Short Communication

Biosynthesis of Cytidine Diphosphate Diglyceride by Cauliflower Mitochondria

S. Sumida and J. B. Mudd
Department of Biochemistry and Statewide Air Pollution Research Center,
University of California, Riverside, California 92502
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The presence of phosphatidyl inositol and phosphatidyl glycerol in plant tissue is well authenticated, but the pathways of biosynthesis are unknown. Cytidine diphosphate diglyceride has been established as an intermediate in the biosynthesis of phosphatidyl inositol in chicken liver (4) and in the biosynthesis of phosphatidyl glycerol in chicken liver (3). The enzymic formation of cytidine diphosphate diglyceride from CTP and phosphatidic acid in guinea pig liver (2) and embryonic chick brain (5) has been described in detail.

From the point of view of comparative biochemistry we have been interested to see if comparable reactions take place in plant tissues.

Cauliflower (Brassica cauliflora) was purchased at local markets. The cauliflower inflorescence was cut up and 100 g homogenized in a mortar and pestle with 100 ml of a solution 0.5 M with respect to sucrose and 0.01 M with respect to tris-HCl, pH 7.5. The homogenate was pressed through cheesecloth. Fifty ml of the homogenate was centrifuged at 800 × g for 10 minutes and the pellet discarded. The supernatant was centrifuged at 18,000 × g for 30 minutes. The supernatant was decanted and the pellet resuspended in 10 ml of the homogenizing medium. All preparative procedures were at 0°C.

In the formation of cytidine containing lipids the reaction mixture contained 1.30 ml of the enzyme source (homogenate, mitochondria, or supernatant) in the sucrose-tris medium, 30 μmole MgCl₂, and 0.306 μmole CTP-¹⁴C (222,000 dpm), in a final reaction volume of 1.52 ml. Reactions were stopped by the addition of 4.5 ml of methanol/chloroform (2/1) which was 0.1 M with respect to HCl. Chloroform and aqueous layers were separated essentially by the method of Bligh and Dyer (1). The residue was treated a second time by the Bligh and Dyer procedure. Chloroform extracts and washings were combined as were the aqueous fractions. An aliquot from the chloroform layer was dried in a scintillation counter vial, the scintillation fluid added and the sample counted.

The aqueous fractions from the reaction mixtures as described above were chromatographed on paper using propanol/water/conc. NH₄OH (66/33/1) adjusted to pH 3.7 with HCl as solvent. This solvent gave good separation of CMP and CTP which were visualized by spraying with a molybdate reagent consisting of 5.0 ml 30% perchloric acid, 10 ml 1 N HCl, 25 ml 4% ammonium molybdate and 60 ml water. After spraying, the paper was dried in a hot air stream and the spots then visualized by ultraviolet irradiation. When radioactive samples were chromatographed strips of the filter paper were cut and counted in the scintillation counter.

Figure 1 shows the incorporation of radioactivity into lipid as a function of time in the 3 fractions of cauliflower. The supernatant is equivalent to the homogenate since there was little volume change after centrifuging down the mitochondria. The mitochondrial fraction was resuspended in 10 ml of the homogenizing medium and so it is 6 times concentrated with respect to the original homogenate. Even when this concentration factor is taken into account, it is apparent that the ability to utilize CTP in the formation of lipid soluble compound is mainly located in the mitochondria. The expected product of the reaction is cytidine disphosphate diglyceride:

\[ \text{phosphatidic acid} + \text{CTP} \rightarrow \text{CDP-diglyceride} + \text{P}_{1} \]  

\[ (1) \]

Fig. 1. Incorporation of radioactivity from CTP-¹⁴C into lipid. Subcellular fractionation and experimental procedure were as described in the text.
CDP-diglyceride was prepared enzymically by the method of Carter and Kennedy (2). The lipid soluble product of \(^{14}\)C-CTP incubation with cauliflower mitochondria was compared with the reference CDP-diglyceride in 3 different chromatographic systems. In all 3 systems the product of the incubation with cauliflower mitochondria co-chromatographed with the reference CDP-diglyceride. The first system employed thin layer chromatography using silica gel G and a solvent composed of chloroform/pyridine/formic acid (50/20/7). The radioactive lipids were located by radioautography. Except for a trace amount left on the start line, the products of the guinea pig liver microsomal biosynthetic system and the cauliflower mitochondrial system chromatographed as a single spot with an \(R_f\) of 0.04. The second system of thin layer chromatography on silica gel G used chloroform/methanol/acetic acid/water (50/25/7/3) as solvent. The products chromatographed in this system as a single spot with an \(R_f\) of 0.28. The third chromatographic system employed formaldehyde treated paper prepared as described by Hendrickson and Ballou (6), and the solvent system was n-butanol/acetic acid (4/1/1) as described by Petzold and Agranoff (5). After development the paper was dried and cut into 1 cm strips which were counted in the scintillation counter. The product from cauliflower mitochondria co-chromatographed in this system with the CDP-diglyceride as prepared by the method of Carter and Kennedy (2). Further utilization of the CDP-diglyceride could give rise to phosphatidyl glycerol or phosphatidyl inositol:

\[
\text{CDP-diglyceride} + \alpha\text{glycerol phosphate} \rightarrow \text{phosphatidyl glycerol phosphate} + \text{CMP} \quad (\text{II})
\]

\[
\text{phosphatidyl glycerol phosphate} + \text{H}_2\text{O} \rightarrow \text{phosphatidyl glycerol} + \text{P}_1 \quad (\text{III})
\]

\[
\text{CDP-diglyceride} + \text{inositol} \rightarrow \text{phosphatidyl inositol} + \text{CMP} \quad (\text{IV})
\]

The shape of the curves in figure 1 indicate the formation of CDP-diglyceride and its subsequent utilization, possibly by way of reactions (II) and (IV). The decline in the radioactivity in the cytidine containing lipid is accelerated when either inositol or \(\alpha\)-glycerophosphate is added to the reaction mixture. If the supply of CTP were in excess, one might expect to achieve a steady state concentration of CDP-diglyceride rather than the rise and decrease actually observed. It has been determined that the availability of CTP was limiting and so prevented this achievement of a steady state concentration. Figure 2 shows the disappearance of CTP from the reaction mixture as determined by chromatography of the water soluble fraction. Practically all of the CTP has been utilized at the end of 2 minutes and this is precisely the time that the radioactivity in CDP-diglyceride starts to decline.

If the CTP is being utilized by reactions (I)-(III), we would expect the radioactivity to appear in CMP. Our experiments show that substantial amounts of CDP are formed as well as CMP, indicating that the CTP is being degraded by a phosphatase. This competing reaction could explain the relatively low incorporation of CTP into a lipid soluble form. Utilization of the radioactive CDP-diglyceride should depend on the availability of inositol, \(\alpha\)-glycerol phosphate and possibly serine. Initial experiments indicate that radioactive inositol and \(\alpha\)-glycerophosphate are incorporated into lipid by mitochondrial preparations from cauliflower. In the case of inositol it has been demonstrated that this incorporation is dependent on added CTP.

The evidence presented in this paper indicates that the pathways of lipid synthesis by way of CDP-diglyceride in plants are similar to the pathways found in other organisms.

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Literature Cited

