Phytanic acid α-oxidation: accumulation of 2-hydroxyphytanic acid and absence of 2-oxophytanic acid in plasma from patients with peroxisomal disorders

H. J. ten Brink,* D. S. M. Schor,* R. M. Kok,* B. T. Poll-The,† R. J. A. Wanders, and C. Jakobs[1,*]**

Department of Pediatrics,* Free University Hospital, Amsterdam, The Netherlands; University Children's Hospital,† Het Wilhelmina Kinderziekenhuis, Utrecht, The Netherlands; and Department of Pediatrics,** Academic Medical Center, Amsterdam, The Netherlands

Abstract A stable isotope dilution method was developed for the measurement of 2-hydroxyphytanic acid and 2-oxophytanic acid in plasma. In plasma from healthy individuals and from patients with Refsum's disease, 2-hydroxyphytanic acid was found at levels < 0.2 μmol/l, whereas the acid accumulated in plasma from patients with rhizomelic chondrodysplasia punctata, generalized peroxisomal dysfunction, and a single peroxisomal β-oxidation enzyme deficiency. In plasma from both healthy controls and patients with peroxisomal disorders, 2-oxophytanic acid was undetectable. Four different groups of diseases were characterized with a defective phytanic acid α-oxidation and/or pristanic acid β-oxidation: *1)* Refsum's disease, with a defect at phytanic acid α-hydroxylation; *2)* rhizomelic chondrodysplasia punctata, with a defect at 2-hydroxyphytanic acid decarboxylation; *3)* generalized peroxisomal disorders, with defects at 2-hydroxyphytanic acid decarboxylation and at pristanic acid β-oxidation; *4)* single peroxisomal β-oxidation enzyme deficiencies, with a defect at pristanic acid β-oxidation, resulting in an impaired phytanic acid α-oxidation by inhibition. The results indicate that 2-hydroxyphytanic acid decarboxylation and pristanic acid β-oxidation take place in peroxisomes.—**ten Brink, H. J., D. S. M. Schor, R. M. Kok, B. T. Poll-The, R. J. A. Wanders, and C. Jakobs.** Phytanic acid α-oxidation: accumulation of 2-hydroxyphytanic acid and absence of 2-oxophytanic acid in plasma from patients with peroxisomal disorders. *J. Lipid Res.* 1992. **33:** 1449–1457.

Supplementary key words pristanic acid β-oxidation • stable isotope dilution analysis • pentafluorobenzyl esters • Refsum's disease • rhizomelic chondrodysplasia punctata • generalized peroxisomal disorders • single peroxisomal β-oxidation enzyme deficiencies

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid only found in trace amounts in healthy human beings. Dietary intake, estimated at 50–100 mg/day is the main source of the acid in humans. As a consequence of the presence of a methyl group at the β-position of its fatty acid chain, phytanic acid cannot be degraded through classical β-oxidation. Instead, the degradation pathway of phytanic acid is believed to involve an initial α-hydroxylation, followed by decarboxylation to yield pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which can be degraded through successive cycles of β-oxidation (**Fig. 1,** see (1) for a review).

Healthy human beings have a large capacity to dissimilate phytanic acid and to prevent its accumulation even at high levels of intake (1). However, a number of diseases is known to be associated with a disturbed phytanic acid degradation, leading to accumulation of the acid in tissues and body fluids. Refsum's disease (heredopathia atactica polyneuritiformis) is the long-known classical example of a "phytanic acid storage disease" (1). A new variety of inherited diseases has been defined, associated with a disturbed function of peroxisomes (see (2) for a review). These so-called peroxisomal disorders can be classified into three groups: *1)* disorders with a generalized peroxisomal dysfunction due to a deficiency of peroxisomes [Zellweger syndrome (ZS), infantile Refsum's disease (IRD), neonatal adrenoleukodystrophy (NALD), hyperpipecolic acidaemia]; *2)* disorders with loss of multiple peroxisomal function [rhizomelic chondrodysplasia punctata (RCDP), Zellweger-like syndrome]; and *3)* disorders with loss of a single peroxisomal function [X-linked adrenoleukodystrophy (X-linked ALD), peroxisomal acyl-CoA oxidase deficiency (pseudo-NALD), bifunc-

Abbreviations: IRD, infantile Refsum's disease; RD, Refsum's disease; RCDP, rhizomelic chondrodysplasia punctata; ECNCI, electron capture negative chemical ionization; SIM, selected ion monitoring; ALD, adrenoleukodystrophy; VLCFA, very long-chain fatty acids.

[1]To whom correspondence should be addressed at: Dept. of Pediatrics, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.

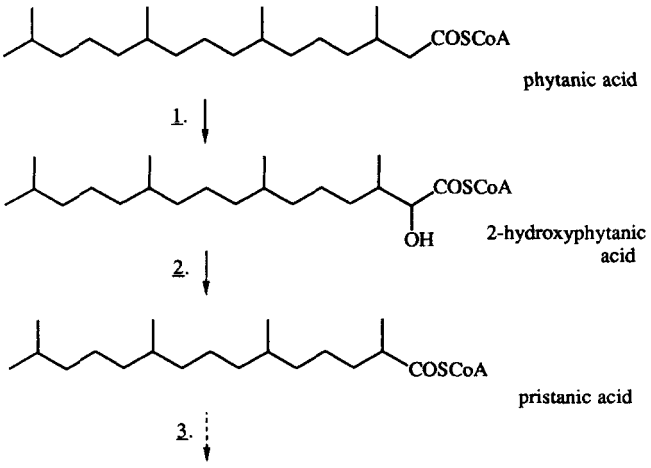

Fig. 1. Metabolism of phytanic acid. 1. α-hydroxylation; 2. decarboxylation; 3. β-oxidation.

tional protein deficiency, peroxisomal 3-oxoacyl-CoA thiolase deficiency (pseudo-ZS), hyperoxaluria type I, acatalasaemia]. Plasma phytanic acid accumulation has been demonstrated to occur not only in generalized peroxisomal disorders, RCDP, and Refsum's disease, but also in disorders with a defect in the peroxisomal β-oxidation at the level of bifunctional protein and/or peroxisomal 3-oxoacyl-CoA thiolase (3).

