypotheses or experimental data which attempt to explain the highly unsaturated fatty acid composition of this glycerophospholipid, except for the data of Holub [34].

3. PROPOSAL OF NEW PATHWAYS

In view of the insufficient specificities of the known pathways to synthesize highly acyl-specific glycerophospholipids it is necessary to postulate new pathways.

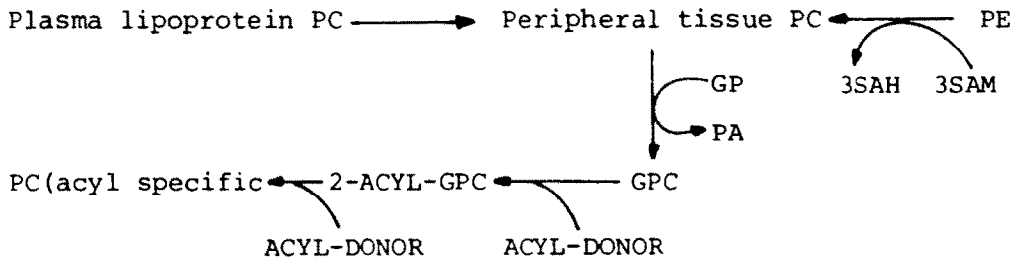
3.1. *De novo biosynthetic pathways*

Three de novo new pathways are proposed for the acyl-specific synthesis of PC, PE, PI, PS, PG and Cl species (schemes 1–3). The hypothetical reactions depicted are the minimum number of steps required, but the actual pathways may have



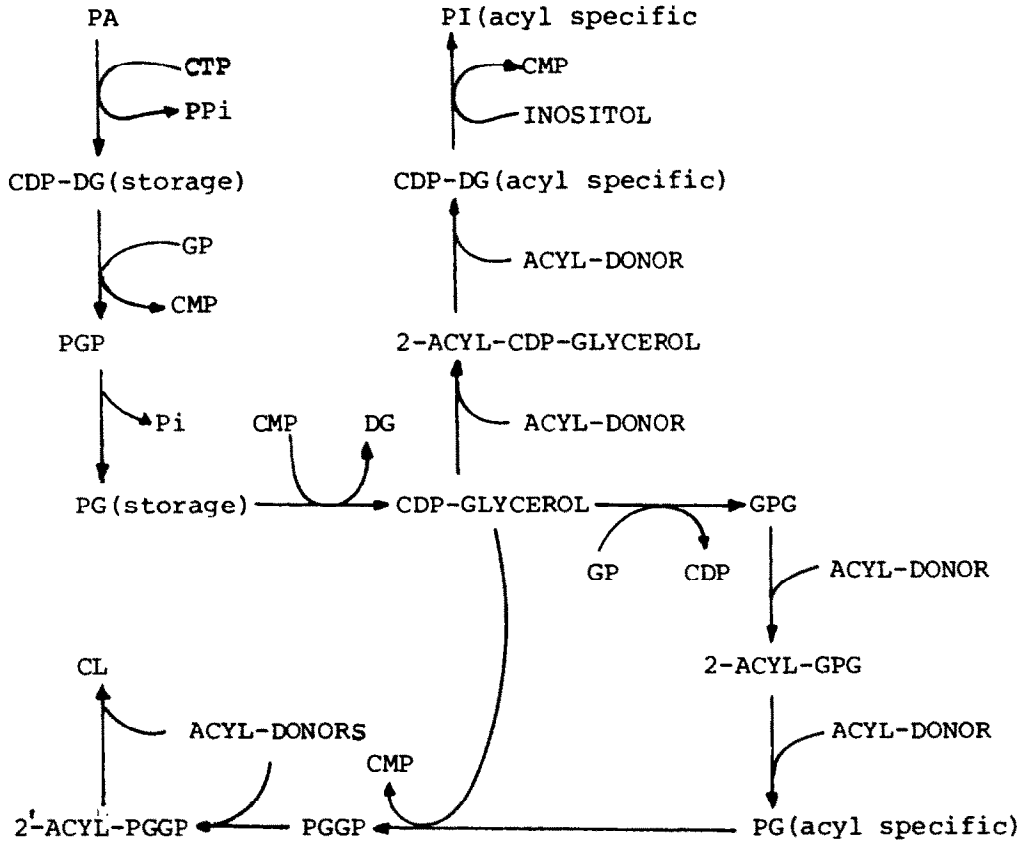
Scheme 1. Terminal reactions of the cytidine pathways, PE methylation, PS decarboxylation and serine exchange sequences, and proposed de novo pathways for the biosynthesis of acyl-specific PC, PE and PS.

