Lipid Biosynthesis in Peroxisomes[a]

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INTRODUCTION

In 1977, it was discovered that peroxisomes contain dihydroxyacetone phosphate acyltransferase (DHAPAT), a lipid biosynthetic enzyme.[1] This was surprising because until then, it was thought that peroxisomes contained mainly catabolic enzymes, especially the oxidases which catalyze the oxidation of various metabolites with molecular oxygen to form H_2O_2.[2] The function of peroxisomes was thought to compartmentalize these enzymes with a high concentration of catalase, which catalyzes the decomposition of H_2O_2. However, our finding regarding the presence of DHAPAT (and other lipid synthesizing enzymes) in peroxisomes[3] and the findings from other laboratories that bile acids[4] and steroids[5] are formed in peroxisomes, indicated that peroxisomes are also involved in cellular biosynthetic reactions. Results obtained in different laboratories have now firmly established that glycerolipid precursors are biosynthesized in all animal peroxisomes. These results and the implications of such peroxisomal lipid synthesis are reviewed here.

ACYL DIHYDROXYACETONE PHOSPHATE PATHWAY

Acyl dihydroxyacetone phosphate (acyl DHAP) was discovered in 1966 as a rapidly labeled lipid formed from $^{32}P_i$ in guinea pig liver crude mitochondrial fraction.[6] It was later shown to be synthesized by enzymatic acylation of DHAP with long chain acyl coenzyme As (acyl-CoAs).[7,8] Over the years, this lipid has been established as an important precursor of glycerolipids, especially glycerol ether lipids.[9] The biosynthesis of acyl DHAP and its conversion to different types of glycerolipids are summarized in FIGURE 1. As shown, acyl DHAP is either enzymatically (acyl DHAP reductase)[10] reduced by NADPH to 1-acyl-*sn*-glycerol-3-phosphate (1-acyl-Gro-3-P or lysoPtdOH), the precursor of non-ether glycerolipids, or is converted to 1-*O*-alkyl DHAP via a novel biochemical reaction catalyzed by alkyl DHAP synthase in which the acyl group is substituted by a long-chain alcohol.[9,11] Alkyl DHAP, like acyl DHAP, is enzymatically reduced by NADPH to 1-*O*-alkyl-Gro-3-P, the precursor of all glycerol ether lipids, including plasmalogens.[9] The same enzyme catalyzes the reduction of both acyl DHAP and alkyl DHAP.[12] The products (1-*O*-acyl-Gro-3-P and 1-*O*-alkyl-Gro-3-P) are then acylated to form phosphatidate (PtdOH) or its ether

[a]This work was supported by the National Institutes of Health grants NS 08841 and NS 15747.

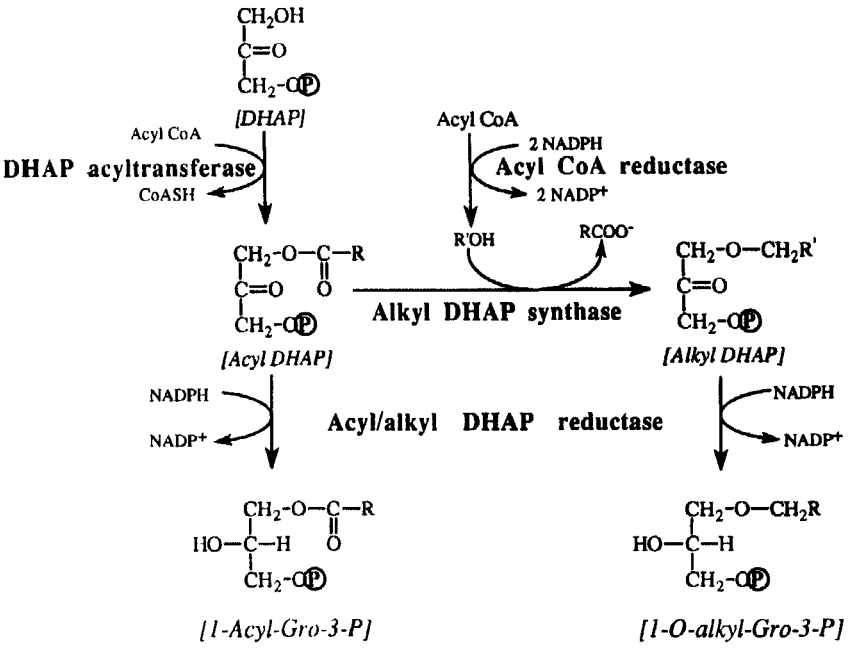

FIGURE 1. Pathways of peroxisomal lipid synthesis.

