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Urinary Bile Acids and Peroxisomal Bifunctional Enzyme Deficiency

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The biosynthesis of normal bile acids involves beta-oxidation of the 8-carbon side-chain of cholesterol, in addition to numerous modifications of the sterol nucleus. Because beta-oxidation of the sterol side-chain has been localized to the peroxisome, bile acid analysis has been suggested to be useful in the diagnostic evaluation of individuals suspected of having peroxisomal disorders. Although data from subjects with generalized peroxisomal disorders support this, few data exist regarding the bile acids in individuals having single peroxisomal beta-oxidation enzyme disorders. In this study, we analyzed the urinary bile acids from 12 patients with peroxisomal bifunctional protein deficiency using continuous flow fast atom bombardment mass spectrometry. All 12 patients had abnormal spectra, although their ion profiles and rank order of intensity of ions varied considerably. Ten of 12 individuals had abnormal spectra with presence of taurine-conjugated tetrahydroxycholestenoates, allowing a definite diagnosis of a peroxisomal beta-oxidation defect and a presumptive diagnosis of bifunctional protein deficiency; the other two cases were nondiagnostically abnormal. The strengths and limitations of urinary bile acid analysis for the diagnosis of peroxisomal beta-oxidation disorders are discussed.

KEY WORDS: peroxisome, peroxisomal disease, bifunctional enzyme, bile acids, cholestanoates, cholestenoates

INTRODUCTION

The peroxisomal disorders constitute a large and still expanding category of inborn errors of metabolism. These disorders are clinically, genetically, and biochemically heterogeneous. To date, genetic abnormalities involving many of the metabolic pathways in peroxisomes have been described. These include defects in the catabolism of very long-chain fatty acids, phytanic acid, and pipecolic acid, as well as abnormalities in the biosynthesis of plasmalogens and bile acids [Wanders et al., 1990b; Kelley, 1991; Brown et al., 1993; Moser, 1993; Lazarow and Moser, 1995; Moser et al., 1995].

The biosynthesis of bile acids is complex, involving numerous enzymatic reactions in different subcellular compartments [Hofmann, 1988; Balistreri, 1991; Clayton, 1991; Setchell, 1991; Russell and Setchell, 1992]. The two primary bile acids in humans, chenodeoxycholate and cholate, are 24 carbon cholanoates derived from cholesterol, through 27 carbon intermediates, the cholestanoates. This metabolic pathway involves modifications of both the sterol nucleus and the 8-carbon side-chain of cholesterol (Fig. 1). The latter is shortened to a 5-carbon side-chain through a beta-oxidation reaction sequence mediated by the sequential actions of 4 enzymes: bile acid coenzyme A ligase, bile acid oxidase, peroxisomal hydratase/dehydrogenase (bifunctional enzyme), and peroxisomal thiolase (Fig. 2). Several lines of evidence have established that the biosynthesis of normal bile acids and, more specifically, the side-chain shortening process, occurs in peroxisomes. These include both in vitro and in vivo studies that showed defective production of bile acids in cells and tissues from patients with various peroxisomal disorders, as well as studies that showed that the final three enzymes of the beta-oxidation sequence are localized in peroxisomes [Kase, 1989; Russell and Setchell, 1992].

Due to the importance of peroxisomes in the biosynthesis of normal bile acids, several investigators initiated preliminary studies to evaluate the utility of bile acid analysis of blood or urine for the diagnosis and characterization of various peroxisomal disorders [Eyssen et al., 1985; Lawson et al., 1986; Clayton et al.,

