Localization of the Oxidative Defect

in Phytanic Acid Degradation

in Patients with Refsum's Disease

CHARLES E. MIZE, JAMES H. HERNDON, JR., JOHN P. BLASS, G. W. A.
MILNE, CHARLOTTE FOLLANSBEE, PHILIPPE LAUDAT, and
DANIEL STEINBERG

*From the Molecular Disease Branch, National Heart Institute, National
Institutes of Health, Bethesda, Maryland 20014*

A B S T R A C T The rate of oxidation of phytanic acid-U-^{14}C to ^{14}CO$_2$ in three patients with Refsum's disease was less than 5% of that found in normal volunteers. In contrast, the rate of oxidation of α-hydroxyphytanic acid-U-^{14}C and of pristanic acid-U-^{14}C to ^{14}CO$_2$, studied in two patients, while somewhat less than that in normal controls, was not grossly impaired. These studies support the conclusion that the defect in phytanic acid oxidation in Refsum's disease is located in the first step of phytanic acid degradation, that is, in the alpha oxidation step leading to formation of α-hydroxyphytanic acid.

The initial rate of disappearance of plasma free fatty acid radioactivity after intravenous injection of phytanic acid-U-^{14}C ($t_{\frac{1}{2}} = 5.9$ min) was slower than that seen with pristanic acid-U-^{14}C ($t_{\frac{1}{2}} = 2.7$ min) or palmitic acid-1-^{14}C ($t_{\frac{1}{2}} = 2.5$ min). There were no differences between patients and normal controls in these initial rates of free fatty acid disappearance for any of the three substrates tested.

There was no detectable lipid radioactivity found in the plasma 7 days after the injection of palmitic acid-1-^{14}C or pristanic acid-U-^{14}C in either patients or controls. After injection of phytanic acid-U-^{14}C, however, the two patients showed only a very slow decline in plasma lipid radioactivity (estimated $t_{\frac{1}{2}} = 35$ days), in contrast to the normals who had no detectable radioactivity after 2 days. Incorporation of radioactivity from phytanic acid-U-^{14}C into the major lipid ester classes of plasma was studied in one of the patients; triglycerides accounted for by far the largest fraction of the total present between 1 and 4 hr.

INTRODUCTION

Heredopathia atactica polyneuritiformis (HAP, Refsum's disease) (1) has been shown to be an inherited lipid storage disease in which large amounts of an unusual fatty acid, phytanic acid (3, 7, 11, 15-tetramethyl-hexadecanoic acid), accumulate in all body tissues (2, 3). The clinical manifestations have been recently reviewed (4–6). The accumulation of phytanic acid has been shown to be due to a defect in its catabolism (5, 7–9).

A major pathway of phytanic acid metabolism has recently been elucidated in animal studies (10–12), and the occurrence of this pathway in man has been confirmed in tissue cultures of skin fibroblasts (12, 13). These findings have made possible an investigation of the specific site of the defect in this disease. In the current studies, patients with HAP have been studied to compare their oxidative capacity for phytanic acid, α-OH-phytanic acid, and pristanic acid (2,6,10,14-tetramethyl-pentadecanoic acid) with that of controls. Preliminary reports of some of these results have appeared elsewhere (14–16).

METHODS

Two of the patients with HAP studied here in the Clinical Center (K. S. and J. S.) are a brother and sister from Northern Ireland who were transferred to the NIH through

the cooperation of Dr. J. H. D. Millar. Their case histories have been presented in detail elsewhere (5, 17). A third HAP patient (M. G.) was studied in the research unit of Dr. Philippe Laudat at the Institute National de la Santé et de la Récherche Medicale in Paris. Her clinical features have been reported by Bonduelle et al. (18). The control subjects studied in the Clinical Center were normal volunteers except for one patient with type IV hyperlipoproteinemia in whom palmitate oxidation was studied.