Measurement of a reduced release of $^{14}CO_2$ in cultured skin fibroblasts incubated with [1-^{14}C]phytanic acid is accepted to be diagnostic of defective phytanic acid α-oxidation. It has been well established that in RD the biochemical defect is located at the first step in the α-oxidation of phytanic acid, i.e., its conversion into 2-hydroxyphytanic acid (4, 5). Also, in generalized peroxisomal disorders and RCDP a reduced production of $^{14}CO_2$ is measured in fibroblasts incubated with [1-^{14}C]phytanic acid, suggesting that peroxisomes are involved in phytanic acid α-oxidation (2). However, it has never been demonstrated that peroxisomal activity is required for both individual steps in this latter process.

Recently we observed that orally administered [1-^{13}C]phytanic acid was converted to labeled 2-hydroxyphytanic acid in a chondrodysplasia punctata patient, but not in an RD patient (6). This finding indicates that in these diseases phytanic acid degradation is impaired at a different level, and prompted us to investigate in more detail whether the known impairment of phytanic acid α-oxidation in a number of peroxisomal disorders is associated with a diminished capability to convert phytanic acid into its first metabolite, 2-hydroxyphytanic acid. Although plasma concentrations of metabolites may not fully reflect the rates of biochemical processes involved in their generation, they are useful parameters to provide insight into differences in metabolic capacities.

In this paper we describe the measurement of 2-hydroxyphytanic acid in plasma from patients suffering from various peroxisomal disorders. The method consists of stable isotope dilution analysis of the acid using a $^2H_3$-labeled analogue of the substance as internal standard for quantitation. In addition, a similar method was used to investigate whether 2-oxophytanic acid, a possible intermediate in the conversion of 2-hydroxyphytanic acid into pristanic acid, is present in plasma from these patients.

PATIENTS

Investigations were carried out in plasma from patients whose diagnosis previously had been established based on clinical examination and confirmed biochemically. Classification into three groups was according to Wanders et al. (2). Control plasma was obtained from healthy individuals (age 3 weeks–45 years) without any symptoms of metabolic disease or impaired renal and liver function.

METHODS

Materials

Unlabeled 2-hydroxyphytanic acid was synthesized from phytanic acid. Bromine was introduced into the α-position by Hell-Volhard-Zelinsky reaction (7), and ethanolysis of the reaction mixture yielded 2-bromophytanic acid ethyl ester. Bromide was substituted by acetate using sodium acetate in dimethylsulfoxide, and the resulting diester was saponified with potassium hydroxide in ethanol giving 2-hydroxyphytanic acid after acidification.

2-Oxophytanic acid was synthesized from 2-hydroxyphytanic acid. The acid function was first protected by esterification with ethanol, and subsequently the resulting ethyl ester was oxidized with chromium(VI) oxide to 2-oxophytanic acid ethyl ester. 2-Oxophytanic acid was stored as its ethyl ester. Its identity was confirmed by GC–MS analysis of the PFB-quinoxalinol derivative prepared by reaction with 1,2-diaminobenzene (8).

In an analogous way [3-methyl-$^2H_3$]-labeled 2-hydroxyphytanic and 2-oxophytanic acids were synthesized from [3-methyl-$^2H_3$]phytanic acid (9).

Plasma analysis

Plasma analysis of pristanic and phytanic acids as their pentafluorobenzyl (PFB) esters was as described previously (3). Analyses of 2-hydroxyphytanic acid and 2-oxophytanic acid were performed in 200 μl plasma. After addition of the internal standards (0.05 nmol [3-methyl-$^2H_3$]2-hydroxyphytanic acid and 0.01 nmol [3-methyl-$^2H_3$]2-oxophytanic acid ethyl ester) to the samples, hydrolysis was performed by first adding 2.0 ml 0.5 M HCl/acetonitrile and heating for 45 min at 100°C, fol-

lowed by addition of 2.0 ml 1 M NaOH/methanol and heating for 45 min at 100°C. The cooled solutions were acidified with 6 N HCl to pH 1 and extracted with hexane (3 × 3 ml). The combined hexane extracts were evaporated to dryness at 40°C under a stream of nitrogen. Carboxylic acids were converted to PFB esters by treating the residue with 100 μl 7% PFB-Br/acetonitrile and 10 μl triethylamine for 15 min at room temperature. After adding 150 μl 0.5 N HCl, PFB esters were extracted into 1 ml hexane, which was concentrated to 50 μl. From this fraction 1 μl was used for GC-MS analysis of 2-oxophytanic acid.

Prior to analysis of 2-hydroxyphytanic acid, the remaining hexane extract was purified over a silica solid phase extraction column (volume 2.8 ml, Bond Elut, Analytichem Int. Inc.). The column was solvated with 10 ml hexane, and the extract containing PFB esters was introduced onto the column. After rinsing with 2 ml hexane and 10 ml 1% ethylacetate/hexane the column was eluted with 10 ml 5% ethylacetate/hexane. The eluent was evaporated to dryness at 40°C under a stream of nitrogen. Hydroxyl groups were derivatized by adding 100 μl bis-trimethylsilyltrifluoroacetamide (BSTFA) and 100 μl pyridine to the residue and heating for 60 min at 80°C. From this mixture 1 μl was used for GC-MS analysis of 2-hydroxyphytanic acid.