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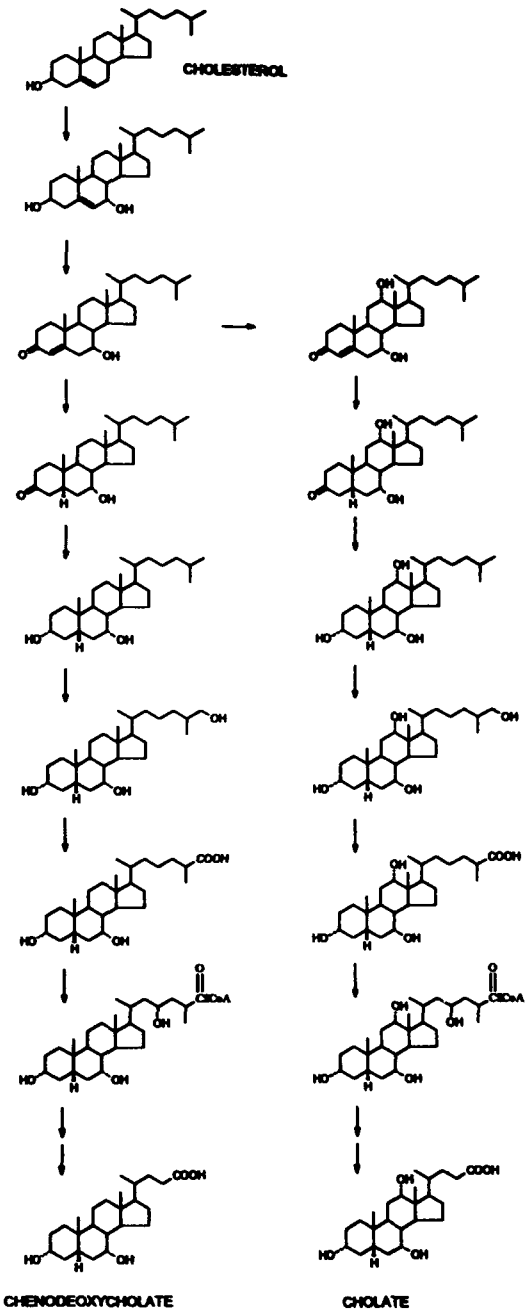

Fig. 1. An overview of the bile acid biosynthetic pathway from cholesterol to the two primary bile acids, cholate and chenodeoxycholate. The presence of multiple arrows indicates several enzymatic reactions.

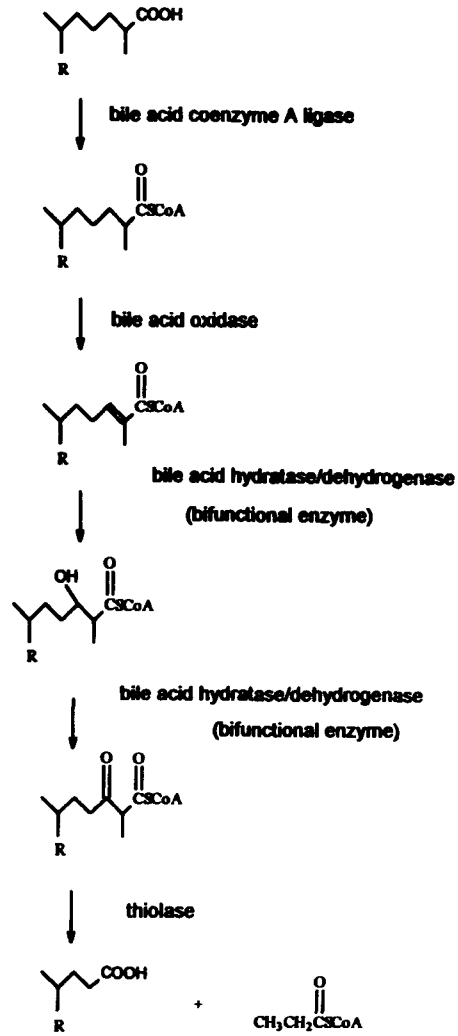

Fig. 2. An overview of the beta-oxidation reaction sequence of the 8-carbon side-chain of cholesterol. The bifunctional and thiolase enzymes participate in the beta-oxidation of both very long-chain fatty acids and bile acids; bile acids but not very long-chain fatty acids are substrates for the bile acid coenzyme A ligase and bile acid oxidase.