D,L-phytanic acid-U^{14}C (SA = 17 μc/μmole; radiopurity > 98%) and D,L-pristanic acid-U-^{14}C (SA = 4.5 μc/μmole; radiopurity > 97%) were prepared from algal phytol-U-^{14}C and purified by preparative gas-liquid chromatography (GLC) as described previously (13, 19). The synthesis of D,L-α-hydroxy-D,L-phytanic acid-U-^{14}C from D,L-phytanic acid-U-^{14}C has been previously described (12) and is discussed in detail in the accompanying paper (13). The methyl ester of the D,L-α-hydroxy-D,L-phytanic acid-U-^{14}C was collected from the single mass peak found on preparative GLC (14% ethylene glycol succinate [EGS]) and shown to be 96% radiopure (SA = 6.9 μc/μmole). The two isomeric fractions resolved by thin-layer chromatography (TLC) are inferred to represent, respectively, a mixture of 2D, 3L plus 2L, 3D, and a mixture of 2D, 3D plus 2L, 3L (13). Palmitic acid-1-^{14}C was purchased from the New England Nuclear Corp., and was at least 98% radiopure by GLC. Radioactive fatty acids were prepared as potassium salts and adsorbed to human serum albumin. The preparations were passed through a Millipore filter and checked for sterility and freedom from pyrogens before use.

The patients were fasted for 12–16 hr before injection of labeled fatty acids, and food was withheld for the 1st 3 hr after injection. Patients receiving labeled branched-chain fatty acids were placed on a low-phytol, low–phytanic acid diet for 24 hr before the study and for at least 2 days thereafter.

The labeled fatty acids were administered by rapid intravenous infusion (10–20 sec) through an indwelling catheter in an antecubital vein. Venous blood samples were withdrawn at intervals for the 1st 12 hr from a plastic catheter in the opposite antecubital vein; later samples were obtained by venipuncture. For measurements of $^{14}CO_2$ production, expired respiratory gases were collected into Douglas bags over exactly measured 2-min intervals. Collections were made every 30 min for the 1st 2 hr, then hourly to 6 hr, and subsequently every 2 hr to 12 hr. Total urine and fecal collections were usually made for at least 4 days. The methods of analysis of plasma and fecal lipid radioactivity, total urinary radioactivity, and radioactivity in respiratory CO_2 have been described (9, 21).

RESULTS

$^{14}CO_2$ *production after intravenous injection of U-^{14}C-labeled fatty acids (Fig. 1).* Phytanic acid was rapidly oxidized by normal controls, 26% of the dose on the average (range 13–39%) being converted to $^{14}CO_2$ in 12 hr. In sharp contrast, the three patients converted only 0.9% (0.2–2.1%) of the dose to $^{14}CO_2$. The time course of $^{14}CO_2$ production in two patients and in four controls is shown in Fig. 2.

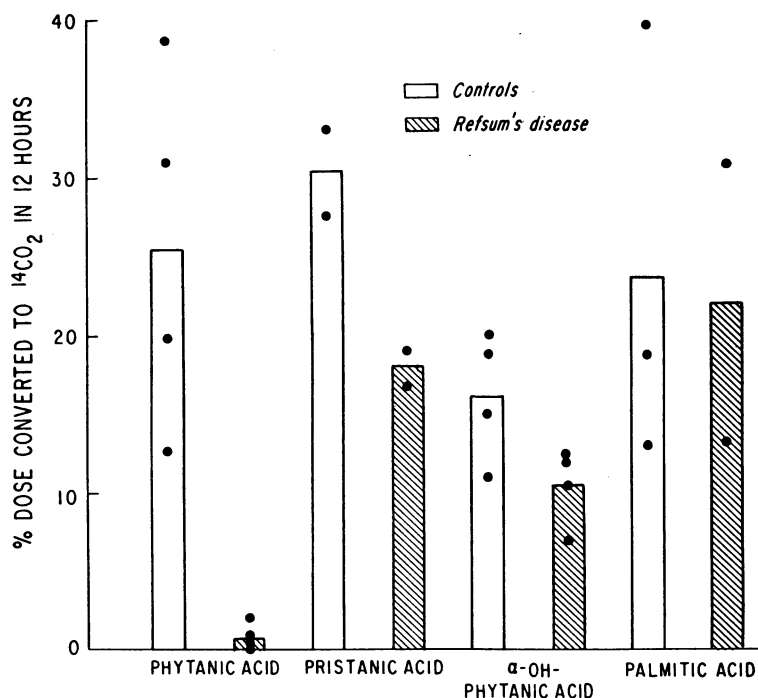Patients K. S. and J. S. were studied twice. On the