GC-MS was performed on a Kratos MS 80 mass spectrometer equipped with a Data General 120S/Eclipse data system and a Carlo Erba HRGC 5300 gas chromatograph. Chromatographic separation was achieved on a CP-Sil 19 CB capillary column (25 m × 0.22 mm, film thickness 0.21 μm, Chrompack, Middelburg, The Netherlands) which was inserted directly into the ion source. Samples (1 μl) were introduced onto the column using a glass falling needle injector operating at 320°C. A GC oven temperature program was applied: the initial temperature of 150°C was raised to 250°C in 30°/min, kept constant for 1 min, and raised to 320°C in 10°/min. The GC-MS interface temperature was 280°C. Helium was used as carrier gas with a linear flow rate of 30 cm/sec.

The operating conditions for electron capture negative chemical ionization (ECNCI) detection were as follows: source temperature 280°C, moderating gas methane at optimized source pressure, electron energy 40 eV, filament emission current 1.5 mA. The resolving power was 1000 (10% valley). Mass spectra were recorded at a scan speed of 1 sec/decade.

Substances were analyzed by selected ion monitoring measurement of carboxylate anion fragments (loss of the PFB moiety) with – *m/z* values 399 (2-O-TMS-2-hydroxyphytanate) and 325 (2-oxophytanate) relative to those of the labeled internal standards (– *m/z* 402 and 328, respectively), with operator-controlled determination of peak areas. Calibration curves were developed using a series of standard solutions containing the labeled internal standards and variable amounts of 2-hydroxyphytanic acid (0.01 nmol–0.1 nmol) and 2-oxophytanic acid ethyl ester (0.005 nmol–0.1 nmol) carried through the entire procedure. Linear regression analysis was used to calculate plasma concentrations of the acids.

RESULTS

In **Fig. 2** the ECNCI mass spectra of 2-O-TMS-2-hydroxyphytanic acid and 2-oxophytanic acid PFB esters and their $^2H_3$ labeled analogues are depicted. Fragmentation almost exclusively consists of loss of the PFB moiety, resulting in the generation of the respective carboxylate anions. Selected ion monitoring (SIM) measurement of these ions was performed for quantitative analysis of the analytes. Calibration curves of both acids yielded straight lines (correlation coefficients 0.998–0.999).

Typical SIM chromatograms obtained from a 200 μl control plasma sample are shown in **Fig. 3.** A substance with a mass fragment identical to the carboxylate anion of 2-oxophytanic acid (– *m/z* 325, Fig. 3b) was observed almost co-eluting with the labeled internal standard. However, the difference in retention time of both compounds was sufficient to establish that this substance was not 2-oxophytanic acid PFB ester. Although the method enables the measurement of concentration values down to 0.003 μmol/l for both acids, 2-oxophytanic acid appeared to be undetectable in the control plasmas, or in plasma from all patients investigated.

Concentrations of phytanic acid, 2-hydroxyphytanic acid, and pristanic acid were determined in plasma from healthy persons and from patients with various peroxisomal disorders and RD. The results are shown in **Table 1.** The 2-hydroxyphytanic acid concentrations measured in control plasma were in the percentage range of phytanic acid levels. In plasma from a number of patients with a generalized peroxisomal dysfunction, especially IRD, 2-hydroxyphytanic acid was found elevated. Also, in RCDP and bifunctional protein/peroxisomal 3-oxoacyl-CoA thiolase deficiency, increased levels of 2-hydroxyphytanic acid were measured. In plasma from RD and X-linked ALD patients, levels were within the control range.

In **Fig. 4,** concentrations of 2-hydroxyphytanic acid in plasma from patients with generalized peroxisomal dysfunction, RCDP, and RD are given relative to those of phytanic acid. In generalized peroxisomal diseases these concentrations appear strongly related, whereas in RCDP and RD the ratios between 2-hydroxyphytanic acid and phytanic acid are different, not only from those in the former diseases, but also among themselves.