1987]. An important rationale for this diagnostic approach is based on the fact that some of the enzymes in the peroxisomal beta-oxidation pathway utilize only bile acids or very long-chain fatty acids as substrates, whereas other enzymes (i.e., peroxisomal bifunctional and thiolase enzymes) catalyze the oxidation of either type of substrate [Russell and Setchell, 1992] (Fig. 2). Consequently, analyses of plasma very long-chain fatty acids should detect some peroxisomal beta-oxidation defects not detected by analyses of the bile acids, and analyses of bile acids should enable the detection of some peroxisomal disorders that are not associated with aberrant very long-chain fatty acid metabolism. To date, the published data regarding bile acids in peroxisomal disorders are limited and pertain primarily to the generalized peroxisomal disorders such as Zellweger syndrome and, to a much lesser extent, a few cases of single peroxisomal enzyme deficiencies. Thus far, the data indicate that the analysis of bile acids is useful for defining disorders in which there is peroxisomal dysfunction of bile acid biosynthesis [Eyssen et al., 1985; Clayton et al., 1987] and, further, that fast atom bombardment-mass spectrometry (FAB-MS) of bile acids may be a rapid and effective diagnostic screening test for many peroxisomal disorders [Lawson et al., 1986; Clayton et al., 1990; Libert et al., 1991]. In this study we sought to determine the efficacy of FAB-MS of urinary bile acids for the diagnosis of a specific peroxi-

somal disorder, bifunctional enzyme deficiency. In addition, because bile acids have not been studied in this disorder except in isolated cases, we sought to provide a preliminary cataloging of the variety of urinary bile acid abnormalities in a large cohort of individuals with this diagnosis.

MATERIALS AND METHODS

Patients

Urine specimens from 12 patients with bifunctional enzyme deficiency were analyzed in this study. All 12 patients had extensive peroxisomal metabolic testing in the laboratories of The Kennedy Krieger Institute, Baltimore, MD, that established that they had deficient function of a single peroxisomal beta-oxidation enzyme, in contrast to the multiple peroxisomal enzymatic deficiencies present in cells from individuals with Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. The analyses included determinations of plasma very long-chain fatty acids and phytanic acid; fibroblast very long-chain fatty acids; fibroblast oxidation of very long-chain fatty acids and phytanic acid; fibroblast plasmalogen biosynthesis; and fibroblast catalase latency [Watkins et al., 1995]. Immunoblot analyses of peroxisomal beta-oxidation activities and cell hybridization/genetic complementation analyses were then performed to establish the diagnosis of bifunctional enzyme deficiency [Watkins et al., 1989; McGuinness et al., 1993]. Complementation studies involving cells from individuals 90-762, 90-773 and 94-280 were reported previously; those individuals were designated P3, P2 and P4, respectively [McGuinness et al., 1993]. Clinical and some biochemical information about the individuals studied here was recently published [Watkins et al., 1995].

Analysis of Urinary Bile Acids

The analysis of urinary bile acids and their conjugates was accomplished by direct injection-continuous flow FAB-MS. Briefly, the bile acids from 2 ml of urine were extracted by passage over a C-18 extraction cartridge, which was washed with water and hexane and eluted with methanol. The eluates were dried and redissolved in 0.1 ml of 50% methanol containing 5% glycerol. The extracts from each urine were injected into the FAB-MS probe for FAB analysis using a VG 70-250 SE double focusing mass spectrometry system equipped with an Ion Tech saddle field fast atom source. Negative ion spectra were acquired by scanning from mass:charge ratios (m/z) 360 to 710. A more detailed description of the FAB-MS method and the method for reduction of bile acids using sodium borodeuteride have been described previously [Evans et al., 1993; Natowicz and Evans, 1994]. The rank order of intensity of the 10 most abundant $[M-H]^-$ ions was established for each individual's urine, after subtracting the contribution of ^{13}C isotope-containing ions.

RESULTS AND DISCUSSION

A diagnosis of peroxisomal bifunctional enzyme deficiency can be accomplished by several means: (1) a determination of bifunctional enzyme activity [Wanders et al., 1990a]; (2) metabolic labeling and immunoblot analyses of the bifunctional protein [Watkins et al., 1989; Suzuki et al., 1994]; (3) cell hybridization/genetic complementation analysis [Wanders et al., 1992; McGuinness et al., 1993; Suzuki et al., 1994]; and (4) analysis of bile acids [Libert et al., 1991]. Each of these methods has strengths and limitations. The complexity and limited availability of each of these assays as well as the relatively low frequency of the disorder accounts for the small number of cases of peroxisomal bifunctional enzyme deficiency that have been reported [Naidu et al., 1988; Watkins et al., 1989; Wanders et al., 1990a, 1992; Libert et al., 1991; McGuinness et al., 1993; Suzuki et al., 1994]. Minimal or no data were reported regarding the bile acids in the individuals with bifunctional enzyme deficiency who were the subjects of those reports, with the exception of 2 siblings discussed by Clayton et al. [1990] who were subsequently reported to have bifunctional enzyme deficiency [Wanders et al., 1990a] and one patient reported by Libert et al. [1991]. Our FAB-MS analysis of the bile acids of 12 individuals with peroxisomal bifunctional protein deficiency, therefore, represents the largest study of urinary bile acids in bifunctional enzyme deficiency.