FIGURE 1 Percentage conversion of injected phytanic acid-U-^{14}C, pristanic acid-U-^{14}C, α-hydroxyphytanic acid-U-^{14}C, and palmitic acid-1-^{14}C to $^{14}CO_2$ in 12 hr by controls and by patients with HAP. Each point represents results of a single study, and the heights of the bars represent mean values.
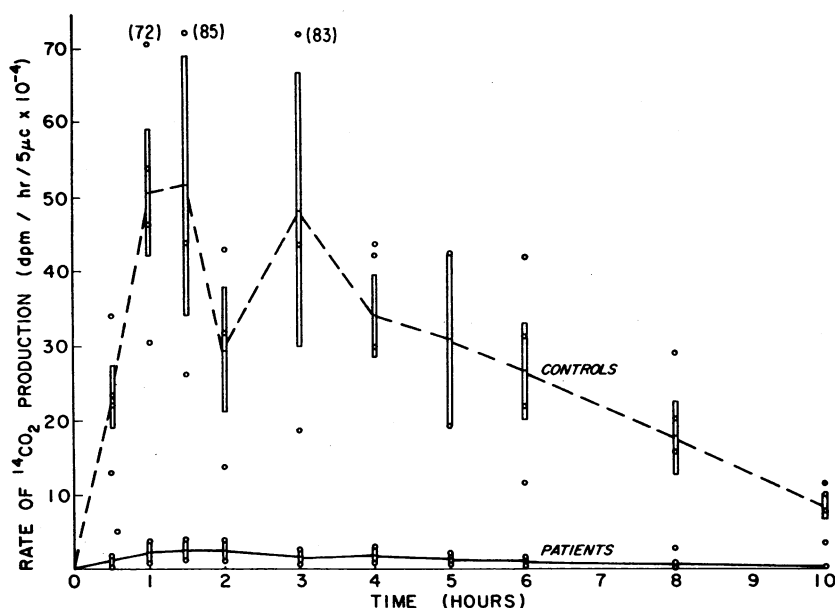
FIGURE 2 Rates of $^{14}CO_2$ production at intervals after injection of phytanic acid-U-^{14}C in three controls and in two patients with HAP. The dashed and solid curves connect the mean values for normals and patients, respectively.

first occasion, shortly after admission, their plasma phytanate levels were very high, about 1000 μg/ml. At that time, the values for fractional oxidation were 0.4 and 1.8%. The second tests were carried out after the patients had been maintained for almost a year on a low-phytol, low-phytanic acid diet, by which time their plasma phytanate levels had dropped by more than 50%. Fractional oxidation values at this time were 0.23 and 0.25%, i.e., lower than the values obtained when the endogenous pools of phytanic acid were larger.

The third patient, with the classical clinical features of Refsum's disease (18) and with high plasma phytanate levels (10–13% of total fatty acids), was studied in collaboration with Dr. Philippe Laudat at the Institut National de la Santé et de la Récherche Médicale in Paris. She oxidized 2.1% of the intravenous dose of phytanic acid-U-^{14}C in 12 hr.

Pristanic acid was oxidized to $^{14}CO_2$ by two normal controls at rates comparable to those observed for phytanic acid, 28 and 33% in 12 hr (Fig. 1). The corresponding values in K. S. and J. S., 17 and 19%, were somewhat lower but the significance of the difference remains uncertain with the limited data available. In the context of the present studies, it is important to note particularly the relative rates of phytanate and pristanate oxidation. In normal subjects, the rate of phytanate oxidation was 82% of that of pristanate, whereas in the HAP patients it was only 3.3%.

α-hydroxyphatanic acid-U-^{14}C was resolved by TLC into two isomeric mixtures (see Methods), and the rates

of oxidation of the labeled material in the upper and lower bands were studied separately (Table I). Both in controls and patients, the material in the upper band was oxidized about 50% more rapidly than that in the lower band. Similar relative rates of oxidation of upper and lower bands have been observed also in human fibroblast cultures (13) and in isolated rat liver mitochondria.[1] Both in patients and in controls, hydroxyphytanate was oxidized less rapidly than pristanate (Fig. 1). Since the rate of oxidation of the two isomeric mixtures, relative to each other, were the same in controls and in patients, the results have been combined in Fig. 2. Again, the most relevant comparison may be between the rates of phytanate and hydroxyphytanate oxidation. In normal subjects, pooling all the data, the rate of phytanate

TABLE I

Oxidation of α-Hydroxyphytanate-U-^{14}C Isomers Injected Intravenously in Normal Subjects and in HAP Patients

| | Percentage of injected dose converted to $^{14}CO_2$ in 12 hr | |
Substrate	Normal subjects	HAP patients
Upper band of isomers	19, 20	12, 12
Lower band of isomers	11, 15	7, 10