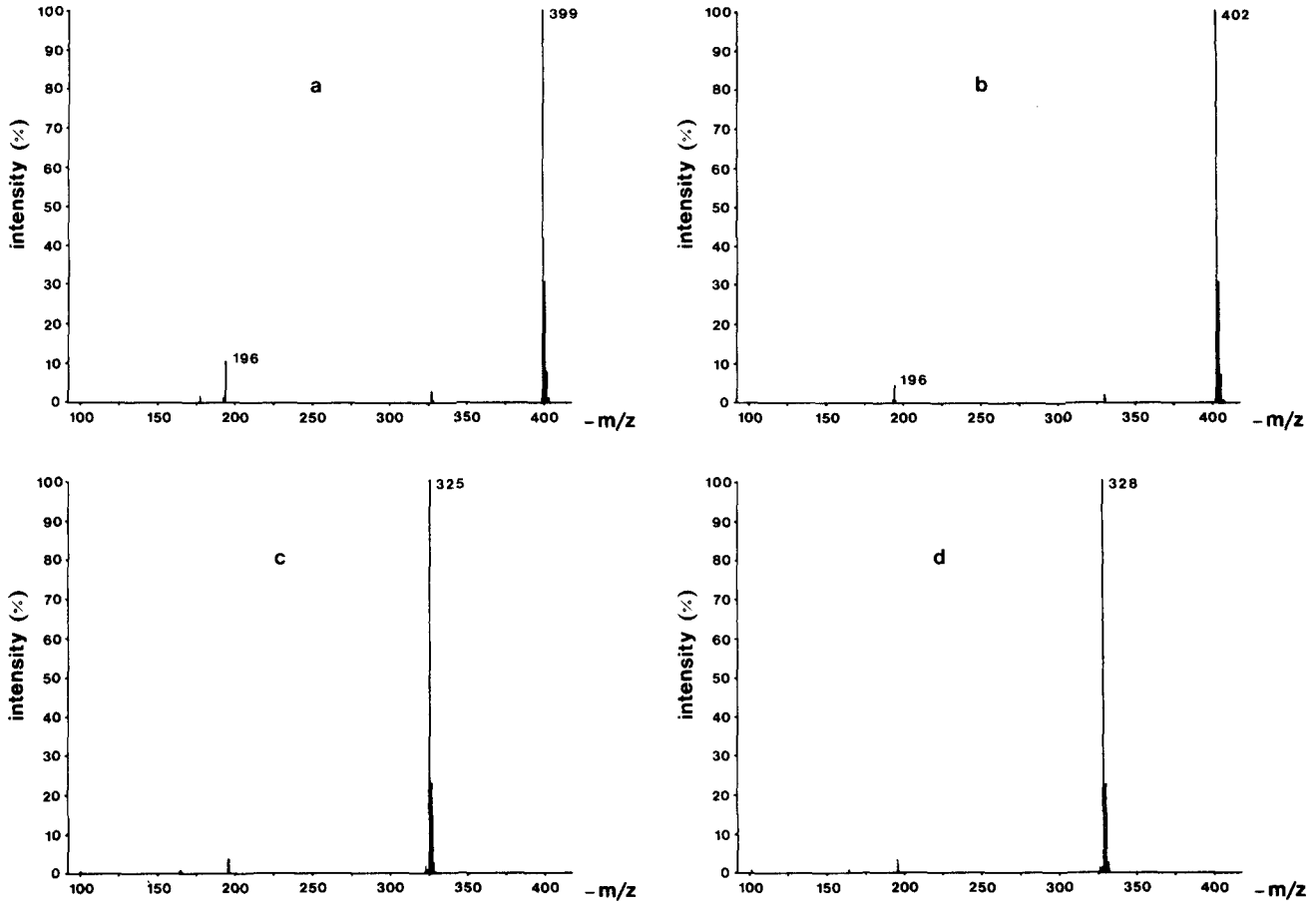

Fig. 2. ECNCI mass spectra of PFB esters of: (a) 2-O-TMS-2-hydroxyphytanic acid; (b) [3-methyl-$^2H_3$]2-O-TMS-2-hydroxyphytanic acid; (c) 2-oxophytanic acid; (d) [3-methyl-$^2H_3$]2-oxophytanic acid.

DISCUSSION

Reduced production of $^{14}CO_2$ in cultured skin fibroblasts incubated with [1-^{14}C]phytanic acid is observed in the generalized peroxisomal disorders, RCDP and Refsum's disease. This phenomenon is generally accepted to be indicative of a defect in the α-oxidation of phytanic acid, and the suggestion that this latter process takes place in peroxisomes is obvious. However, the finding alone that conversion of phytanic acid into pristanic acid and CO_2

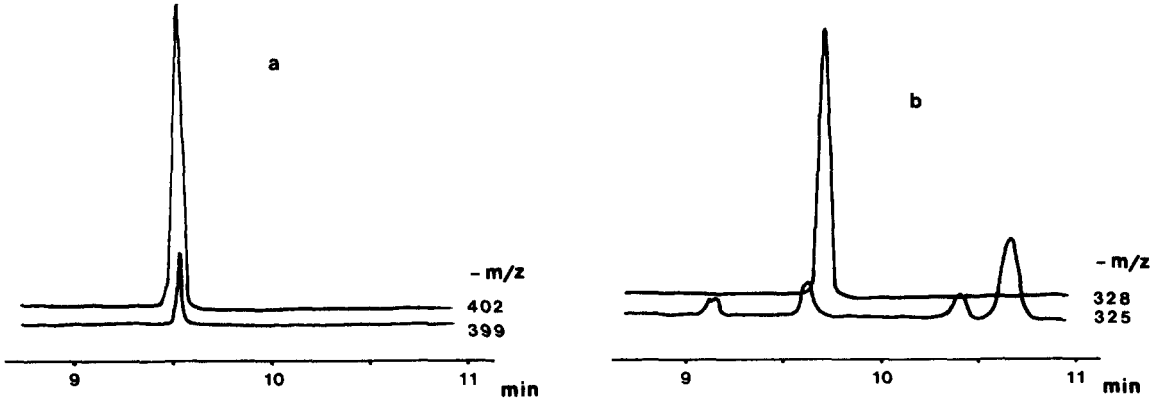

Fig. 3. Mass fragmentogram of PFB esters of unlabeled and [3-methyl-$^2H_3$]-labeled 2-O-TMS-2-hydroxyphytanic (a) and 2-oxophytanic (b) acids, derived from 0.2 ml of control plasma.