analog, the precursor of all glycerides and phosphoglycerides.[13] Thus the acyl DHAP pathway is an alternate pathway (as opposed to direct enzymatic acylation of Gro-3-P) for the synthesis of lysoPtdOH, and is an obligatory pathway for the synthesis of all glycerol ether lipids in animals.[3,9]

PEROXISOMAL LOCALIZATION OF THE ACYL DHAP PATHWAY ENZYMES

The enzymes of the acyl DHAP pathway (FIG. 1) are present in a membrane-bound form in all animal cells so far investigated. Initially, these enzymes were thought to be present in mitochondria, as was found in liver[7]; however, in brain they were found to be mainly localized in the microsomal fraction.[14] It was later shown, by careful subcellular fractionation of rodent livers using a combination of differential and density gradient centrifugations, that these enzymes co-sedimented with peroxisomal marker enzymes such as particulate bound catalase and urate oxidase.[1,15] The peroxisomal localization of these enzymes in many tissues has been confirmed by the development of a density-gradient centrifugation procedure using iodinated density gradient materials such as Metrizamide or Nycodenz (Accudenz)[3,16,17]; this procedure is now widely used to isolate pure peroxisomes from many eukaryotes. These compounds, having relatively higher molecular weight (~800) than sucrose or cesium chloride, can penetrate peroxisomal membranes, but not lysosomes or inner

mitochondrial membrane, so that on density gradient centrifugation in vertical rotors the peroxisomes quickly sediment to the position of their intrinsic density, leaving the other organelles behind on the top of the gradient. However, on prolonged centrifugation, other organelles, such as endoplasmic reticulum (ER) vesicles, also sediment to a density close to that of peroxisomes. Therefore, the separation of peroxisomes from other organelles in Nycodenz gradient is better accomplished by rate-density centrifugation rather than under isopycnic conditions.

Using this separation method, we have established that DHAPAT, alkyl DHAP synthase and most of acyl/alkyl DHAP reductase are localized in the peroxisomal membrane.[3,15,16,18] A part of the reductase, however, was also shown to be present in the ER membrane.[18]

We have recently shown that a fourth lipid biosynthetic enzyme, acyl-CoA: NADPH reductase (long-chain alcohol forming) (FIG. 1) is also present in peroxisomes. This enzyme has long been known as a microsomal enzyme[19]; however, from the similarity of subcellular distribution of particles containing catalase and acyl-CoA reductase in rat brain, it was postulated that long-chain alcohols are synthesized in peroxisomes.[20] This was found to be true when Burdett *et al.*,[21] using Nycodenz density gradient centrifugation of guinea pig intestine mucosal cell homogenate, showed that the enzyme is indeed localized in microperoxisomes. FIGURE 2 illustrates