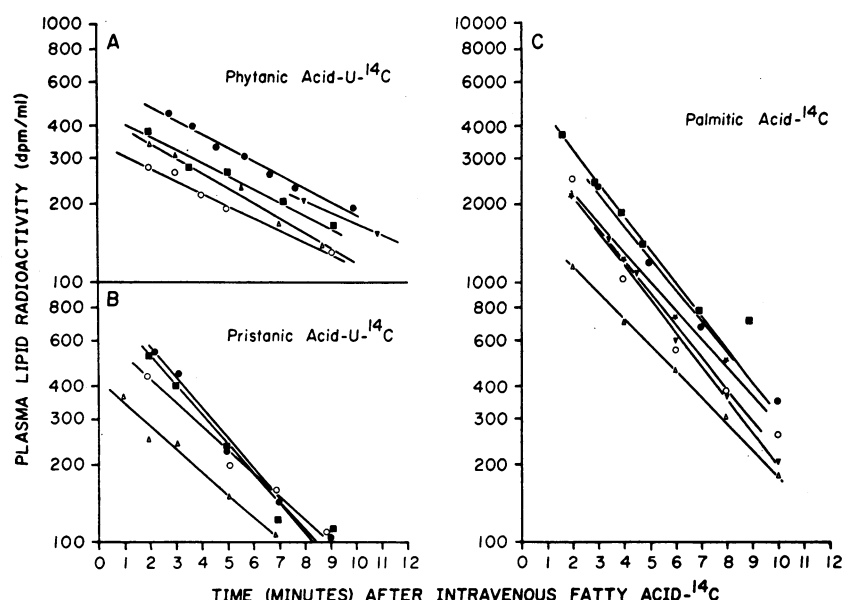[1] Tsai, S-C., J. Avigan, and D. Steinberg. Manuscript in preparation.

FIGURE 3 Semilogarithmic plot of the lipid radioactivity in plasma after intravenous injection of fatty acid-^{14}C in normal volunteers and in two patients with HAP. (A) Phytanic acid-U-^{14}C: O,△,▼, normal controls; ●,■, HAP. (B) Pristanic acid-U-^{14}C: O,△, normal controls; ●,■, HAP. (C) Palmitic acid-1-^{14}C: O,△,▼,★, normal controls; ●,■, HAP.

oxidation was 162% that of hydroxyphytanate oxidation; in the HAP patients it was 6%. Separate consideration of the results obtained with the individual isomeric does not alter the fundamental point: whereas phytanate oxidation was grossly impaired, hydroxyphytanate oxidation was only modestly impaired in the patients.

Palmitic acid was oxidized at the same rapid rate by both patients and controls (Fig. 1). The patients appear to have no block in the catabolism of straight-chain fatty acids.

Decay of plasma radioactivity after intravenous injection of U-^{14}C–labeled fatty acids (Fig. 3 and Table II).

TABLE II

Half-Time of Initial Disappearance of Plasma Radioactivity after Injection of Labeled Free Fatty Acids Bound to Serum Albumin[*]

Fatty acid injected	Half-time	
	Normal	HAP
	min	*min*
Phytanic acid-U-^{14}C	5.9	5.8
Pristanic acid-U-^{14}C	3.1	2.4
Palmitic acid-1-^{14}C	2.5	2.4

* The initial disappearance curves from which these values were calculated are shown in Fig. 3.

The decline in total plasma lipid radioactivity during the 1st 10 min after injection of albumin-bound phytanic acid-U-^{14}C is shown in Fig. 3. Subfractionation showed that over 90% of the total plasma radioactivity was still in the free fatty acid (FFA) fraction at the end of 15 min. The initial rate of decay of free phytanate radioactivity was first-order and essentially the same in three normal subjects and in the two patients (K. S. and J. S.), the apparent half-life of disappearance calculated from the initial slope being approximately 6 min. The initial rates of removal for pristanic acid and palmitic acid were again comparable in patients and controls (Fig. 3), half-life of disappearance being 2–3 min. Thus the values found for t₁ of palmitate were comparable to those previously reported by others, and pristanate disappeared at a similar rapid rate. However, phytanate clearly disappeared more slowly both in controls and in patients (Table II).