more intermediates. In the conceptual development of the proposed pathways, the following design features are included. First, in tissues which have the cytidine pathways these are integrated into the proposed biosynthetic pathways as sources of bases or alcohols, i.e., choline, ethanolamine, inositol, and glycerol, which are esterified by GP to form the corresponding GP derivatives. The cytidine pathways are proposed to have a primary role in the salvage, storage, and conservation of phospholipid bases or alcohols. These cytidine pathways may also have a role in short-term storage of essential fatty acid precursors, such as C18:2*n*–6 and C18:3*n*–3 acids. Second, the de novo pathways may also be integrated with plasma lipoprotein phospholipids in certain tissues (scheme 2). These lipoproteins are assumed to transport phospholipid bases and essential fatty acids (both dietary precursors and desaturated and elongated metabolites) from the liver to peripheral tissues such as muscle, testes, brain, and retina. This is particularly important for these highly specialized tissues, which may have little or no activity of the storage-cytidine pathways. Third, the esterification of the phospholipid bases to GP to form glycerophosphodiesters has a central role in establishing the specific acyl and alkyl composition of the final phospholipid species, except for that of PI. The postulated glycerophosphodiesters circumvent the need for common acylated metabolites or intermediates, such as DG or CDP-DG, for the synthesis of specific phospholipid species. The acyl



Scheme 2. Proposed sequences for the synthesis of acyl-specific glycerophospholipids from plasma lipoprotein glycerophospholipids, with PC depicted as an example.

specificity is postulated to be achieved by two specific acyl transferases or one acyl and one alkyl transferase, which would have recognition sites for both acyl or alkyl donors and acceptors. The first acyl transferase would recognize the glycerophosphodiester and acylate the *sn*-2 position, which is the position found to be most specific for its acyl moiety. A second acyl or alkyl transferase would then recognize the 2-acyl-*sn*-glycerophosphodiester and acylate or alkylate the *sn*-1 position, forming the final product. Fourth, the phosphoryl group in the final glycerophospholipid is postulated to