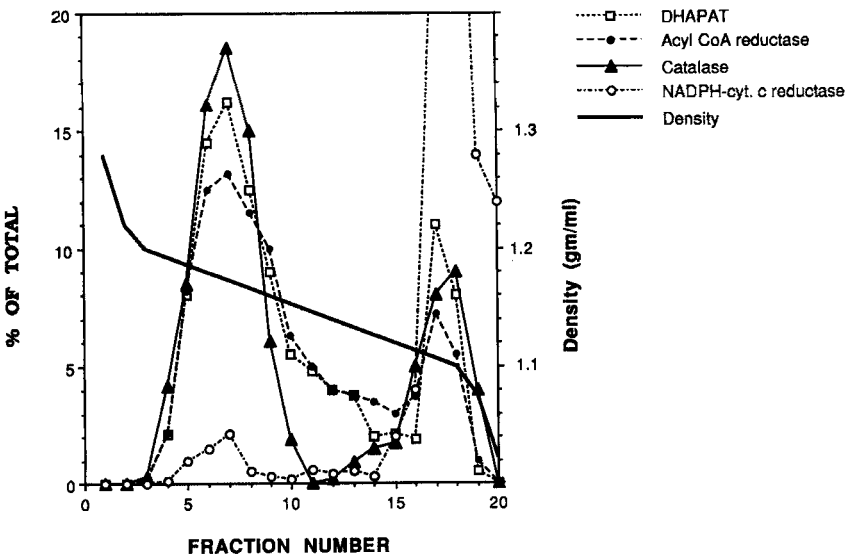

FIGURE 2. Subcellular distribution of acyl-CoA reductase and DHAPAT in guinea pig intestine mucosal cells. Light mitochondrial fraction of the mucosal cells was subjected to Nycodenz density gradient centrifugation in a vertical rotor and fractions were collected from the bottom of the tube. The activity of catalase (marker enzyme for peroxisomes), NADPH-cyt. C reductase (ER marker enzyme), DHAPAT and acyl-CoA reductase in these fractions, along with the density of each fraction are shown. The microperoxisomes (catalase-containing particles) banded at a density of 1.18 g/mL. (Adapted from Burdett *et al.*[21]).

the separation of intestinal subcellular organelles using Nycodenz (Accudenz) density gradient centrifugation, and the co-localization of acyl-CoA reductase with DHAPAT and catalase-containing microperoxisomes.

These results show that, starting from acyl-CoA, DHAP and NADPH, lysoPtdOH or its ether analog can be biosynthesized in peroxisomes. However, the enzymes catalyzing the conversion of lysoPtdOH to PtdOH and then to other membrane glycerolipids are found to be absent in peroxisomes.[3,22] These enzymes are all present in ER,[23] indicating that the glycerolipid precursors formed inside peroxisomes are exported to ER to form storage triglycerides and membrane phosphoglycerides.[13,23,24]

TOPOGRAPHY OF ACYL DHAP PATHWAY ENZYMES ON PEROXISOMAL MEMBRANE

The topography of these four biosynthetic enzymes on peroxisomal membranes was determined by studying the lability of these enzymes to protease treatment under permeable and non-permeable conditions.[8,18,21,25] A composite diagram is shown (FIG. 3) which represents the summary of experimental results obtained in different systems. As shown in this diagram, the two NADPH-requiring reductases are labile to brief trypsin treatment in the absence of the detergent Triton-X-100. In contrast, DHAPAT and alkyl DHAP synthase are stable to proteolysis in the absence of the detergent (FIG. 3). These results were interpreted to mean that the re-

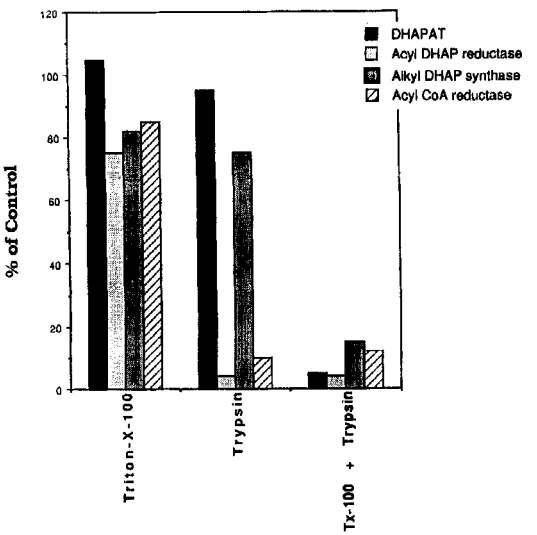

FIGURE 3. Lability of peroxisomal acyl DHAP pathway enzymes to trypsin. Isolated peroxisomes (from brain, liver, or intestinal mucosa) were treated with trypsin (20–50 μg/mg peroxisomal protein) at 4 °C with or without Triton-X-100 (0.01%–0.05%) as described previously.[21,25] After the incubation the activity of the enzymes was determined.

ductases are localized on the outer surface (cytosolic side) whereas the two synthetic enzymes (DHAPAT and alkyl DHAP synthase) are on the inner surface (luminal side) of the peroxisomal membrane (see FIG. 7).

