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# Bile Acid Profiles in a Peroxisomal D-3-Hydroxyacyl-CoA Dehydratase/D-3-Hydroxyacyl-CoA Dehydrogenase Bifunctional Protein Deficiency

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**Bile acid profiles in serum, urine and bile from an infant with a peroxisomal D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein) deficiency were analyzed by means of gas-liquid chromatography, gas-liquid chromatography-mass spectrometry, and high-performance liquid chromatography. As in such several peroxisomal disorders as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease, the accumulation of $C_{27}$-bile acid intermediates was also demonstrated in the infant with D-bifunctional protein deficiency, accounting for 74% of the total bile acids in serum, 59% in urine, and 35% in bile. In addition, the major constituents of the $C_{27}$-bile acids were (24*R*,25*R*)- and (24*R*,25*S*)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acids along with small amounts of their 24*S* counterparts. Since immunoreactive acyl-CoA oxidase, L-bifunctional protein, and thiolase were all present in the liver, the impairment of the oxidative side-chain cleavage in bile acid biosynthesis is considered to be due to the defect of D-bifunctional protein.**

**Key words: bile acid, bile acid intermediates, D-bifunctional protein, peroxisomal disorder, β-oxidation.**

3α,7α,12α-Trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid are key intermediates in the formation of cholic acid (CA) and chenodeoxycholic acid (CDCA), respectively (*1*). These $C_{27}$-bile acids are thought to be cleaved *via* α,β-unsatulated acid, β-hydroxyl acid, and β-keto acid, in a similar manner to β-oxidation of fatty acid in liver peroxisomes. The enzyme involved in the first reaction in the side-chain cleavage of $C_{27}$-bile acids is coprostanoyl-CoA oxidase, which differs from acyl-CoA oxidase in the β-oxidation system of fatty acid (*2, 3*). The second and third reactions of the peroxisomal β-oxidation are catalyzed by two types of bifunctional enzymes in rat liver, bifunctional enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-bifunctional protein) and D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase (D-bifunctional protein) for fatty acid and bile acid intermediates, respectively (*4, 5*). D-bifunctional protein has also been identified in human peroxisomes (*6, 7*).

Bile acid intermediates are known to be accumulated in such peroxisomal disorders as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease, accompanied by the accumulation of very long chain fatty acids (*8-15*). Recently, we have found two Japanese patients affected by the deficiency of D-bifunctional protein (Suzuki Y., unpublished findings).

In the present study, we analyzed the bile acids in biological fluids in one of these patients to obtain more precise $C_{27}$-bile acids profiles, thereby providing further evidence for the correlation between enzyme deficiency and bile acid profiles.

## MATERIALS AND METHODS

*Subjects*—The patient was a female child of consanguineous Japanese parents. Hypotonia, mild craniofacial dysmorphism, hepatic dysfunction, multifocal convulsions, and calcific stippling were present. Accumulation of very long chain fatty acids and decreased activity of lignocelic acid oxidation in fibroblasts were evident. Enzyme protein of peroxisomal acyl-CoA oxidase, L-bifunctional protein, and 3-ketoacyl-CoA thiolase were detected, but not D-bifunctional protein.

*General*—Gas liquid chromatography (GLC) was carried out on a Shimadzu GC-14A gas chromatograph using a fused silica capillary column (30 m×0.25 mm i.d.) coated (0.15 μm film) with DB-17HT (J&W Scientific, CA). The column temperature was 220-290°C, 2.5°C/min.

Gas-Liquid Chromatography-Mass Spectrometry (GC-MS) was carried out on a Hewlett-Packard 5890 gas chromatograph and a JEOL JMS-SX 102 mass spectrometer under the following conditions: column, a fused silica capillary column (15 m×0.32 mm i.d.) coated (0.15 μm film) with DB-17HT (J&W Scientific); column oven temperature, 200-280°C at a rate of 2.5°C/min; injection port temperature, 280°C; ion source temperature, 250°C; flow

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rate of helium carrier gas, 2.0 ml/min; ionizing energy, 70 eV; and ionizing current, 300 $\mu$A.