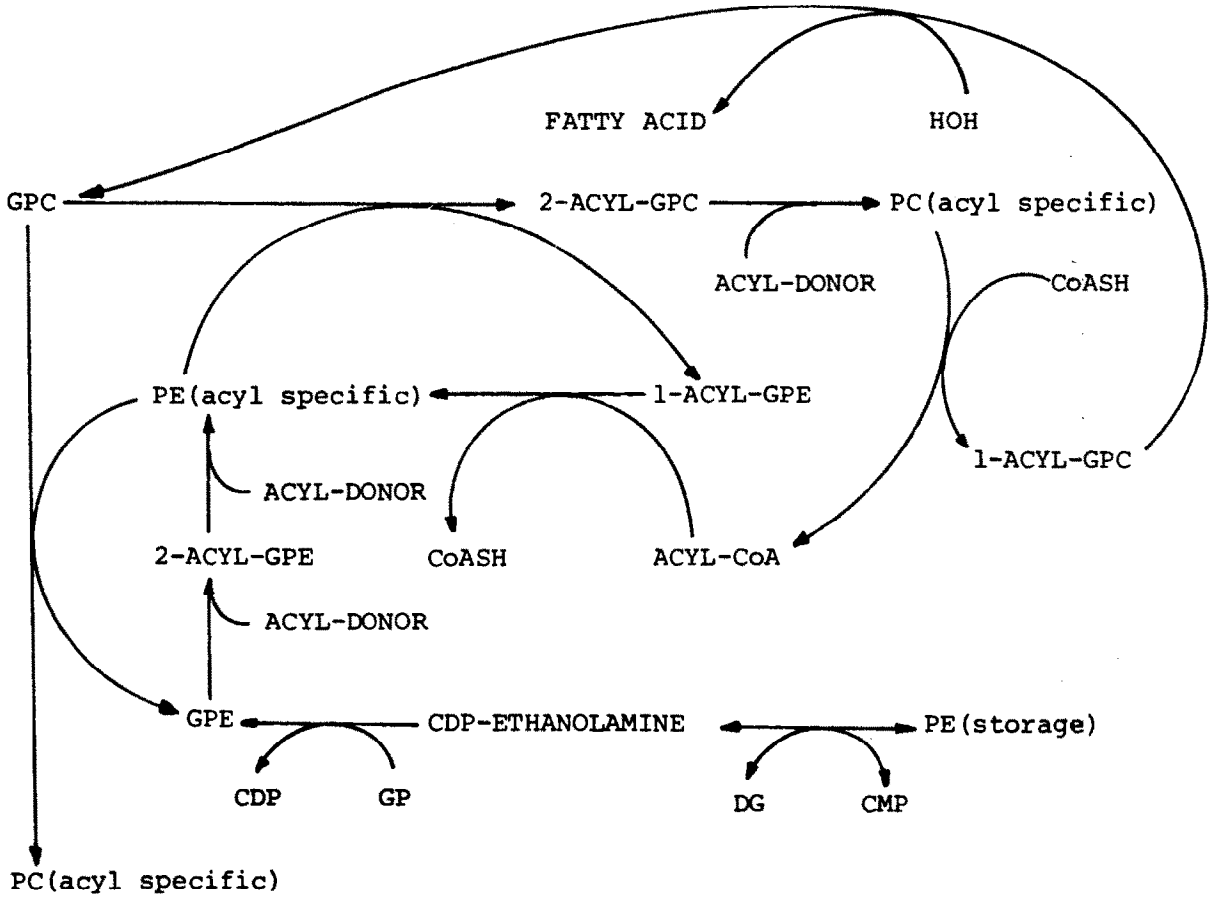
Scheme 3. Proposed pathways for the biosynthesis of acyl-specific PG and PI, and their integration with the storage, liponucleotide sequence.

originate from GP and not from the phosphorylated base, as is the case for the cytidine pathways for PC and PE synthesis. The GP is therefore incorporated as a unit, and this could be tested by a GP incorporation experiment with double-labeled GP. Consequently, when the CDP-esters are integrated with the proposed de novo pathways they do not donate the phosphorylated base as a unit but are only base donors for GP.

The biosynthesis of PI is proposed to be achieved by an acyl-specific CDP-DG which transfers its PA moiety to inositol. This CDP-DG is postulated to be synthesized by two specific acylations of CDP-glycerol (scheme 3). The synthesis of acyl-specific PG is proposed to proceed as for the other phospholipids, by specific acylations of its glycerophosphodiester precursor. Cardiolipin, however, is postulated to be synthesized from an acyl-specific PG via a transfer of GP from CDP-glycerol to this PG in a reaction analogous to that of phosphorylcholinetransferase. The PGGP intermediate would then be specifically acylated to form the final CL product (scheme 3). In these hypothetical pathways, PI, PG and CL do not share any common acylated intermediates.

3.2. *Glycerophosphodiester salvage pathways*

Dawson [62] has shown that the catabolism of phospholipids involves the formation of glycerophosphodiesters. This occurs by the combined ac-



Scheme 4. Proposed glycerophosphodiester recycling pathway (with GPC as an example), and fatty acid (*sn*-2 acyl moiety) recycling pathway, integrated with the proposed de novo and salvage GPE sequence.

tion of phospholipases A_1 or A_2, and lysophospholipases A_1 or A_2. The resulting glycerophosphodiesters may be further hydrolyzed to GP and free base by the corresponding glycerophosphodiesterases [63]. I postulate that in conditions of metabolic stress, such as in choline deficiency, the catabolism of acyl-specific phospholipids is decreased, and the glycerophosphodiesterase activities are decreased, to allow an increased molar fraction of the catabolic phosphodiesters to be reutilized or recycled to form specific glycerophospholipids. Tissues which have low activities of the base conservation-storage pathways (i.e., the cytidine pathways), or are devoid of them, should have very low phosphodiesterase activities. This would allow for a high flux of reutilization of glycerophosphodiesters.