TABLE 1. Concentrations of phytanic acid (PhA), pristanic acid (PrA) and 2-hydroxyphytanic acid (OH-PhA) in plasma from patients with various peroxisomal disorders and Refsum's disease

Patients	PhA	PrA	OH-PhA
		μmol/l	
1. Generalized peroxisomal dysfunction			
Zellweger syndrome			
1	12.2	4.29	0.039
2	148	34.7	0.562
3	28.2	6.87	0.348
4	n.d.	n.d.	0.229
5	n.d.	n.d.	0.420
Infantile Refsum's disease			
1	177	38.6	0.737
2	232	83.3	1.355
3	13.1	3.86	0.417
4	135	65.6	1.181
NALD			
1	76.6	21.0	0.117
1[a]	5.05	1.25	0.015
2	25.3[b]	n.d.	0.190
3	73.4[b]	n.d.	0.220
4	68.5[b]	n.d.	0.533
2. Multiple peroxisomal dysfunction			
RCDP			
1[c]	232	1.17	0.405
2	373[b]	n.d.	0.168
3	336[b]	n.d.	0.414
4	699[b]	n.d.	0.425
3. Single enzyme defects			
X-linked ALD			
1	4.91	1.04	0.077
2	5.91	1.49	0.102
3	5.00	0.42	0.108
Bifunctional protein/peroxisomal 3-oxoacyl-CoA thiolase deficiency			
1	5.45	33.1	0.317
2	50.3	68.6	0.462
Refsum's disease			
1	875	0.59	0.030
2	868	0.01	0.050
3	372	0.11	0.087
Controls (n)	(90)	(90)	(13)
Mean ± S.D.	3.22 ± 2.67	0.45 ± 0.50	0.099 ± 0.038
(Range)	(0.04-9.88)	(0.01-2.98)	(0.022-0.188)

[a]After prescription of a diet containing low levels of phytanic acid.
[b]Determined elsewhere (10).
[c]Non-rhizomelic CDP, featuring with biological findings associated with peroxisomal dysfunction (impaired phytanic acid α-oxidation capacity, impaired plasmalogen biosynthesis, unprocessed 3-oxoacyl-CoA thiolase); her case has been described (11).
n.d., not determined.

is disturbed in these diseases provides no information about the possible differences in the individual enzymatic steps constituting the phytanic acid α-oxidation, nor about the subcellular location of the individual enzymes involved. For this purpose more detailed studies are required, similar to those that have resulted in the elucidation of the enzyme defect in RD (1).

In this paper a mass spectrometric method is introduced to measure 2-hydroxyphytanic acid and 2-oxophytanic acid in plasma. Electron capture negative chemical ionization of PFB esters is a highly efficient ionization process. In combination with the low fragmentation due to the generation of a stable carboxylate anion, this leads to a very sensitive detection method, increasingly applied in the analysis of acidic substances in body fluids (3, 12–15). A high degree of accuracy and specificity is further obtained by the use of stable isotopically labeled analogues of the analytes as internal standards.

In plasma from healthy persons, 2-hydroxyphytanic acid was demonstrated to be present in readily measura-

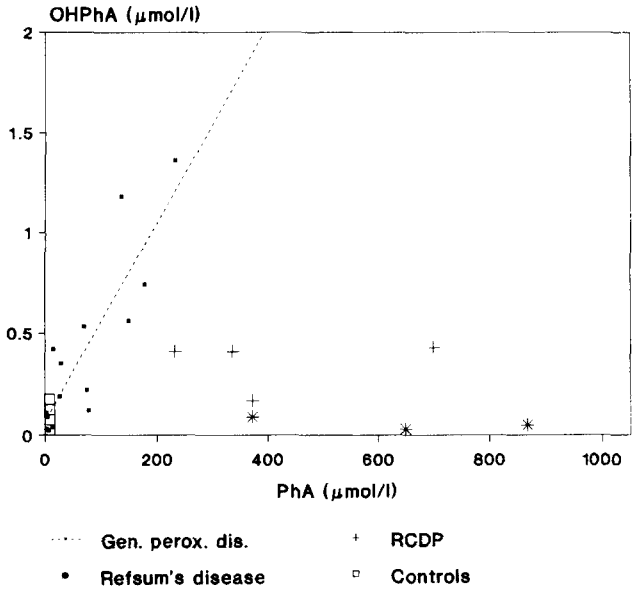

Fig. 4. Relative concentrations of phytanic acid (PhA) and 2-hydroxyphytanic acid (OHPhA) in plasma from patients with generalized peroxisomal disorders, RCDP and RD.

ble amounts. Control plasma values of 2-hydroxyphytanic acid were substantially lower than phytanic acid and pristanic acid levels. This may reflect that α-hydroxylation of phytanic acid is the rate-limiting step in phytanic acid α-oxidation; alternatively, this may be the consequence of the higher polarity of the hydroxyacid, resulting in a less ready release from the cells or a more rapid clearance from the plasma.

In order to investigate in detail the individual steps in phytanic acid α-oxidation, quantitative analysis of phytanic acid, 2-hydroxyphytanic acid, and pristanic acid was performed in plasma from patients suffering from various peroxisomal disorders and RD. All these diseases are associated with a disturbed degradation of phytanic acid and/or pristanic acid, except X-linked ALD.

The finding of normal plasma concentrations of both pristanic acid and 2-hydroxyphytanic acid in RD demonstrates that in this disease the phytanic acid degradation pathway is not disturbed beyond 2-hydroxyphytanic acid, establishing an impaired α-hydroxylation of phytanic acid as the only biochemical defect. The low amounts of 2-hydroxyphytanic acid found in plasma from RD patients indicate that the net flux of accumulating phytanic acid to 2-hydroxyphytanic acid is in the normal range, resulting from the residual activity of the enzyme catalyzing phytanic acid α-hydroxylation. Indeed, measurements of [1-^{14}C]phytanic acid degradation in cultured skin fibroblasts have revealed that the deficiency is not complete, residual activities being about 5% of control values (1). The pristanic acid levels measured reflect the balance between an undisturbed degradation of the acid and its supply by dietary intake and degradation of the residual amounts of 2-hydroxyphytanic acid.