All 12 individuals studied here had abnormal urinary bile acid profiles when evaluated by FAB-MS, although their FAB-MS profiles (Fig. 2) and rank order of intensity of ions (Table I) varied considerably. Previous investigators established that the presence of an $[M-H]^-$ ion at m/z 554, 570, or 586 is strongly suggestive of taurine-conjugated tri, tetra, and pentahydroxycholestenoates, respectively, and can be diagnostic of peroxisomal bifunctional enzyme deficiency [Libert et al., 1991]. Using this as a guide, we noted that 10 of the 12 individual's urine specimens were suspicious or diagnostic of a defect in the peroxisomal beta-oxidation of bile acids since those 10 individuals had abnormally increased urinary FAB-MS signals at m/z 570 (Table I). That it was not an oxocholestanoate of identical m/z was established by its lack of reduction by sodium borodeuteride in each of the 3 cases where this reaction was performed (data not shown). However, in contrast to the 3 cases reported in the literature [Clayton et al., 1990; Libert et al., 1991], 2 of our patients did not have urinary $[M-H]^-$ ions at m/z 570 or any other ions known to be diagnostic of a peroxisomal beta-oxidation disorder (Table I). These data indicate that most (approximately 80–85%) but not all individuals with peroxisomal bifunctional protein deficiency can be detected by FAB-MS analysis of urinary bile acids.

Nine of the 12 patients studied had additional ions of considerable abundance that not only supported the diagnosis of a peroxisomal disorder, but also supported the possibility of a bifunctional enzyme deficiency. Some of these $[M-H]^-$ ions (Table I) and their possible identities (Table II) are at m/z 415, 447, 463, 479, 511, and 527. Each of these ions is consistent with either a cholestenoate or an oxocholestanoate and not a cholanoate or a conjugate of a cholanoate. Other structures are also possible and some of these ions may represent di- and trihydroxylated fatty acid glucuronides observed in generalized peroxisomal disorders [Street

TABLE I. Major Urinary FAB-MS $(M-H)^-$ Ions in Bifunctional Deficiency

	Mass/charge (m/z)[a]										
Patient	1	2	3	4	5	6	7	8	9	10	570?[b]
92-558	429	386	627	445	443	607	433	415	643	461	+
92-255	411	413	429	481	383	409	410	463	503	461	+
92-247	429	627	386	445	583	539	643	525	541	607	−
93-216	530	480	528	546	514	464	531	512	469	544	+
92-258	417	429	419	421	463	641	461	627	445	405	+
90-762	627	509	463	527	525	511	429	411	469	570	+
94-280	411	429	527	463	641	465	413	625	621	443	+
93-207	505	489	461	433	415	463	487	417	443	627	+
91-219	530	480	481	570	464	463	469	627	528	393	+
90-773	481	463	511	509	479	527	641	545	497	627	+
93-203	481	463	417	419	465	461	641	415	479	447	+
93-211	530	480	528	514	510	494	464	429	478	427	−

[a] The FAB-MS $[M-H]^-$ ions are listed in rank order of intensity with m/z #1 indicating the ion of greatest intensity and 10 indicating the ion with the tenth most intense response.
[b] The 570? indicates whether an ion at m/z 570 was present in the spectra, regardless of whether it was one of the 10 most abundant ions, with (+) indicating that the ion was present above background and (−) indicating an absence of that ion in the scan.

et al., 1995]. Of the 3 urine specimens that were reduced with borodeuteride, ions at m/z 447, 463, and 479 did not reduce, ions at m/z 511 and 527 had partial shifts, and there were inadequate intensities of ions at m/z 415 to monitor any potential shifts (data not shown). This indicates that ions at m/z 447, 463, and 479 are not oxocholestanoates and that ions at m/z 511 and 527 are probably mixtures of cholestenoates and oxocholestanoates. Of these ions, the $[M-H]^-$ ion at m/z 463 was particularly common and helpful in establishing the diagnosis of bifunctional enzyme deficiency, although its identity is not known. This ion was noted to be a prominent abnormal species in the bile acid analyses in 3 cases of bifunctional enzyme deficiency described previously [Clayton et al., 1990; Libert et al., 1991].