Two salvage pathways are proposed (scheme 4), particularly for GPC, which includes a recycling of C22:6*n*−3 from PC turnover by phospholipases. In these proposed salvage pathways, part of the PE pool is considered to have a reservoir role for C22:6*n*−3, and other important fatty acids, for other glycerophospholipids such as PC. These fatty acids may be transferred from PE singly or as diglyceride units.

The glycerophosphodiester recycling pathway may be segregated from the de novo GPC pathway (which may be in a different organelle) to allow independent control of the glycerophosphodiesters according to their metabolic origin or location. An alternative way to control the catabolism of the phosphodiesters may entail the design of a high-K_m phosphodiesterase operating on a single pool of glycerophosphodiesters. Catabolic activity would be high only above a certain concentration of the particular phosphodiester. Unique fatty acids such as C22:6*n*−3 resulting from the phospholipase or lysophospholipase turnover of the functionally active glycerophospholipids, such as PC, would then be conserved by reacylation into PE via de novo or recycled GPE, or acylation of the 1-acyl-*sn*-glycerophosphorylethanolamine. In addition, dietary C22:6*n*−3 would be stored in PE via the cytidine salvage-conservation pathway through the somewhat C22:6*n*−3-specific phosphorylethanolaminetransferase reaction. These conservation pathways may be responsible for the tenacious retention of C22:6*n*−3 observed by Tinoco et al. [8] in C18:3*n*−3-deficient rats.

4. RE-INTERPRETATION OF LITERATURE DATA ACCORDING TO THE PROPOSED PATHWAYS

4.1. *Precursor-product studies*

The demonstration, in early studies, that GPC and GPE could be isolated from mammalian tissues [63–66] suggested the hypothesis that these glycerophosphodiesters might be intermediates in the synthesis of PC and PE, respectively. The availability of the analysis of precursor-product data from pulse-chase experiments, according to the criterion proposed by Zilversmit et al. [67], prompted its use to test the above hypothesis. Ansell and Norman [66], in experiments with rat brain, observed that after the intraperitoneal or intracisternal injection of $^{32}P_i$ the specific activity of brain GPE was always greater than that of PE during a 24-h chase. Similar results were obtained by in vitro incubations. The authors, however, concluded that a biosynthetic pathway involving GPE as a possible intermediate was unlikely to be a major pathway. They concluded this because the difference in specific activity between GPE and PE was not large enough considering the small pool size of GPE and the higher specific activity observed in P-ethanolamine. However, if the immediate precursors of P-ethanolamine and GPE are not the same, and the specific activity of the GPE precursor is lower than that of the P-ethanolamine precursor (scheme 1), then these data are compatible with the hypothesis that the proposed de novo pathway carries a large fraction of the biosynthetic flux for PE synthesis in this tissue. In this pathway the immediate ^{32}P-labelled precursor for GPE and GPC is GP, while that for phosphorylcholine and phosphorylethanolamine is ATP. The specific activity of GPE will be much lower than that of P-ethanolamine because most of the GP pool is derived from the glycolytic pathway [68]. The fairly large pool sizes of the 4 phosphorylated glycolytic intermediates between ATP and GP, plus several convergent and divergent branches, will greatly decrease the ^{32}P specific activity of GP with respect to P-ethanolamine. Therefore, when the specific activity of the total pool of PE is determined, as Ansell and Norman did, the molecular species of PE synthesized by the cytidine pathway will contribute more radioactivity to total PE than their share of the biosyn-

thetic flux. Since I propose that the de novo GPE pathway has a role to synthesize specific molecular species of PE, the high levels of C20:4*n*−6 and C22:6*n*−3 PE species formed in the brain [69] will require a high biosynthetic flux through the de novo GPE pathway, at least during growth and development. In the adult, the requirements for de novo synthesis may be less if they are mostly determined by slow turnover rates, which may utilize glycerophosphodiester recycling pathways.