Both in normal controls and in patients, lipid radioactivity appeared in ester form within a few hours after injection of labeled phytanate. In normal controls, this lipid ester radioactivity disappeared quite rapidly, falling to undetectable levels within 5–7 days. In the patients, on the other hand, lipid ester radioactivity disappeared much more slowly, some radioactivity being demonstrable as long as 130 days after injection (Fig. 4). The semilogarithmic plot shows that the disappearance curve was roughly linear between 30 and 120 days,
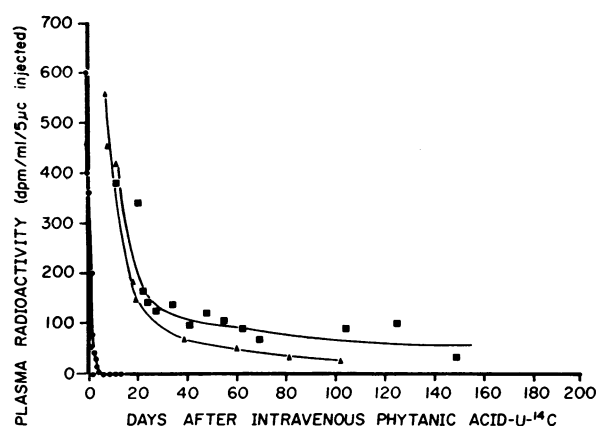
FIGURE 4 Lipid radioactivity in the plasma after intravenous injection of phytanic acid-U-^{14}C in a normal volunteer and in two patients with HAP. ●, normal volunteer; △, HAP (patient K. S.); ■, HAP (patient J. S.). The plasma radioactivity as a function of time in two additional normals was essentially identical to that shown for above normal volunteer; their overlapping patterns are not included for reason of graphical clarity.

and the slope corresponded to an apparent half-life of disappearance of about 35 days. To what extent this disappearance of radioactivity from the plasma reflects irreversible loss and to what extent equilibration with tissue pools is not known.

After injection of pristanate-^{14}C, lipid radioactivity disappeared rapidly from the plasma of both controls, falling to barely detectable levels within 5 days. The late time course of plasma lipid radioactivity was studied in one patient (K. S.) and showed persistence of significant levels for at least 4 days. Taken together with the lower yield of ^{14}CO$_2$ obtained from pristanate, these data suggest that the patients do indeed metabolize pristanate somewhat more slowly than do the controls.

Early incorporation of phytanic acid-U-^{14}C into plasma lipid esters. The distribution of radioactivity among the various major lipid classes in plasma was determined at intervals during the 1st 4 hr after injection of phytanate in one of the HAP patients (J. S.). As FFA radioactivity declined, the largest and most rapid incorporation occurred into the triglyceride fraction. The radioactivity in phospholipids during the 1st 4 hr never accounted for more than 5% of the total; that in cholesterol esters never exceeded 10% of the total. Radioactivity was also incorporated into an as yet unidentified TLC component migrating with a polarity intermediate between that of cholesterol and free fatty acids. At 4 hr, the radioactivity in this component, currently under further study, accounted for 16% of the total plasma lipid radioactivity.

Urinary and fecal radioactivity after intravenous injection of ^{14}C-labeled fatty acids. Total and ether-ex-

tractable urinary radioactivity was measured in normal subjects and in patients given intravenous phytanic acid-U-^{14}C or pristanic acid-U-^{14}C. The rate of appearance of radioactivity in the urine was maximal at 6–8 hr after injection, and significant radioactivity continued to be excreted for 12–24 hr. After the first day, urinary excretion fell to negligible levels in all cases (less than 0.1% of injected dose per 24 hr).

The total urinary radioactivity appearing in the 1st 24 hr is shown in Table III. Only a very small fraction of the injected dose appeared in the urine, less than 6%. The yield of urinary radioactivity in the patients appeared to be distinctly below that in the normal controls. Of the total radioactivity appearing in the urine, less than 5% was extractable from acidified urine into ether; the remainder was in water-soluble products not yet identified. Thus, only a small fraction of the urinary radioactivity could have been due to unchanged radioactive phytanic acid.

After intravenous injection of pristanic acid-U-^{14}C, a somewhat larger fraction of the dose appeared in the urine (Table III). The yield of urinary radioactivity was roughly comparable in controls and patients.

Total fecal lipid radioactivity was determined at 24 hr, 48 hr, and then at 2-day intervals. Only traces of radioactivity were recovered at any time. The patients excreted less than 0.001% of the administered dose of phytanic acid-U-^{14}C in the fecal lipids during the 1st 48 hr. A somewhat larger fraction of the injected dose was found in fecal lipids after injection of pristanic acid or palmitic acid, but this was still less than 0.02% of the injected dose. Trace amounts of radioactivity continued to appear in fecal lipids for 4–8 days, and there was no difference between normals and patients in this regard. These very small fractions of the injected radioactivity could represent traces of impurities in the GLC purified fatty acids. They might also represent products of phytanic acid metabolism excreted by the liver or across the gut wall.