ENZYME DEFICIENCIES IN PEROXISOMAL DISORDERS

In 1973, Goldfischer *et al.*[26] reported that in Zellweger cerebrohepato-renal syndrome (ZS), the tissues of the patients are deficient in peroxisomes. From this report and our finding that the acyl DHAP pathway enzymes are localized in peroxisomes, Borst[27] suggested that ZS patients may suffer from ether lipid deficiency. Following that suggestion, Heymans *et al.*[28] reported that the tissues of the ZS patients are indeed deficient in plasmalogens, the major mammalian ether lipid. When the activity of the acyl DHAP pathway enzymes were determined in the liver, leukocytes, and cultured skin fibroblasts of these patients, it was found that DHAPAT activity was very low (10 to 20% of normal controls).[29–31] This DHAPAT deficiency is seen not only in ZS, but also in other genetic peroxisomal disorders such as neonatal adrenoleukodystrophy,[32] infantile Refsum disease,[33] and in different forms of chondrodysplasia punctata[34,35] and is now used for the diagnosis of such congenital diseases.[36]

Besides DHAPAT, alkyl DHAP synthase activity was also found to be partially deficient in the tissues of these patients[19,30,37]; however, the activities of acyl/alkyl DHAP reductase and acyl-CoA reductase are not diminished.[37] It may be surmised then, that the activity of the enzymes which are localized on the lumenal surface of the peroxisomal membrane are reduced in these cells because they are not imported inside the peroxisomes, and thus are exposed to cytosolic proteases. Defects in the import of peroxisomal proteins are believed to be the main cause of such genetic diseases.[19,38] In contrast to the enzymes normally localized on the cytosolic side of the membrane, these lumenal enzymes are probably labile to the cytosolic proteases, and thus their activity is reduced when they are not protected inside the organelle. It will be of interest to find out whether or not the reductases, whose activities are not affected, are localized on the defective or "ghost" peroxisomes shown to be present in cells of patients with these diseases.[39]

PROPERTIES AND PURIFICATION OF ACYL DHAP PATHWAY ENZYMES

The acyl DHAP pathway enzymes have been solubilized from the membrane and purified, and their properties have been extensively studied in different laboratories. DHAPAT was shown to be solubilized from peroxisomal membrane with different detergents at high ionic strength.[40] Recently, this enzyme was purified to homogeneity from guinea pig liver after solubilization with the detergent CHAPS, followed by gel filtration, ion exchange, and hydroxylapatite chromatographies and chromatofocusing.[41,42] The enzyme was identified as a 69 kDa band on an sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretogram. The purified enzyme (4.0 μmol/min/mg) is activated by membrane phospholipids, especially phosphatidyl-

choline.[40,42] Ofman and Wanders[43] recently reported purification of this acyltransferase from human placenta employing similar methods, and identified the enzyme (0.6 μmol/min/mg) as a 65 kDa protein.

Brown and Snyder[44] reported solubilization and partial purification (100–200-fold) of alkyl DHAP synthase from Ehrlich ascites tumor cell microsomes. Using this partially purified enzyme (0.02 μmol/min/mg protein), they confirmed the findings from other laboratories[45,46] that there is a stereospecific exchange of the C-1 hydrogen of the DHAP moiety of acyl DHAP during the formation of the ether bond. Horie *et al.*[47] solubilized and partially purified (7 nmol/min/mg protein) the synthase from guinea pig liver peroxisomes, and studied its properties and inhibition by analogs of acyl DHAP. Zomer *et al.*[48] recently reported purification of the enzyme to homogeneity (10.8 nmol/min/mg protein) from the same source and identified it to be a 65 kDa band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Acyl/alkyl DHAP reductase has also been purified from guinea pig liver peroxisomes. A Nycodenz step gradient centrifugation was used to isolate peroxisomes in bulk quantities.[17,41] The enzyme was solubilized with detergents at high ionic strength in the presence of NADPH and then purified by differential solubilization and gel filtration, followed by NADPH-affinity chromatography.[12] The purified enzyme of high specific activity (40–60 μmol/min/mg) was identified as a 60 kDa protein band on SDS-PAGE.[12]

Recently, starting from peroxisomes prepared from commercially available guinea pig liver (shipped unfrozen on ice), we used a simplified method to purify the reductase. The modified method involved the same differential solubilization for the partial purification of the enzyme followed directly by affinity chromatography, omitting the gel filtration step. The enzyme was eluted from the affinity column with a NADPH gradient. The purified enzyme on SDS-PAGE migrated as a 40 kDa band instead of the 60 kDa band previously reported. Antibodies were raised in mice against the pure enzyme and the polyclonal antiserum used for immunoblot analysis of the purified enzyme also showed a single 40 kDa band (FIG. 4). However, with guinea pig liver peroxisomes, three bands, (60 kDa, 45 kDa, and 40 kDa) were seen after immunoblotting (FIG. 4). The same three bands were also observed with guinea pig liver microsomal fraction (FIG. 4). It is not clear at present whether the multiple bands represent isozymes or degradation products formed by proteolysis of the parent 60 kDa enzyme. The polyclonal antibodies generated against the guinea pig liver enzyme also cross-reacted strongly with rat tissue enzyme(s) (45 kDa, 40 kDa, and 35 kDa proteins) and very weakly with the human enzyme (FIG. 4).

