PART VI: METABOLISM OF INOSITOL PHOSPHATES

BIOSYNTHESIS OF PHOSPHATIDYLINOSITOL*

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Interest in our laboratory in the inositol lipids derives from the presence of relatively high amounts of phosphatidylinositol phosphate (PhIP) and phosphatidylinositol diphosphate (PhIPP) in excitable tissue. It was with this relationship in mind that we embarked on a study of biosynthetic pathways of inositol lipids several years ago.

A first step in the elucidation of biosynthetic pathways of phospholipids goes back to 1953, when Kornberg and Pricer1 showed that a liver microsomal fraction catalyzed the acylation of glycerol-3-phosphate (G-3-P, L-α-glycerophosphate) to diacyl-G-3-P (phosphatidate), a lipid that was not previously known to be a biosynthetic intermediate substance. Subsequent studies indicated that phosphorylcholine was a precursor in the synthesis of phosphatidylcholine.4 Kennedy and Weiss, in 1955, found that ATP stimulated the incorporation of phosphorylcholine into lipid.4 An important step forward was taken when they learned that the stimulation by ATP was due to the presence of trace amounts of cytidine nucleotide.5 From this experiment evolved the role of CDP-choline and CDP-ethanolamine in phospholipid synthesis. A DPNH-linked dehydrogenase was found that reduced DHAP produced in glycolysis to G-3-P,6 which, in turn, could be acylated by the microsomal enzyme1 and fatty acyl-CoA to phosphatidate. Phosphatidate phosphohydrolase7 was shown to cleave phosphatidate to P1 and 1,2-diglyceride, which then reacted with CDP-choline or CDP-ethanolamine to yield phosphatidylcholine and phosphatidylethanolamine, respectively. A pathway for phospholipid synthesis was thus proposed, beginning with the reduction of DHAP to G-3-P, acylation to phosphatidate, dephosphorylation to diglyceride, and, finally, to yield phosphatidylcholine or phosphatidylethanolamine by reaction with the appropriate cytidine diphosphate derivative.8

By analogy, we began to look for a similar system that would synthesize phosphatidylinositol (PhI). We looked in vain for an inositol kinase and for the presence of CDP-inositol, using inositol labeled with tritium by the Wilzbach technique or by reduction of scyllo-inosose.9 Although there was no evidence for inositol phosphate or CDP-inositol formation, we observed that cytidine nucleotides did significantly stimulate inositol incorporation into lipid in incubations with particulate fractions from kidney.10 When inositol was omitted, radioactivity from labeled CMP accumulated, as a function of time, into a

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This observation, as well as related findings, led us to postulate the formation of a liponucleotide, CDP-diglyceride, and its subsequent cleavage by inositol to PhI and CMP. A clue to this pathway was present in the experiments of Dawson and of Hokin and Hokin. They showed that the specific activity of phosphatidate and PhI were higher following incubation with $^32P$ than were other phospholipids. In the CDP-diglyceride pathway, phosphorus in phosphatidate enters the phospholipid, while in the diglyceride pathway, it does not. The role of CDP-diglyceride was substantiated by the studies of Paulus and Kennedy, who synthesized CDP-dipalmitin via dicyclohexylcarbodiimide. They proposed that the liponucleotide was formed by the action of a CTP:phosphatidate transferase:

$$\text{CTP} + \text{phosphatidate (PhA)} \Leftrightarrow \text{CDP-diglyceride} + \text{PP}_1$$  \hspace{1cm} (1)

The reaction was not unequivocally proven. For example, it had been shown that CTP reacted with G-3-P in bacterial systems to yield CDP-glycerol. One might visualize that CDP-glycerol is acylated to form CDP-diglyceride.

That CTP:phosphatidate transferase was in fact the reaction by which CDP-diglyceride forms was demonstrated much later in liver and in chicken brain. In each instance, it was possible to demonstrate dependency on exogenously added phosphatidate for the formation of CDP-diglyceride. Although enzyme activity appears to be primarily in the mitochondrial fraction in chick brain, it is most active in the microsomal fraction of guinea pig liver.