Similar precursor-product experiments reported by Dawson [62] in rat liver failed to confirm the data for the brain reported by Ansell and Norman [66]. Rather, Dawson's data showed lower specific activities for GPE than PE, and thus was consistent with GPC and GPE being catabolic products of their respective glycerophospholipids. The inconsistency between the brain and liver experiments can readily be understood if one assumes that the biosynthetic flux of the cytidine pathway in the liver is much higher than that of the de novo GPC and GPE pathways, and that the catabolic flux of GPC and GPE in the liver is higher than that in the brain. When this is coupled with a higher ^{32}P specific activity of the cytidine-originated PC and PE than that originated by the de novo GPC and GPE pathways, the invisibility of the latter pathway by the pulse-chase method is expected when the specific activity of the total pools of PC and PE *vs* GPC and GPE are being measured. Reiner [70] has written a lucid discussion of other metabolic conditions which do not comply with the precursor-product criterion of Zilversmit et al. [67].

A condition which greatly decreases the flux of PC synthesis via the cytidine pathway, in the liver, is a choline-methionine deficient diet. Such a dietary manipulation can produce a nearly 5-fold decrease in the P-choline concentration in rat liver [71,72]. If choline kinase (EC 2.7.1.32) catalyzes the flux-generating step of this cytidine pathway [73–75], then a similar decrease in this biosynthetic flux to PC should occur. Since PC can also be synthesized via the sequential methylation of PE [76], a compensatory increase in the synthesis of PE occurs. This leads to its accumulation because the concomitant methionine deficiency impairs the methylation of PE to PC [77]. Most of the expansion of the PE pool is due to an increase in the C22:6*n*−3 species of PE [77]. Since there is a large increase in the intermediates of the cytidine pathway for PE synthesis in choline-methionine deficiency [72], it may be inferred that the increased flux through this pathway may be partially responsible for the higher levels of C22:6*n*−3 PE. This is possible because the phosphorylethanolaminetransferase has some specificity for the hexaenoic diglycerides [24,26,27]. More significant, however, is a 3-fold increase in GPE concentrations seen in choline-methionine deficiency [72]. In agreement with the earlier report of Dawson [62], pulse-chase studies with [^{14}C]ethanolamine with the choline-methionine sufficient animals showed lower specific activity of GPE when compared to that of PE at all times of the chase [72]. In the choline-methionine deficient animals, however, the specific activity of GPE was higher than that of PE in the early chase times. These data support the proposed pathway whereby GPE is a precursor of PE. I postulate that the major reason for the increased flux through the PE-cytidine pathway is to provide CDP-ethanolamine for the de novo acyl-specific GPE pathway (scheme 1). I suggest that in the choline-methionine deficient animals the GPE pathway becomes visible by the pulse-chase technique because of its greatly increased flux. This hypothesis is further consistent with the fact that the C22:6*n*−3 PE species account for most of the increased biosynthetic flux, since the proposed GPE pathway is largely responsible for the synthesis of this highly unsaturated PE species.

4.2. *GPC-recycling and fatty acid salvage-conservation pathways*

I suggest that an additional reason for the increase in the de novo GPE pathway in the choline-methionine deficient state is to activate the GPC recycling pathway (scheme 4). By this mechanism, the catabolically originated GPC (the flux of which should also be decreased by the deficiency) is salvaged by re-acylation by the C22:6*n*−3 PE. In addition, the GPC phosphodiesterase activity should be decreased by the deficiency to maximize GPC's reutilization. The postulation of a decreased catabolic production of GPC, concomitant with a decrease in GPC phosphodiesterase in the deficient state, is consistent with the observation that the liver pool size of GPC in the deficient animals is not significantly different from that of the control animals [72]. This hypothesis predicts

that a pulse-chase study with $^{32}P_i$ in choline-methionine deficient animals should show a precursor-product relationship for the GPC-PC pair, since the cytidine pathway and catabolic production of GPC should be drastically decreased by this dietary condition. The GPC-recycling pathway should also become visible by the pulse-chase technique under this dietary deficiency.