PHYSIOLOGICAL IMPORTANCE OF LIPID BIOSYNTHESIS IN PEROXISOMES

Peroxisomes mainly contain H_2O_2-producing oxidases and catalase, which catalyzes the degradation of H_2O_2. It is not clear why the biosynthetic acyl DHAP pathway enzymes are also localized in peroxisomes. These membrane-bound enzymes, of course, should be topographically separated so that the biochemical reactions they catalyze can proceed at optimum rates. For example, because acyl/alkyl DHAP re-

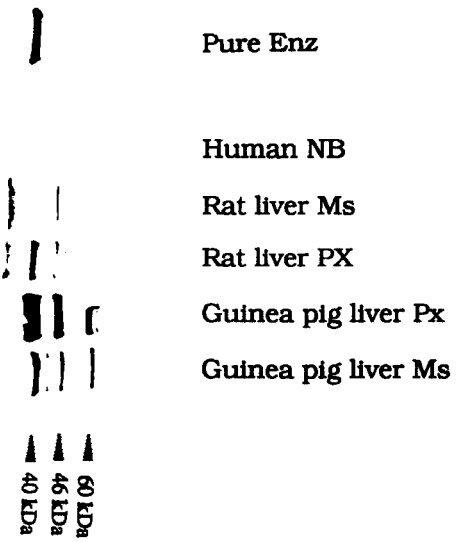

FIGURE 4. Immunoblot analysis of acyl/alkyl DHAP reductase. Different protein samples were subjected to SDS-PAGE followed by electroblot transfer of the separated proteins onto PVDF membrane. After blocking the unoccupied sites with bovine serum albumin, the blot was incubated with the mouse antiserum raised against purified guinea pig liver acyl/alkyl DHAP reductase. After washing, the blot was incubated with goat antimouse IgG coupled to alkaline phosphatase (Promega). After further incubation and washings, the bands were developed by incubating with the chromogenic substrates (bromochloro-indolyl phosphate plus nitrotetrazolium blue). Pure enzyme, purified guinea pig liver acyl/alkyl DHAP reductase; NB, SK-N-SH neuroblastoma cells; Ms; microsomes; Px, peroxisomes. Note: A faint band of 40 kDa was seen in the human NB lane, which is not reproduced in this photograph.

ductase catalyzes the reduction of both acyl DHAP and alkyl DHAP, the formation of alkyl DHAP will be hindered if DHAPAT and acyl DHAP reductase are present together. This is seen *in vitro* by the inhibition of the biosynthesis of alkyl DHAP by NADPH.[9] Therefore, the topographic segregation of the reductase from DHAPAT and alkyl DHAP synthase is necessary for the smooth operation of the pathway. Also, because the NADPH-requiring reductases are present on the outer surface of the peroxisomal membrane, cytosolic NADPH is utilized for the reduction of the keto lipids and therefore the generation of NADPH inside peroxisomes is not necessary. However, with such distribution of these enzymes, it is necessary to supply DHAP inside the peroxisomes for the operation of the pathway. It was found that the membrane of isolated peroxisomes is permeable to relatively large molecules of up to 2,000 Da, allowing exogenous DHAP to easily penetrate for the reactions to proceed. However, this may be artifactual, and there is evidence that *in situ* peroxisomes are not permeable to such large molecules.[19] Therefore, physiologically it is necessary to generate (or transport) DHAP in peroxisomes for the operation of the acyl DHAP pathway.