Our results show that elevated plasma levels of 2-hydroxyphytanic acid are found in all disorders investigated except RD and X-linked ALD. Apparently, although the total phytanic acid α-oxidation, measured as the production of $^{14}CO_2$ in cultured skin fibroblasts incubated with [1-^{14}C]phytanic acid, is disturbed in generalized peroxisomal disorders and RCDP, patients with these diseases show a considerable capacity to convert phytanic acid to 2-hydroxyphytanic acid. This suggests that in these disorders the primary defect in phytanic acid α-oxidation is not located in the α-hydroxylation step, but in the conversion of 2-hydroxyphytanic acid into pristanic acid. The finding that this observation is encountered in disorders known to be defective in peroxisomal function suggests that the conversion of 2-hydroxyphytanic acid into pristanic acid involves peroxisomes.

RCDP seems to be a distinct group when phytanic acid α-oxidation is considered in detail. In RCDP high levels of phytanic acid are found in plasma. However, plasma pristanic acid levels are normal, leading to low pristanic acid/phytanic acid ratios (3). This indicates that the pristanic acid β-oxidation system is not disturbed, and that the defect is located within the phytanic acid α-oxidation itself. The accumulation of 2-hydroxyphytanic acid observed in RCDP indicates that RCDP differs from RD in the first step of phytanic acid α-oxidation. This suggests that in RCDP the primary defect in phytanic acid α-oxidation is located in the second step, being a defective degradation of 2-hydroxyphytanic acid. The high phytanic acid levels observed in plasma from RCDP patients are probably due to strong product inhibition by 2-hydroxyphytanic acid upon its formation from phytanic acid.

In generalized peroxisomal disorders both phytanic acid and pristanic acid accumulate, but pristanic acid/phytanic acid ratios in plasma from these patients are within the control range (3). This indicates that both phytanic acid α-oxidation and pristanic acid β-oxidation are blocked, due to defects in either of the two pathways. In plasma from these patients, levels of 2-hydroxyphytanic acid are also found to be elevated. A strong relationship is found between plasma phytanic acid and 2-hydroxyphytanic acid concentrations in these diseases. Apparently phytanic acid can be degraded to 2-hydroxyphytanic acid, suggesting that the degradation of phytanic acid is disturbed at the stage of the conversion of 2-hydroxyphytanic acid into pristanic acid.

In the disorders with a defect in peroxisomal β-oxidation at the level of bifunctional protein and/or peroxisomal 3-oxoacyl-CoA thiolase, accumulation of phytanic acid is observed. However, accumulation of pristanic acid is still more pronounced, leading to strongly increased pristanic acid/phytanic acid ratios (3). Apparently the