There are other data consistent with the decreased catabolic production and increased reutilization of GPC in the choline-methionine deficient state. GPC is known to be present in high concentrations in sheep liver [78], which also has extremely low GPC phosphodiesterase (EC 3.1.4.2) activity [79], and very low choline oxidation rates as well [80]. Moreover, dietary choline is almost totally degraded in the sheep rumen by microbial demethylation [81], which is consistent with the finding that long-term feeding of labelled choline produced only insignificant labelling of plasma PC [82]. Therefore the sheep, and perhaps other ruminants as well, may normally have a functional dietary choline deficiency. I postulate that, in addition to the high flux of PE methylation observed in the liver of this species [80], the sheep does not develop signs of choline deficiency because it has a high flux of the de novo acyl-specific GPC pathway and uses the PC synthesized from PE methylation as a choline donor for GP (scheme 2), and that the sheep also has a high flux of GPC recycling (scheme 4). Therefore, the sheep liver should be a very good tool to study the de novo GPC pathway, as well as the GPC salvage pathways.

Data consistent with the proposed conservation-salvage pathway of C22:6*n*−3 (scheme 4) are reported by Tinoco et al. [83]. These authors showed that hexaenoic PE incorporated and lost labelled ethanolamine rapidly. Despite a small proportion of C22:6*n*−3 in the diglycerides, the rat livers incorporated a large fraction of the label into C22:6*n*−3 PE species. Estimations of the rate of loss of radioactivity in the C22:6*n*−3 PE and its appearance in the C22:6*n*−3 PC indicated that the docosahexaenoic acid is conserved, possibly by reincorporation into PE after becoming part of the hexaenoic PC species. The tenacious conservation of C22:6*n*−3 in C18:3*n*−3-deficient rats [8] is consistent with the proposed conservation schemes.

4.3. *Connections with the cytidine pathways*

The cytidine sequences for PC and PE synthesis have unusual thermodynamic profiles and associated kinetic properties. The first two reactions, catalyzed by the kinases and cytidylyltransferases, are far from equilibrium, while the last transfer of the phosphorylated base to the diglyceride is near equilibrium [73]. This reaction lacks the far-from-equilibrium directional force normally seen in the final reaction of biosynthetic pathways. Computer simulations with a thermodynamic design of this type show a kinetic behavior which tends to maintain a constant concentration of the cytidine intermediates, i.e., CDP-choline and CDP-ethanolamine (unpublished). Consistent with this kinetic property is the fact that the liver pool size of CDP-choline is by far the least affected by a choline-methionine deficient diet [71,72,84]. In addition, when rat hepatocytes are subjected to increasing concentrations of ethanolamine, they maintain a nearly constant CDP-ethanolamine concentration, whereas the pool sizes of ethanolamine and P-ethanolamine are drastically increased [85]. Simulation studies of the cytidine pathway for PC synthesis, at various levels of choline, show that at normal cellular concentrations of choline the pool size of CDP-choline is maintained by a forward net flux through the two far-from-equilibrium reactions catalyzed by choline kinase and the corresponding cytidylyltransferase (EC 2.7.7.15). In the choline-methionine deficient state, however, the level of CDP-choline is maintained by a net reverse flux of the phosphorylcholinetransferase, with the far-from-equilibrium condition of the first two steps assuring that the net flux is reversed only up to the CDP-choline intermediate (unpublished). I conclude that CDP-choline and CDP-ethanolamine must have fundamental metabolic roles other than being only intermediates of the cytidine pathway. I suggest that the PC and PE species of the cytidine pathway may act as reservoirs of choline or ethanolamine units to form the donor species (CDP-choline or CDP-ethanolamine) for the de novo GPC and GPE pathways (scheme 1). The cytidine PC and PE species may act as reservoirs of dietary essential fatty acids as well.