One possible enzymatic reaction to form DHAP inside the peroxisomes is the oxidation of Gro-3-P by NAD^+. Tolbert and co-workers have shown that a large fraction

of cellular NAD-linked glycerophosphate dehydrogenase is present inside peroxisomes.[49] The function of this enzyme is not clear, though Tolbert speculated that it may catalyze the oxidation of NADH (formed by the peroxisomal β-oxidation pathway) via a Gro-3-P shuttle.[49] We have, however, provided evidence that though the equilibrium of the reaction catalyzed by this dehydrogenase is more towards the reduction of DHAP, oxidation of Gro-3-P can be accomplished by removing NADH, a product of the reaction. This was shown by the formation of DHAP from Gro-3-P in peroxisomes when pyruvate and lactic dehydrogenase are added to the reaction mixture.[24] We also recently observed a rapid oxidation of Gro-3-P to DHAP in peroxisomes when cytochrome C was added to the reaction mixture by measuring the formation of acyl DHAP from Gro-3-P and also by directly measuring the formation of DHAP from Gro-3-P in peroxisomes under similar conditions in the presence of NAD^+ and cytochrome C (Fig. 5). It is possible that, physiologically, DHAP is formed from Gro-3-P in peroxisomes by this mechanism (Fig. 6). Cytochromes are known to be present in peroxisomes, and a NADH-cytochrome C reductase has also been shown to be present on peroxisomal membrane.[49,50] The fate of the reduced cytochrome C in peroxisomes, however, is not clear.

Based on these findings, a model is proposed (Fig. 7) for the peroxisomal synthesis of glycerolipids and its regulation. Cellular Gro-3-P, which is present in relatively high concentrations (1–2 mM) compared to DHAP (~ 0.1 mM), is oxidized in peroxisomes by NAD^+, catalyzed by glycerophosphate dehydrogenase in the presence of cytochrome C. DHAP is then acylated and converted to alkyl DHAP inside the peroxisomes. Alkyl DHAP, having relatively high critical micellar concentration, diffuses out from peroxisomes to cytosol, where it is reduced by NADPH, catalyzed by peroxisomal (or microsomal) alkyl DHAP reductase. The product of the reaction, that is, the ether analog of lysoPtdOH, is then enzymatically acylated in ER to form the ether analog of PtdOH, which is the precursor of all cellular glycerol ether lipids and plasmalogens. Such export of lipids from peroxisome to ER and their acylation in ER have been experimentally demonstrated.[51]

CONCLUSIONS

As summarized above, it is now well established that all animal peroxisomes are involved in ether lipid biosynthesis. Other cellular components have also been shown to be biosynthesized in peroxisomes. This was first evident in germinating seeds, where the glyoxylate cycle enzymes, catalyzing the conversion of fats to carbohydrates, were shown to be present in peroxisomes (glyoxysomes). Enzymes of the fatty acid β-oxidation cycle which are universally present in all eukaryotic peroxisomes, are generally believed to participate in the oxidation of unusual fatty acids which are not normally oxidized in mitochondria. However, it is evident that, unlike mitochondrial β-oxidation of fatty acids, which is mainly utilized for cellular energy production, the peroxisomal β-oxidation cycle produces large amounts of acetyl CoA, which is the building block for all fatty acids, steroids and other isoprenoids. Therefore, it seems that the main function of peroxisomal β-oxidation of fatty acids is to supply precursors for cellular anabolic reactions. As mentioned above, bile acids and steroids have also been shown to be formed in peroxisomes. From all of these re-