High-performance liquid chromatography (HPLC) was carried out using a TSK GEL ODS-80TM (4.6 mm i.d.× 150 mm, Tosoh, Tokyo) column with a Waters (Milford, MA) M-45 solvent-delivery system and a Shimadzu (Kyoto) SPD-1 UV detector operated at a wavelength of 254 nm. The bile acid samples were subjected to HPLC after *p*-bromophenacyl ester derivatization. A mixture of methanol and water, 82.5:17.5, was used as the mobile phase at a flow rate of 1 ml/min.

*Cholanoids*—Cholic acid and chenodeoxycholic acid were commercial products. (25*R*)- and (25*S*)-3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholestanoic acid (THCA) (*16*), (24*E*)-3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholest-24-enoic acid [(24*E*)-$\Delta^{24}$-THCA] (*16*), (24*R*,25*R*)-, (24*R*,25*S*)-, (24*S*,25*R*)-, and (24*S*,25*S*)-3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acids [(24*R*,25*R*)-, (24*R*,25*S*)-, (24*S*,25*R*)-, and (24*S*,25*S*)-24-OH-THCA] (*16*), and 3$\alpha$,7$\alpha$,12$\alpha$,26-tetrahydroxy-5$\beta$-cholestanoic acid (26-OH-THCA) (*17*) were chemically synthesized from cholic acid according to the method described previously.

*Analytical Procedure*—Serum (1 ml) and urinary (47 ml) bile acids were extracted through Sep-pak $C_{18}$ cartridges. The extracts were hydrolyzed with 2 N KOH after enzymatic solvolysis. Biliary (0.8 ml) bile acids were obtained by filtration after dilution of bile with ethanol. The biliary bile acids were separated on a PHP-LH-20 column into unconjugated, glycine-conjugated, and taurine-conjugated bile acids, respectively. The conjugated bile acid fractions were hydrolyzed with 2 N KOH. All bile acid samples were analyzed by GLC and GC-MS as their methyl ester-TMS ether derivatives. An aliquot of the unconjugated bile acid fraction was converted to *p*-bromophenacyl ester and analyzed by HPLC (*18*).

## RESULTS

Serum bile acids were analyzed by GLC and GC-MS as methyl ester-TMS ether derivatives after alkaline hydrolysis following to solvolysis. The bile acid composition and concentration are shown in Table I. Total serum bile acid concentration from the infant with D-bifunctional protein deficiency was 25.2 $\mu$g/ml, and the bile acids predominantly consisted of $C_{27}$-bile acids (74%) along with small amounts of cholic acid and chenodeoxycholic acid. The major bile acids were identified as 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholestanoic acid (THCA), 3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid (24-OH-THCA), and 3$\alpha$,7$\alpha$,12$\alpha$,26-

TABLE II. **Biliary bile acid composition in peroxisomal D-bifunctional protein deficiency.**

| Bile acids | Unconjugated | Glycine-conjugated | Taurine-conjugated | Total |
|---|---|---|---|---|
| CA | 0.5 | 12.3 | 6.3 | 8.6 |
| THCA | 2.0 | 2.6 | 6.0 | 3.2 |
| 24-OH-THCA | 84.2 | 6.3 | 2.4 | 22.0 |
| 26-OH-THCA | 6.9 | 1.2 | 3.8 | 2.9 |
| $\Delta^{24}$-THCA | – | 1.6 | 7.7 | 2.5 |
| CDCA | 0.4 | 73.2 | 68.6 | 56.8 |
| DHCA | 6.0 | 2.8 | 5.2 | 4.0 |

CA, cholic acid; CDCA, chenodeoxycholic acid; THCA, 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholestanoic acid; DHCA, 3$\alpha$,7$\alpha$-dihydroxy-5$\beta$-cholestanoic acid; 24-OH-THCA, 3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid; 26-OH-THCA, 3$\alpha$,7$\alpha$,12$\alpha$,26-tetrahydroxy-5$\beta$-cholestanoic acid; $\Delta^{24}$-THCA, 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholest-24-enoic acid.