TABLE II. Possible Identities of Major Diagnostic Urinary FAB-MS $[M-H]^-$ Ions in Bifunctional Enzyme Deficiency

Mass:charge ratio of $[M-H]^-$ ion	Possible identity(ies)
415	monohydroxycholestenoate
417	monohydroxycholestanoate
433	dihydroxycholestanoate
447	trihydroxycholestenoate
463	tetrahydroxycholestenoate
479	pentahydroxycholestenoate
481	pentahydroxycholestanoate
497	hexahydroxycholestanoate
511	dihydroxycholestenoate sulfate
527	trihydroxycholestenoate sulfate
545	tetrahydroxycholestanoate sulfate
570	taurine-conjugated tetrahydroxychlestenoate
625	GlcA[a]-conjugated trihydroxycholestanoate or GlcNAc[b]-conjugated tetrahydroxycholestenoate or GlcA-conjugated pentahydroxycholestenol
641	GlcA-conjugated tetrahydroxycholestanoate or GlcNAc-conjugated pentahydroxycholestenoate or GlcA-conjugated hexahydroxycholestenol

* The ions listed above could also be consistent with other structures since FAB-MS provides molecular mass data but no structural information. Ions listed as cholestenoates could also be consistent with oxocholestanoates containing one less hydroxyl group.
[a] GlcA, glucuronic acid.
[b] GlcNAc, N-acetylglucosamine.

Other urinary $[M-H]^-$ ions that were in abundance and were suggestive of a peroxisomal beta-oxidation disorder include ions at m/z 417, 481, 487, 545, 625, and 641. Each of these ions is consistent with a cholestanoate and not with a cholanoate, although ions at m/z 625 and 641 could also be consistent with cholestenoates, and others such as ions at m/z 417 and 487 could also be consistent with hydroxy fatty acid glucuronides [Street et al., 1995] (Table II). Like m/z 463, ions at m/z 481 and/or 417 were especially prominent in a subset of the patients with bifunctional enzyme deficiency (Table I).

The variability in urinary spectra raises the possibility that distinct biochemical and perhaps clinical or genetic subsets of individuals with bifunctional enzyme deficiency might be recognized by FAB-MS analysis of the urinary bile acids. Figure 3 shows this interindividual variability. In 2 of the 12 individuals, the pattern was indicative of significant liver disease and, because hepatic pathology can be part of the natural history of most peroxisomal beta-oxidation disorders [Kelley, 1991; Roels et al., 1991, 1993], that pattern is likely a secondary phenomenon of little diagnostic utility. Several other spectral patterns were noted including one dominated by ions at m/z 411, 429 and/or 417, one dominated by ions at m/z 481 and/or 463, and one dominated by m/z 489 and 505.

The existence of multiple diagnostic FAB-MS profiles in bifunctional enzyme deficiency is particularly interesting in view of the recent finding of at least 2 distinct genetic complementation groups for this enzyme deficiency [McGuinness et al., 1993]. Human peroxisomal bifunctional protein consists of a single polypeptide and has 2 known enzymatic activities, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities [Reddy et al., 1987]. There is considerable structural and functional similarity between the human enzyme