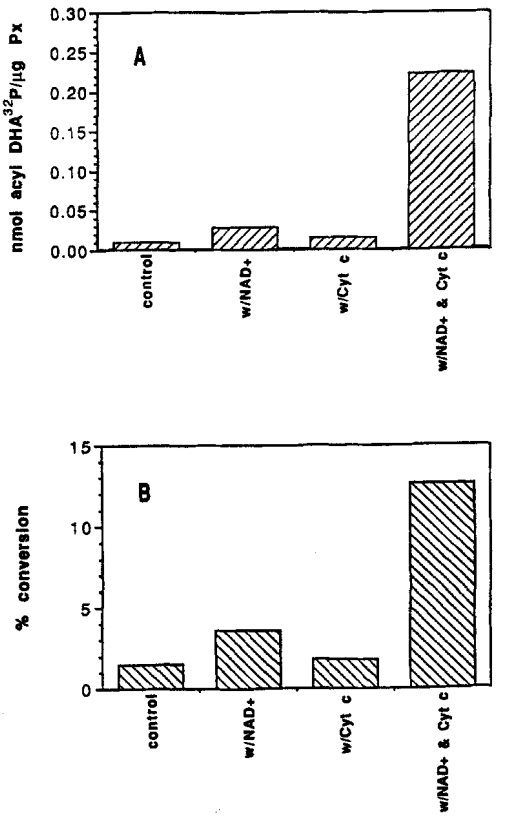

FIGURE 5. Stimulation of enzymatic oxidation of Gro-3-P to DHAP by cytochrome C in rat liver peroxisomes. Rat liver peroxisomes (50 μg protein) were incubated at 37° for 30 min in Tris-HCl buffer (pH 7.4, 75 mM) and Gro-3-^{32}P (5 mM, 14,000 cpm/nmol) with (w) or without NAD^+ (1 mM) and cytochrome C (0.5 mg) in a total volume of 0.4 mL. After incubation aliquots were taken to (A) measure the formation of acyl DHAP by adding palmitoyl-CoA (0.1 mM) and other cofactors or (B) separate $DHA^{32}P$ formed from Gro-3-^{32}P by high voltage paper electrophoresis at pH 1.5. The fractions of Gro-3-P converted to DHAP are shown.

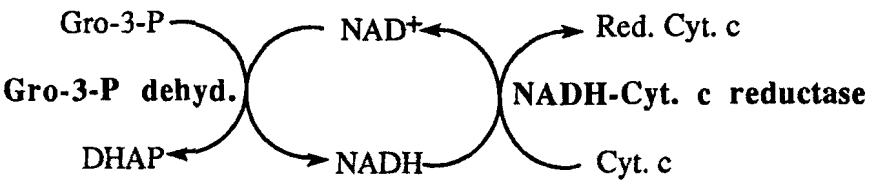

FIGURE 6. Enzymatic oxidation of Gro-3-P in peroxisomes.

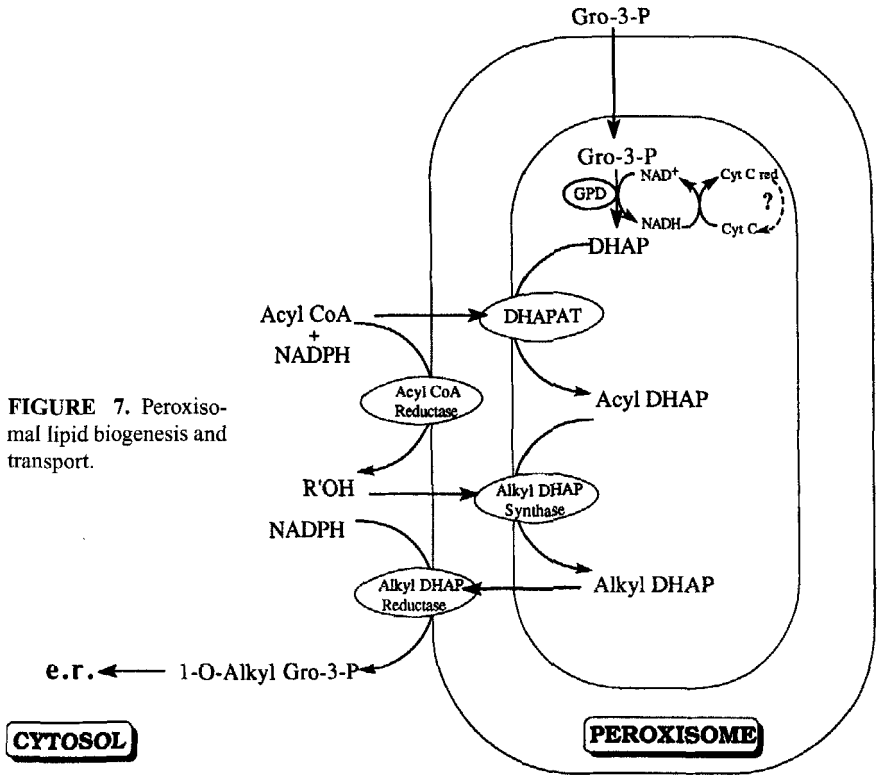

FIGURE 7. Peroxisomal lipid biogenesis and transport.

sults, it may be inferred that the main physiological function of peroxisomes is to generate cellular components by compartmentalization of the enzymes and reactants so that the biochemical reactions can proceed at an appreciable rate and toxic products are immediately removed.

ACKNOWLEDGMENTS

We wish to thank Ms. Leslie Larkins and Ms. Jo Ann Kelsch for editorial assistance in the preparation of this manuscript.

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