TABLE I. **Bile acid composition in a D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase deficiency.**

| | $C_{24}$-Bile acids | | $C_{27}$-Bile acids | | | | |
|---|---|---|---|---|---|---|---|
| | CA | CDCA | THCA | $\Delta^{24}$-THCA | DHCA | 24-OH-THCA | 26-OH-THCA |
| Serum (25.2 $\mu$g/ml) | 9.2 | 16.8 | 22.2 | n.d. | n.d. | 29.7 | 22.1 |
| Urine (4.48 $\mu$g/ml) | 17.5 | 22.9 | 9.3 | 6.2 | n.d. | 29.1 | 15.0 |
| Bile | 8.6 | 56.8 | 3.2 | 2.5 | 4.0 | 22.0 | 2.9 |

CA, cholic acid; CDCA, chenodeoxycholic acid; THCA, 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholestanoic acid; $\Delta^{24}$-THCA, 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholest-24-enoic acid; DHCA, 3$\alpha$,7$\alpha$-dihydroxy-5$\beta$-cholestanoic acid; 24-OH-THCA, 3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid; 26-OH-THCA, 3$\alpha$,7$\alpha$,12$\alpha$,26-tetrahydroxy-5$\beta$-cholestanoic acid.

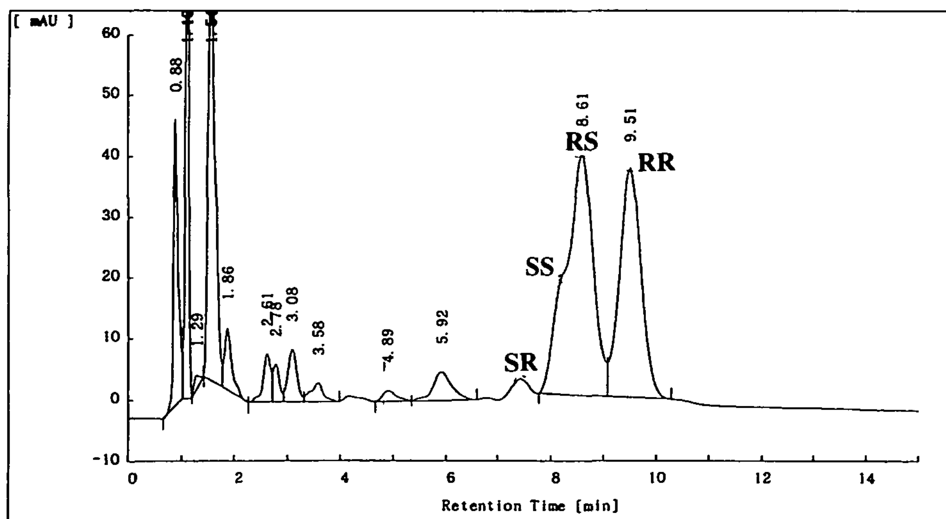


Fig. 1. **HPLC analysis of unconjugated bile acids in bile from D-specific bifunctional protein deficiency.** SR, (24*S*,25*R*)-3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid; SS, (24*S*,25*S*)-3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid; RS, (24*R*,25*S*)-3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid; RR, (24*R*,25*R*)-3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid.



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tetrahydroxy-5$\beta$-cholestanoic acid (26-OH-THCA) by comparing retention times and mass spectra with authentic standards.

The urinary bile acid profile was similar to that of serum. Therefore $C_{27}$-bile acids were the major constituents and comprised 60% of the total bile acids in urine (Table I).

The biliary bile acids were fractionated into unconjugated, glycine-conjugated, and taurine-conjugated bile acids using a PHP-LH-20 column. The amidated bile acids were hydrolyzed with 2 N KOH, and the deconjugated bile acids and unconjugated bile acids were analyzed by GLC and GC-MS after methyl ester-TMS ether derivatization. The results are shown in Table II. Although chenodeoxycholic acid was a major component (57%) in the total biliary bile acids, $C_{27}$-bile acids accounted for 35% of the total. The unconjugated bile acid fraction consisted of primarily $C_{27}$-bile acids, in which 24-OH-THCA comprised 84%. Furthermore, an aliquot of the unconjugated bile acids was converted to *p*-bromophenacyl ester derivatives and analyzed by HPLC, revealing the presence of (24*R*,25*R*)- and (24*R*,25*S*)-24-OH-THCAs along with small amounts of 24*S*-counterparts (Fig. 1).

## DISCUSSION

Bile acid intermediates, $C_{27}$-bile acids, are known to be accumulated in peroxisomal disorders (*8-15*). These disorders are classified based on peroxisomal fatty acid $\beta$-oxidizing enzyme activity (*19-22*). The accumulation of very long chain fatty acids in serum is associated with increased amounts of $C_{27}$-bile acids in serum, urine and bile.