The subsequent formation of phosphatidylinositol via CDP-diglyceride:inositol transferase:

$$\text{CDP-diglyceride} + \text{myo-inositol} \Leftrightarrow \text{PhI} + \text{CMP}$$  \hspace{1cm} (2)

has been studied extensively in kidney, liver, and brain. An improved method for the chemical synthesis of CDP-diglyceride was developed in our laboratory with use of CMP-morpholidate as an intermediate. This substance is commercially available (Calbiochem). For studies on CDP-diglyceride:inositol transferase in guinea pig brain, a variety of CDP-diglycerides including $C_{10}$, $C_{12}$, $C_{18}$ and $C_{22}$ were synthesized. The products were separated by solvent distribution and silica gel column chromatography. The purity was checked by chromatography on formylated paper by which the various CDP-diglycerides were readily separated from their analogous phosphatidates. The CDP-diglyceride:inositol transferase was assayed by a spectrophotometric method based on the inositol-dependent release of CMP. While liver homogenates had the highest total activity, heart, lung, and spleen had the highest specific activities, and brain homogenate had a relatively high activity and a low endogenous (−inositol) rate. Upon fractionation of brain, the highest specific activity was in the 76,000$\times g$ (crude microsomal) fraction. The presence of bovine serum albumin during incubation was found to be necessary for demonstration of full activity. P rottey and Hawthorne have also reported a similar effect with an enzyme preparation from kidney. Subfractionation according to a method of DeRobertis and co-workers revealed that the highest specific activity remained in the microsomal fraction, with rather uniform specific activity in fractions B–E ("nerve endings" and "mitochondria").

Using the brain microsomal preparation, we found that the maximal rate with CDP-didecanoin was about six times higher than with CDP-dipalmitin. The short-chain derivative was therefore used for subsequent studies. In experiments with labeled inositol, stoichiometry of the transferase reaction was
demonstrated and the product was identified as PhI. One-half of the added CDP-DL-didecanoin reacted as expected, since only the D-α,β-isomer (corresponding to G-3-P) should be active.\textsuperscript{20} CMP was identified as a product by electrophoresis at pH 1.5.\textsuperscript{24} The \( K_m \) for inositol was \( 1.5 \times 10^{-8} \text{M} \) and for the active isomer of CDP-didecanoin, \( 8.2 \times 10^{-5} \text{M} \).\textsuperscript{20}

With CDP-didecanoin as the lipid acceptor, various cyclitols were examined as substrates. All nine isomers of hexahydroxycyclohexane were examined, but only myo-inositol was active. \( \text{cis-} \), scyllo- and \( \text{epi-} \)inositol were slightly inhibitory. Mytilitol and isomytilitol\textsuperscript{25} were not available, but 2-O-C-methylenemyo-inositol\textsuperscript{26} was tested and found inactive as a substrate, as was the 2-C-OH-methyl derivative. None of the myo-inositol monophosphates served as substrates.\textsuperscript{20}

Galactinol \((1-O-\alpha,\beta\text{-galactopyranosyl-2-3-mylo-inositol})\)\textsuperscript{27} was a noncompetitive inhibitor of the reaction with 50% inhibition when added at equimolar concentrations with inositol. Two chromatographically purified inososes appeared to react with CDP-diglyceride in the presence of the guinea pig brain microsomal preparation.\textsuperscript{51} Similar results had been previously reported for chick liver.\textsuperscript{14} Since we were using a spectrophotometric method at 280 nm, a question arose concerning the possible contribution to UV absorption from an enediol that could form during incubation from the added inososes. Experiments with inososes incubated with brain preparations in the absence of CDP-diglyceride gave variable results. In order to verify the formation of phosphatidylinososes in brain, the lipid products from incubations with \( \text{epi-} \) or scyllo-inosose were reduced with NaB\(_3\)H\(_4\). In each instance, a radioactive product could be demonstrated that chromatographed near PhI on formylated paper. However, the yields were only 14–21% of that expected on the basis of CMP release.\textsuperscript{21} This might reflect instability of the phosphatidylinosose formed. The use of NaB\(_3\)H\(_4\) should be of general applicability in the determination of putative ketolipids.

In other studies, we have investigated the further metabolism of PhI. In vitro studies\textsuperscript{28,29,30} have suggested the subsequent phosphorylation of brain PhI to PhIP and PhIPP. Colodzin and Kennedy\textsuperscript{31} reported a PhI kinase in brain. This enzyme and PhIP kinase have been studied in brain.\textsuperscript{32,33} Active phosphatases are also present\textsuperscript{34,35,36} and may account for the rapid loss of PhIP from brain following death.\textsuperscript{36,37}

Evidence has accumulated that trace amounts of PhIP are present not only in nerve, but in all animal tissues examined.\textsuperscript{38–40} Mitochondria incubated for short periods of time with \( \text{32P}_i \) accumulate radioactivity in the phosphomonoester position of PhIP.\textsuperscript{41} Using a similar system and \( \gamma-\text{32P-} \text{ATP or 32P}_i \) as precursors, we were able to show that three lipids are selectively labeled: PhIP, phosphatidate, and a new lipid, described below.\textsuperscript{42} Atractyloside blocks \( \text{32P}_i \to \text{PhIP} \), but not \( \text{32P}_i \to \text{AT}^{32P} \) or \( \text{AT}^{32P} \to \text{PhIP}^{32P} \). This result suggests that there is an ATP-mediated PhI kinase and that the PhIP labeling system is not available to intramitochondrial ATP.\textsuperscript{42,43}