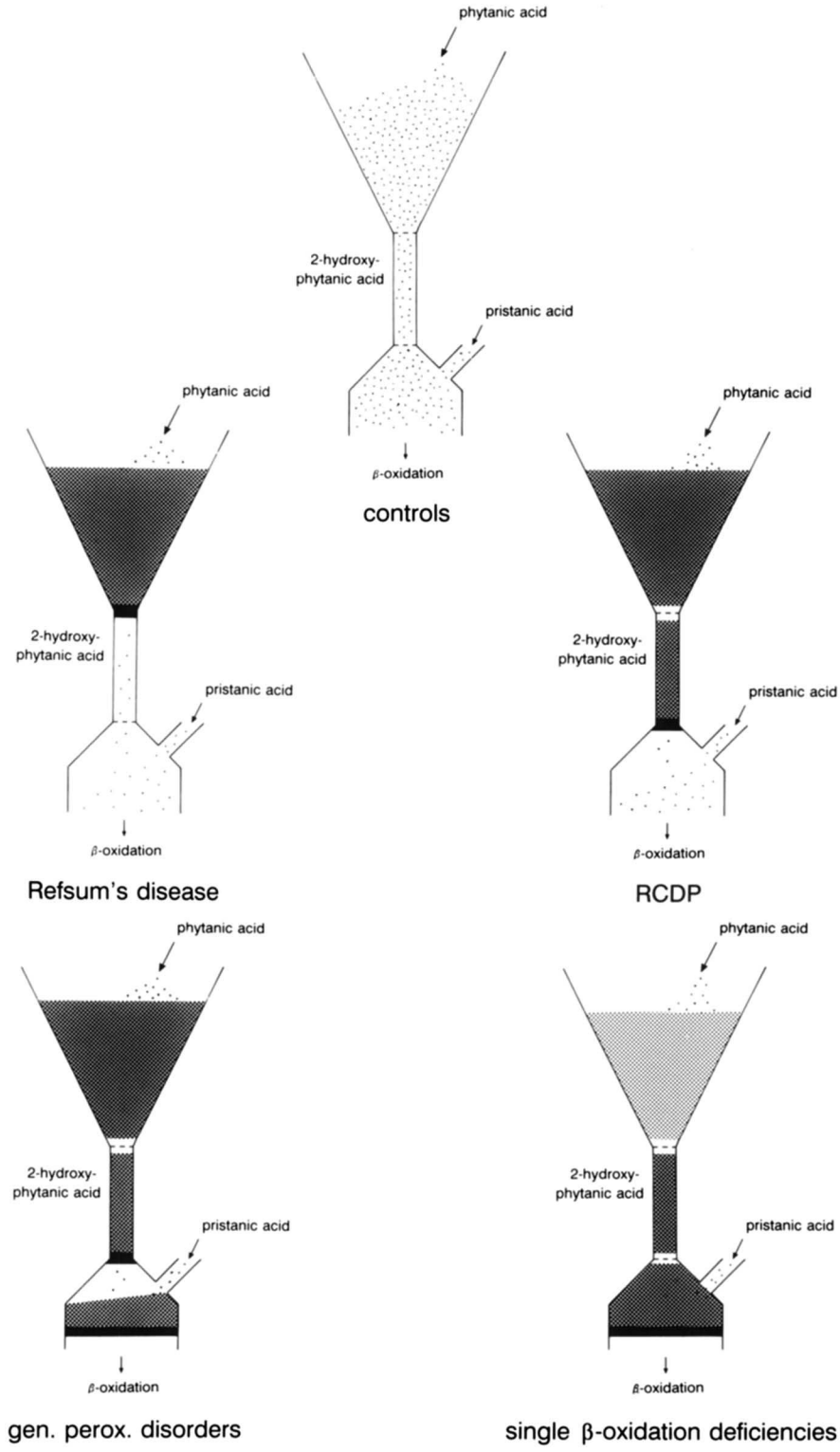

Fig. 5. Model illustrating the proposed localization of metabolic defects in phytanic acid α-oxidation and pristanic acid β-oxidation in various peroxisomal disorders and Refsum's disease. Relative concentrations of substances are depicted with different shades of gray; metabolic blocks are depicted in black.

blocked pristanic acid β-oxidation results in accumulation of this acid, which originates from dietary intake and as a metabolite of phytanic acid. Also, elevated levels of 2-hydroxyphytanic acid are found in these disorders, indicating that phytanic acid α-hydroxylation can proceed. There is no primary enzyme defect in phytanic acid α-oxidation, which was demonstrated in the studies that revealed that bifunctional protein deficiency and peroxisomal 3-oxoacyl-CoA thiolase are single enzyme deficiencies (16, 17). Apparently the accumulating pristanic acid inhibits a normal phytanic acid α-oxidation, probably at the level of the conversion of 2-hydroxyphytanic acid into pristanic acid.

In X-linked ALD, peroxisomal β-oxidation of VLCFA is disturbed due to a deficient VLCFA acyl-CoA synthase (18), resulting in accumulation of VLCFA. In plasma from X-linked ALD patients, normal plasma levels of 2-hydroxyphytanic acid were found. Next to the finding of normal phytanic and pristanic acid plasma levels, this indicates that phytanic acid α-oxidation is not disturbed by a deficient VLCFA acyl-CoA synthase.

Complementation studies on phytanic acid α-oxidation in peroxisomal disorders indicated the existence of four complementation groups: RD, RCDP, NALD, and the group composed of IRD and Zellweger syndrome (19). Our study of plasma concentrations of phytanic acid, 2-hydroxyphytanic acid, and pristanic acid enabled us to distinguish three of the four groups: RD, RCDP, and the group of generalized peroxisomal disorders. Our measurements could not discriminate between NALD and the other two peroxisome deficiency disorders.

It is not sure whether the proposed pathway of phytanic acid α-oxidation depicted in Fig. 1 shows all the intermediates involved in this process. In the second step of this pathway, 2-hydroxyphytanic acid is directly converted into pristanic acid, a decarboxylation step which in this form is unprecedented in nature. It could well be that 2-oxophytanic acid is an intermediate in this process. The formation of this acid has been observed as a product of the peroxisomal oxidation of 2-hydroxyphytanic acid in rat kidney cortex (20, 21). However, although concentrations of 2-oxophytanic acid added to plasma down to 0.003 μmol/l are measurable using the described method, attempts to demonstrate that this substance is present in plasma were unsuccessful, both from controls and from selected patients expected to accumulate this acid due to a defective phytanic acid α-oxidation and/or pristanic acid β-oxidation. The finding that 2-oxophytanic acid does not accumulate under conditions where 2-hydroxyphytanic acid and pristanic acid are elevated might indicate that 2-oxophytanic acid is not an obligate intermediate in phytanic acid α-oxidation. Nevertheless, we cannot completely rule out this possibility. It remains possible that 2-oxophytanic acid is decarboxylated as soon as it is formed, preventing it from being detected in plasma.

In conclusion, our results indicate the existence of four different groups of disorders with a defective phytanic acid α-oxidation and/or pristanic acid β-oxidation: *1)* RD, with a single defect at phytanic acid α-hydroxylation; *2)* RCDP, with a single enzyme defect at 2-hydroxyphytanic acid decarboxylation; *3)* generalized peroxisomal disorders, with a defect at 2-hydroxyphytanic acid decarboxylation and a defect at pristanic acid β-oxidation; and *4)* single peroxisomal β-oxidation enzyme deficiencies with a primary defect at pristanic acid β-oxidation, resulting in an impaired phytanic acid α-oxidation by inhibition. In **Fig. 5** a model is presented in which the above conclusions, drawn from the plasma measurements, are visualized.

Considering that all diseases in which the conversion of 2-hydroxyphytanic acid into pristanic acid is disturbed are peroxisomal disorders, we find strong evidence that this reaction is peroxisomal.

Manuscript received 24 January 1992 and in revised form 6 May 1992.