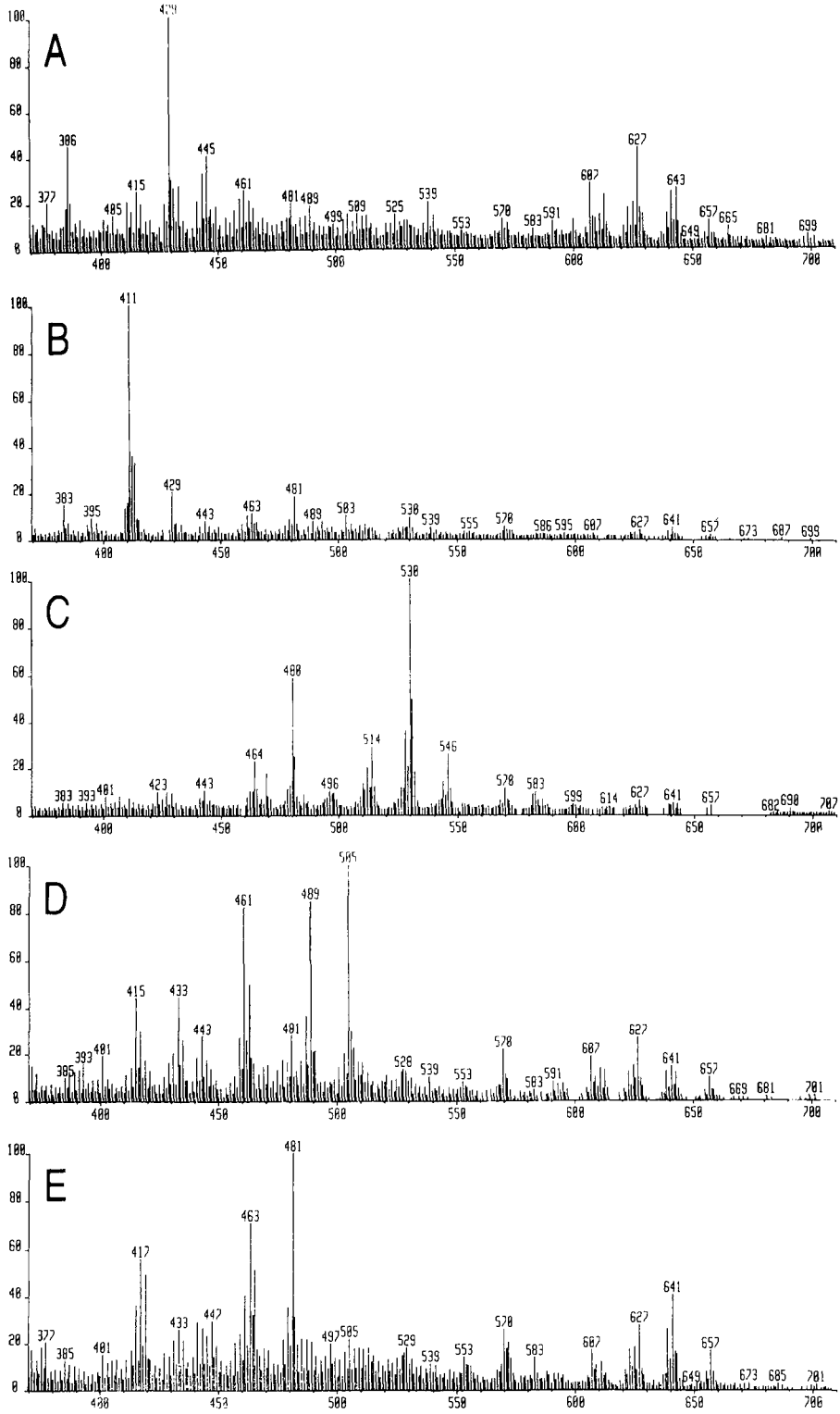

Fig. 3. The direct injection-continuous flow fast atom bombardment mass spectrometry spectra from m/z 370 to m/z 700 of the urine from 5 individuals with peroxisomal bifunctional enzyme deficiency. **A:** Patient 92-558. **B:** Patient 92-255. **C:** Patient 93-216. **D:** Patient 93-207. **E:** Patient 93-203.

and rat bifunctional protein [Reddy et al., 1987], with the latter also possessing both hydratase and dehydrogenase functions [Osumi and Hashimoto, 1979] that are probably localized at separate sites in the protein [Furuta et al., 1980] and gene [Ishii et al., 1987]. More recent data indicate that rat peroxisomal "bifunctional" protein is a trifunctional enzyme that also possesses enoyl-CoA isomerase activity [Palosaari and Hiltunen, 1990] and that the peroxisomal "bifunctional" enzyme from *Candida tropicalis* is a trifunctional enzyme with similar hydratase and dehydrogenase, as well as 3-hydroxyacyl-CoA epimerase activities [Moreno de la Garza et al., 1985]. Taken together, these data suggest the possibility that the presumed interallelic complementation between human bifunctional protein mutants may be due to mutations that affect different active sites, although mutations in noncatalytic regions of the protein such as the peroxisomal targeting domain could be present [Miura et al., 1984; Chen et al., 1991; de Hoop and Ab, 1992].