Since the phosphorylcholinetransferase reaction is near equilibrium, the flux will be very sensitive to changes in the mass action ratio of the reactants involved [73]. A rise in cytosolic CMP will increase

the net reverse flux in the direction of CDP-choline synthesis, which is then channelled into the GPC pathway. Quirk et al. [86] have studied the temporal relationships between the various lung developmental changes and the pool sizes of several cytidine metabolites in rabbit lung. Up to day 26 of gestation there is an increased concentration of CDP-choline coupled with a decrease in the size of the CTP pool, while the CMP concentration remains relatively constant. From day 26 to adulthood there is a drastic decrease in CDP-choline coupled with a 4-fold increase in CMP concentration, whereas the level of CTP remains relatively constant. I propose that these changes are due to an increase in the forward net flux of the cytidine pathway as a temporary storage of choline up to about day 26 of gestation. After this time, the specific GPC pathway for the synthesis of dipalmitoyl-PC surfactant is turned on using CDP-choline as a choline donor for GP (scheme 1). At this stage an increased fraction of the CDP-choline is produced by a reversal of the phosphorylcholine-transferase reaction of the cytidine pathway by an increase in the CMP concentration, in addition to being produced by the forward net flux from choline to CDP-choline. Hence, the increased synthesis of CMP and the activation of the de novo GPC pathway are likely to be the initial responses to the hormonal signals responsible for the activation of the lung surfactant synthesis necessary for lung maturation. Mandel and Edel-Harth [87] found a similar increase in the concentration of CMP coupled with low CDP-choline and CDP-ethanolamine levels in the brains of 1-day old rats. These nucleotide concentrations are associated with a high growth rate of the brain at this stage [69]. I suggest that these nucleotide ratios in the brain are also due to the activation of the de novo GPC and GPE pathways for the synthesis of the C22:6*n*−33 PC and PE species which are being deposited at high levels in the developing brain, particularly in the gray matter [69]. At this developmental stage a large fraction of the base donors, i.e., CDP-choline and CDP-ethanolamine, may originate from the reversal of the terminal reaction in each of the cytidine pathways by the increased concentration of CMP. Upon lactation, dietary choline then restores the forward flux of the cytidine pathway for choline and ethanolamine storage.

Other evidence compatible with the proposed connection between the cytidine pathways and the de novo glycerophosphodiester pathways comes from the analyses of phospholipid metabolites in growing cell cultures. Activation of quiescent Swiss 3T3 mouse fibroblasts by serum has been reported to be accompanied by 3-fold increases of P-choline, P-ethanolamine, and their glycerophosphodiesters [88]. Similar findings have been reported for other cell lines, including cancer cells [89–91]. Since high rates of phospholipid catabolism are unlikely in rapidly growing cells, I suggest that a large fraction of the glycerophosphodiester pools, in these cells, originates from their de novo synthesis for the manufacture of the corresponding acyl-specific phospholipid species.

In the case of the specific acyl composition of PI, the hypothetical metabolic sequence proposed in scheme 3, and its connection with the cytidyl-liponucleotide pathway, also explains the literature data. An acyl-specific pool of CDP-DG formed by specific acylation, i.e., with C20:4*n*−6 and C18:0, of the de novo synthesized CDP-glycerol explains the similar acyl composition of the liponucleotide and PI (both of which are different from that of PA) reported by Thompson and MacDonald [58]. I suggest that the total liponucleotide pool is composed in large part of the acyl-specific species, and a minor fraction comes from the non-specific liponucleotide pathway connected to the PA pool. The difference in fatty acid composition of CL and the liponucleotide, also reported by the above authors, is explained by the pathway proposed in scheme 3. In this hypothetical sequence the CDP-glycerol transfers the glycerol moiety to GP to form glycerophosphorylglycerol (GPG) in a reaction similar to that proposed for the de novo GPC and GPE synthesis (scheme 1). This glycerophosphodiester is then specifically acylated to form the proper phosphatidylglycerol (PG) species. In mitochondria, a GP moiety is then transferred from CDP-glycerol to PG to form the intermediate phosphatidylglyceroglycerophosphate (PGGP), which is specifically acylated to form cardiolipin. Thus, in these proposed pathways, CDP-DG does not function as a source of acyl groups for the synthesis of PG or CL.