REFERENCES

1. Steinberg, D. 1989. Refsum disease. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1533–1550.
2. Wanders, R. J. A., H. S. A. Heymans, R. B. H. Schutgens, P. G. Barth, H. van den Bosch, and J. M. Tager. 1988. Peroxisomal disorders in neurology. *J. Neurol. Sci.* **88:** 1–39.
3. ten Brink, H. J., F. Stellaard, C. M. M. van den Heuvel, R. M. Kok, D. S. M. Schor, R. J. A. Wanders, and C. Jakobs. 1992. Pristanic acid and phytanic acid in plasma from patients with peroxisomal disorders: stable isotope dilution analysis with electron capture negative ion mass fragmentography. *J. Lipid Res.* **33:** 41–47.
4. Steinberg, D., J. H. Herndon, Jr., B. W. Uhlendorf, C. E. Mize, J. Avigan, and G. W. A. Milne. 1967. Refsum's disease. Nature of the enzyme defect. *Science.* **156:** 1740–1742.
5. Herndon, J. H., Jr., D. Steinberg, B. W. Uhlendorf, and H. M. Fales. 1969. Refsum's disease: characterization of the enzyme defect in cell culture. *J. Clin. Invest.* **48:** 1017–1032.
6. ten Brink, H. J., D. S. M. Schor, R. M. Kok, F. Stellaard, J. Kneer, B. T. Poll-The, J. M. Saudubray, and C. Jakobs. 1992. In vivo study of phytanic acid α-oxidation in classical Refsum's disease and chondrodysplasia punctata. *Pediatr. Res.* In press.
7. Mendel, H., and J. Coops. 1939. The gradual decomposition by oxidation of fatty acids into their next lower homologues. *Rec. Trav. Chim.* **58:** 1133–1143.
8. Langenbeck, U., H. U. Möhring, B. Hinney, and M. Spiteller. 1977. Quinoxalinol derivatives of aliphatic 2-oxocarboxylic acids. *Biomed. Mass Spectrom.* **4:** 197–202.
9. ten Brink, H. J., C. Jakobs, J. L. van der Baan, and F. Bickelhaupt. 1989. Synthesis of deuterium labeled analogues of pristanic acid and phytanic acid for use as internal standards in stable isotope dilution analysis. *In* Synthesis and Applications of Isotopically Labeled Compounds. T. A. Baillie, and J. R. Jones, editors. Elsevier, Amsterdam. 717–722.
10. Wanders, R. J. A., W. Smit, H. S. A. Heymans, R. B. H. Schutgens, P. G. Barth, H. Schierbeek, G. P. A. Smit, R.

Berger, H. Przyrembel, T. A. Eggelte, J. M. Tager, P. D. Maaswinkel-Mooy, A. C. B. Peters, L. A. H. Monnens, J. A. J. M. Bakkeren, J. M. F. Trijbels, E. J. P. Lommen, and N. Beganovic. 1987. Age-related accumulation of phytanic acid in plasma from patients with the cerebro-hepato-renal (Zellweger) syndrome. *Clin. Chim. Acta.* **166:** 45–56.

11. Poll-The, B. T., P. Maroteaux, C. Narcy, P. Quetin, M. Guesnu, R. J. A. Wanders, R. B. H. Schutgens, and J. M. Saudubray. 1991. A new type of chondrodysplasia punctata associated with peroxisomal dysfunction. *J. Inher. Metab. Dis.* **14:** 361–363.
12. Waddell, K. A., I. A. Blair, and J. Wellby. 1983. Combined capillary column gas chromatography negative ion chemical ionization mass spectrometry of prostanoids. *Biomed. Mass Spectrom.* **10:** 83–88.
13. de Jong, A. P. J. M., R. M. Kok, C. A. Cramers, and S. K. Wadman. 1986. Determination of acidic catecholamine metabolites in plasma and cerebrospinal fluid using gas chromatography electron capture negative ion mass spectrometry. *J. Chromatogr.* **382:** 19–30.
14. Stellaard, F., S. A. Langelaar, R. M. Kok, and C. Jakobs. 1989. Determination of plasma bile acids by capillary gas–liquid chromatography–electron capture negative chemical ionization mass fragmentography. *J. Lipid Res.* **30:** 1647–1652.
15. Stellaard, F., H. J. ten Brink, R. M. Kok, L. van den Heuvel, and C. Jakobs. 1990. Stable isotope dilution analysis of very long chain fatty acids in plasma, urine and amniotic fluid by electron capture negative ion mass fragmentography. *Clin. Chim. Acta.* **192:** 133–144.
16. Watkins, P. A., W. W. Chen, C. J. Harris, G. Hoefler, S. Hoefler, D. C. Blake, Jr., A. Balfe, R. I. Kelley, A. B. Moser, M. E. Beard, and H. W. Moser. 1989. Peroxisomal bifunctional enzyme deficiency. *J. Clin. Invest.* **83:** 771–777.
17. Goldfischer, S., J. Collins, I. Rapin, P. Neumann, W. Neglia, A. J. Spiro, T. Ishii, F. Roels, J. Vamecq, and F. van Hoof. 1986. Pseudo-Zellweger syndrome: deficiencies in several peroxisomal oxidative activities. *J. Pediatr.* **108:** 25–32.
18. Wanders, R. J. A., C. W. T. van Roermund, M. J. A. van Wijland, R. B. H. Schutgens, H. van den Bosch, A. W. Schram, and J. M. Tager. 1988. Direct demonstration that the deficient oxidation of very long chain fatty acids in X-linked adrenoleukodystrophy is due to an impaired ability of peroxisomes to activate very long chain fatty acids. *Biochem. Biophys. Res. Commun.* **153:** 618–624.
19. Poll-The, B. T., O. H. Skjeldal, O. Stokke, A. Poulos, F. Demaugre, and J. M. Saudubray. 1989. Phytanic acid alpha-oxidation and complementation analysis of classical Refsum and peroxisomal disorders. *Hum. Genet.* **81:** 175–181.
20. Draye, J. P., F. van Hoof, E. de Hoffmann, and J. Vamecq. 1987. Peroxisomal oxidation of L-2-hydroxyphytanic acid in rat kidney cortex. *Eur. J. Biochem.* **167:** 573–578.
21. Vamecq, J., and J. P. Draye. 1988. The enzymatic and mass spectrometric identification of 2-oxophytanic acid, a product of the peroxisomal oxidation of L-2-hydroxyphytanic acid. *Biomed. Environ. Mass Spectrom.* **15:** 345–351.