Structural analyses of the urinary bile acids in individuals with bifunctional protein deficiency potentially offer the opportunity to understand the basis for the interallelic complementation. An absence of functional enzyme should result in an accumulation of cholestenoates with a double bond between C24 and C25, the products of the enzyme that precede the bifunctional protein, bile acid oxidase (Fig. 2). In contrast, the presence of a mutant enzyme that retains hydratase function but lacks dehydrogenase activity would result in an accumulation of hydroxycholestanoates and little or no accumulation of cholestenoates (Fig. 2). The latter, as noted above, are characterized by negative ion mode FAB-MS m/z values that are two units less than their saturated counterparts, the cholestanoates. Our data indicate that at least 10 of the 12 individuals studied here lack functional hydratase enzyme activity since their spectra showed $[M\text{-}H]^-$ ions at m/z 570, instead of 572. Complementation studies of these cases with each other are needed to determine if the presence vs. absence of m/z 570 defines two complementation groups or if there are members of each complementation group represented among the individuals having m/z 570.

It is also possible that the biochemical subsets of bifunctional protein deficiency observed in this study reflect nongenetic parameters. There may be significant effects of diet, medication, or liver disease on the urinary bile acid profile in bifunctional protein deficiency. It is well known that liver disease can affect bile acid metabolism. Limited data also suggest that the metabolism of some of the abnormal bile acids in peroxisomal disorders may be age-related [Eyssen et al., 1985].

The data in this study support the utility of FAB-MS analysis of urinary bile acids for the diagnosis of disorders of peroxisomal beta-oxidation, as has been reported in earlier studies involving fewer subjects. In this study, 80–85% of the individuals were noted to have a defect of peroxisomal beta-oxidation and a deficiency of the peroxisomal bifunctional protein was the provisional or definite diagnosis in each of these cases. This result, together with the inability of the assay for plasma very long-chain fatty acids to detect some of the peroxisomal beta-oxidation disorders such as the presumptive bile acid oxidase deficiency [Christensen et al., 1990] and other bile acid-specific enzyme deficiencies [Przyrembel et al., 1990; Wanders et al., 1991], argues for a role for bile acid analysis in the diagnostic evaluation of suspected peroxisomal disorders.

There are also limitations of FAB-MS analysis of urinary bile acids for the diagnosis of peroxisomal beta-oxidation disorders. FAB-MS of urinary bile acids will not detect cases of peroxisomal fatty acid-specific acyl-CoA oxidase deficiency because bile acids are not a substrate for this enzyme. Our data also show that FAB-MS will not detect all cases of peroxisomal bifunctional protein deficiency for reasons that are unclear. FAB-MS of urinary bile acids also failed to detect a case of peroxisomal thiolase deficiency [Clayton et al., 1990]. These failures may be because some individuals with peroxisomal disorders have very low cholesterol levels and that, in turn, could lead to reduced synthesis of bile acids. Moreover, the interpretation of FAB-MS spectra requires a cautious analysis. Whereas most of our subjects had ions at m/z 570, in many instances the signal at m/z 570 was weak and could easily have been regarded as a minor and unimportant spectral component. In addition, some ions of abundance could be diagnostically misleading because of the inability of FAB-MS to differentiate between stereoisomers, positional isomers and structurally unrelated compounds having the same mass. These limitations of FAB-MS analyses of bile acids for the clinical diagnosis of peroxisomal beta-oxidation defects and other inborn errors of bile metabolism should be readily overcome through the use of HPLC/FAB-MS [Evans et al., 1993]. The latter should also prove helpful in elucidating the structures of other compounds of abundance noted in this study whose structures are currently unknown.

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