Hubscher et al. [92] were the first to report that the incorporation of labelled serine into mitochondrial phospholipids was increased by CMP, ATP,

CoASH, GP, and Mg^{2+}, whereas CTP was less effective than CMP and was inhibitory at higher concentrations. These data were corroborated by later reports [93–95], which showed that stimulation by GP did not occur in the absence of CMP. Since the phosphatidyltransferase has been shown to exchange CMP with CDP-diglyceride, and serine with PS [96], the enzyme may follow a ping-pong kinetic mechanism. Therefore, the stimulation of serine incorporation by CMP may be produced by this enzymatic substitution-catalyzed exchange, which may also show a phospholipase D-like activity by hydrolysis of the probably-unstable phosphatidyl-enzyme form. However, stimulation by CoASH, ATP, and GP cannot be explained by this mechanism. The need for these reactants is explained by the pathway postulated in scheme 1. This sequence proposed the formation of CDP-serine by a CMP-dependent phosphorylserine transfer from the non-acyl-specific PS pool derived from the base-exchange reaction of PE, similar to the phosphorylcholinetransferase reverse reaction. The serine moiety is then further transferred to GP to form glycerophosphorylserine (GPS), which is then acylated by acyl-specific acyltransferases to synthesize the acyl-specific PS. Although attempts to isolate labelled CDP-serine have not been successful in pulse-chase experiments with labelled serine [97], this may be so because CMP was not added to the incubation medium. Baranska [95] also searched for a mechanism to explain the ATP-dependent, and CoASH-, GP-, and CMP-stimulated incorporation of labelled serine into mitochondrial PS. She concluded that none of the known pathways of PS biosynthesis could explain the data. Among several hypotheses which were tested, she ruled out the CDP-serine pathway on the basis that no radioactivity from [β,γ-^{32}P]ATP was recovered in PS. The pathway that I propose explains these data as well, since the ^{32}P label appearing in PS does not originate from CDP-serine, but from GP. In the experiments reported by Baranska [95], unlabelled GP was part of the experimental protocol, so that any endogenous phosphorylation of endogenous glycerol by labelled ATP would be trapped by isotope dilution. ATP was then required only for acyl-CoA synthesis for specific acylation of GPS, and the label from [^{32}P]ATP would not be incorporated into CDP-serine. One direct approach to test the postulated pathway for the synthesis of acyl-specific PS (scheme 1) might include the use of labelled GP and/or labelled CMP in the protocol design.

4.4. *In vivo incorporation studies with labelled choline and phosphate*

A fundamental prediction of the cytidine pathway for the synthesis of PC is that the ratio of labelled choline and phosphate incorporated into this phospholipid should be equal to that of the P-choline precursor, since in this pathway the phosphorylated base is incorporated as a unit into the PC product. An analogous prediction is also valid for the synthesis of PE via the cytidine pathway. A corollary of this prediction is that the isotope incorporation and label ratio of choline *vs* phosphate should be equal for all the molecular species of PC and PE when measured before catabolism occurs, if they are synthesized via the cytidine pathways. The de novo GPC and GPE pathways, on the other hand, predict that the label ratios of choline/phosphate found in PC and PE should be higher than that of P-choline and P-ethanolamine, respectively, for the phosphoglyceride species synthesized by these pathways. As indicated before, this is because the ^{32}P specific activity of GP will be lower than that of P-choline.

The operation of de novo pathways other than the cytidine sequences was first suggested from the studies of Balint et al. [98] who found higher choline/phosphate label ratios for hepatic tetraenoic PC than for the dienoic species. These ratios were also higher for mitochondrial than for microsomal PC. These and other data led these authors to suggest that choline could be incorporated into PC via a pathway independent of CDP-choline. Studies reported by Treble et al. [99] showed that the label ratio of choline/phosphate in PC was much higher than that of P-choline. In agreement with Balint et al. [98], this ratio was higher for the arachidonoyl than for the linoleoyl species. On this basis, Treble et al. [99] suggested the existence of an unknown de novo pathway for PC synthesis involving a source of phosphate with a specific activity much lower than that of the phosphate of P-choline, or that choline was entering PC by base exchange. Since Dils and Hubscher [100] had demonstrated that such an exchange occurs in vitro, this was the preferred hypothesis. Similar

results and interpretations were reported by others [101,102]. However, Bjerve and others [103–105] have shown that in vivo operation of the base-exchange mechanism for PC and PE synthesis is negligible.

The above results are predicted by the proposed pathway (scheme 1), since the specific activity of the phosphate moiety of GP will be much lower than that of the phosphate moiety of P-choline because of dilution of the former by the larger pool of glycolytic intermediates.

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