The novel lipid labeled under these conditions has been identified as acylidihydroxyacetone phosphate (acyl-DHAP).\textsuperscript{44} The labeling by \( \text{32P}_i \) is apparently the result of the combined action of an acyl-DHA kinase and an acyl-DHAP phosphatase. The biosynthesis of acyl-DHAP was investigated and found to be catalyzed by a mitochondrial preparation in the presence of acyl-CoA. Relatively high rates were found for palmitoyl- and stearoyl-CoA,\textsuperscript{45} compared with oleoyl-CoA. This is of interest, since it is known that the acylation of
G-3-P via the Kornberg-Pricer enzyme does not permit sufficient specificity for positions 1 or 2 of phospholipids to account for differences found between the two positions on analysis of phospholipids. It seemed possible that acyl-DHAP might constitute part of an alternative pathway that could permit this specificity. This proposal is given credence by our subsequent finding that mitochondria catalyze the TPNH-dependent reduction of acyl-DHAP to lysophosphatidate. The product is 1-acyl-G-3-P, as demonstrated by deacylation and oxidation of the glycerophosphate formed with the DPN-dependent enzyme. Acylation of this lysophosphatidate to phosphatidate is rapidly cata-

![Diagram]

**Figure 1.** Scheme of known pathways for the formation of inositol lipids. Dihydroxyacetone phosphate arises from glucose via glycolysis or the oxidative shunt pathway. It can be acylated and reduced via mitochondrial enzymes to lysophosphatidate and further acylated to phosphatidate. The previously known mechanism for phosphatidate synthesis involved reduction to glycerophosphate via a cytoplasmic enzyme and two subsequent acylations. Phosphatidate may be cleaved to form diglyceride and be subsequently converted into phosphatidyl choline or phosphatidyl ethanolamine. For inositol lipid formation, phosphatidate reacts with CTP to form CDP-diglyceride and pyrophosphate. CDP-diglyceride is then cleaved in the presence of myo-inositol to form CMP and phosphatidyl inositol. Di- and triphosphoinositide are produced by the action of kinases.

Lyzed by both mitochondrial and microsomal fractions of guinea pig liver, as has previously been reported when 1-acetyl-lysophosphatidate or L-lysophosphatidate (1-acyl-G-3-P) is used as a substrate. Specificity of chain length and degree of saturation of the fatty acid for the second acylation is, in our hands, dependent on the concentration of 1-acyl-G-3-P and fatty acyl-CoA.

The importance of this new pathway is at present not known. The levels of activity present in liver are comparable to or greater than that found for the
Kornberg-Pricer system. Since G-3-P is not an intermediate in the new pathway, the role of the acyl-DHAP pathway must be investigated under conditions such as hyperthyroidism or fasting, in which G-3-P dehydrogenase is known to be altered.\textsuperscript{51}

The known reactions in the biosynthesis of phosphatidylinositol are summarized in Figure 1.

**Summary**

Phosphatidylinositol is synthesized by the following reactions:

\[
\text{CTP + phosphatidate (PhA) } \rightleftharpoons \text{CDP-diglyceride + PP}_1 \quad (1)
\]

\[
\text{CDP-diglyceride + myo-inositol } \rightleftharpoons \text{PhI + CMP} \quad (2)
\]

Guinea pig brain microsomes show specificity for myo-inositol, although inososes also appear to act as substrates. Synthetic CDP-DL-didecanoin has been used as a model substrate. Galactinol (1-\text{O-\text{a,\text{b}-galactopyranosyl-2,3-myoinositol})} is an effective inhibitor of the reaction.

The subsequent phosphorylation of PhI to PhIP by ATP has been studied in guinea pig liver mitochondria with \(\gamma^{32}\text{P-ATP}\). Evidence is obtained that intramitochondrial ATP is in a compartment separate from the PhIP labeling system. In the course of these studies, we have gained new information on the biosynthesis of PhA as well. A novel pathway is proposed for the biosynthesis of PhA via a new lipid, acyldihydroxyacetone phosphate. The transfer of fatty acid from CoA to dihydroxyacetone phosphate is catalyzed by a mitochondrial preparation, which reduces the product to lysophosphatidate in the presence of TPNH. Thus glycerophosphate may not be an obligatory intermediate in the formation of glycerolipids.

**References**