Side-chain cleavage of the intermediates in bile acid formation is considered to proceed analogously to fatty acid $\beta$-oxidation in mammalian liver. A separate acyl-CoA oxidase was shown to catalyze the first step of bile acid formation in rat (*2*) and human liver peroxisomes (*3*). However, it remained uncertain whether or not the bile acid and fatty acid were catalyzed by a common bifunctional enzyme (hydratase, dehydrogenase) and a thiolase.

In the course of the study of the side-chain cleavage in bile acid formation, we investigated the stereochemistry of the intermediates formed from 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholestanoic acid (THCA) and showed the formation of 24*E*-isomer in two geometric isomers of 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholest-24-enoic acid ($\Delta^{24}$-THCA) and (24*R*,25*R*)-isomer among the four stereoisomers at C-24 and C-25 of 3$\alpha$,7$\alpha$,12$\alpha$,24-tetrahydroxy-5$\beta$-cholestanoic acid (24-OH-THCA) (*23-25*). The intermediates formed during the fatty acid $\beta$-oxidation have been found to be (*trans*)-2-enoyl-CoA and L-3-hydroxyacyl-CoA (*26*). Although the stereochemistry of both $\alpha$,$\beta$-unsaturated acids is identical, the configuration at the carbon of the $\beta$-position is opposite, *R* for bile acid and *S* for fatty acid. This dissociation may suggest the presence of separate enzymes for the bifunctional protein.

Recently it has been shown that rat liver peroxisomes contain two multifunctional proteins, an L-specific hydratase/3-hydroxyacyl-CoA dehydrogenase and a D-specific dehydratase/3-hydroxyacyl-CoA dehydrogenase (*5*). These enzymes are considered to be involved in the $\beta$-oxidation of fatty acid and the side chain of $C_{27}$-bile acid intermediates, respectively. Furthermore, it has become evident that the D-specific bifunctional protein, but not the L-bifunctional protein, acts on the intermediates of bile acid biosynthesis (*5, 27-29*). It has also been shown that separate bifunctional proteins are present in human, as was the case of acyl CoA oxidase (*6, 7*). Thus, it became possible to investigate separately D-bifunctional protein deficiency and L-bifunctional protein deficiency.

In this study, we found the accumulation of $C_{27}$-bile acid intermediates in D-bifunctional protein deficiency. This finding confirmed the significant role of the D-bifunctional protein, but not the L-bifunctional protein, in side-chain cleavage in bile acid formation. It was also compatible with the results obtained from *in vitro* studies (*27-29*).

In the present patient, both the activity and the immunoreactive material of D-bifunctional protein were absent. Thus, the hydratase activity must be absent, whereas 24-OH-THCA accumulated in the biological fluids. When the unconjugated bile acids in bile were analyzed using HPLC, it was found that both (24*R*,25*S*)- and (24*R*,25*R*)-24-OH-THCAs along with small amounts of their 24*S*-counterparts were predominant. It seems likely that the 24-OH-THCAs are formed *via* direct hydroxylation of both (25*R*)- or (25*S*)-THCA, not *via* a hydratase action on $\Delta^{24}$-THCA. We recently noted the formation of 24-OH-THCA from THCA by direct 24*R*-hydroxylation in *Bombina orientalis* (*25*). Furthermore, a number of hydroxylation products of THCA at the side chain as well as at the nucleus were identified in several peroxisomal disorders (*11, 12*). Regarding the accumulation of 24-OH-THCAs, the present findings closely resemble those of a thiolase deficiency reported by Clayton *et al.* (*11*), in which the presence of two isomers of 24-OH-THCAs was confirmed. However, their isomers were (24*R*)- and (24*S*)-24-OH-THCAs and were considered to be formed by the reduction of 24-oxo-THCA.

It is also of interest that chenodeoxycholic acid was a major constituent of biliary bile acids, despite the accumulation of the $C_{27}$-intermediates of the cholic acid-type of nucleus in both thiolase deficiency and bifunctional protein deficiency. This may suggest that an alternative route of CDCA formation exists in the subcellular fraction other than peroxisomes in some peroxisomal disorders.

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