TABLE III
Total Urinary Radioactivity Excreted in 24 hr after Intravenous Injection of Labeled Phytanic Acid or Pristanic Acid

	Percentage of injected dose in 24-hr urine	
Injected substrate	Normal subjects	HAP patients
Phytanic acid	3.8	1.8
	5.9	0.09
	4.2	—
Pristanic acid	4.4	11.6
	10.8	14.0

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DISCUSSION

Three patients with HAP showed a marked deficiency in their ability to oxidize tracer doses of intravenously administered phytanic acid-U-^{14}C to $^{14}CO_2$. These results are consistent with those previously obtained in two other HAP patients using orally administered phytol-U-^{14}C, a precursor of phytanic acid (7, 9).

The interpretation of the comparative data on phytanate-^{14}C oxidation in controls and patients is complicated because the patients have large endogenous stores of unlabeled phytanate that could dilute the injected radioactivity. Several lines of evidence suggest, however, that the extremely low $^{14}CO_2$ output is not primarily due to such an effect. In the course of the present studies, the opportunity arose to check for possible effects of the patient's endogenous pool size. As reported previously (20), treatment with a diet low in phytol and phytanic acid causes the plasma phytanate levels in patients with HAP to fall progressively. Similar diet treatment in patients J. S. and K. S. during the present admission decreased phytanate levels to less than 50% of the initial values.[2] Furthermore, analysis of adipose tissue biopsies in one patient (K. S.) showed that tissue stores were also reduced (by approximately 40%). Fractional oxidation of phytanate-^{14}C was redetermined after these reductions in blood and tissue stores had been effected and was found to be, if anything, smaller on the repeat determination. A second line of evidence against a major dilutional effect comes from studies in experimental animals previously reported (19). It was shown that the fractional oxidation of intravenously injected phytanic acid-U-^{14}C was comparable in control rats and in rats on a phytol-rich diet. The latter accumulate large endogenous stores of phytanic acid (15–20% of total fatty acids in blood and liver), relative concentrations approaching those reported in patients with HAP. Nevertheless, the fractional oxidation of phytanate was not depressed. Finally, tissue culture studies provide an independent demonstration that oxidation is drastically impaired in fibroblasts derived from skin biopsies of the three HAP cases reported here even though in culture they contain *no* abnormal stores of phytanic acid (13, 14).

In considering this potential problem of isotopic dilution, it is important to note that much of the $^{14}CO_2$ production during the early hours after intravenous administration of the free fatty acid may be occurring before very much dilution into lipid ester stores can occur. In the present studies, the rate of $^{14}CO_2$ production reached a peak in the 1st 30–60 min after injection. Because of the significant size and appreciable turnover time for the body pool of bicarbonate, the peak rate of generation of CO_2 from fatty acid at the tissue level

[2] Unpublished results.

must have occurred even earlier, possibly within the very first few minutes. Phytanate in free form would tend to dilute the administered phytanic acid-^{14}C, but the large endogenous pool of phytanate esters in the patients may not importantly influence the observed initial rate of $^{14}CO_2$ production.

In contrast to the results with phytanate, the oxidation of α-hydroxyphytanate was almost the same in HAP patients and in controls. The mean value was somewhat lower but the results overlapped those in normal subjects (Fig. 1). As mentioned above, the striking and most relevant comparison is that between phytanate oxidation and α-hydroxyphytanate oxidation. In normal subjects, the rate of phytanate oxidation was on the average 162% that of hydroxyphytanate oxidation; in HAP patients the rate of phytanate oxidation was less than 10% of hydroxyphytanate oxidation. Oxidation of pristanate was also somewhat lower in the HAP patients but again, when compared with the drastically reduced rate of phytanate oxidation, this was a relatively small difference. There are not enough data to conclude that the difference is in fact real, but in view of the marked defect in phytanate oxidation it would not be surprising to find some decrease in the activity of enzyme systems involved in later steps. Also, it is possible that the phytanate accumulated in these patients acts to inhibit oxidation of these closely similar compounds (but not palmitate) in a specific manner or indirectly, related possibly to the toxic effects seen on feeding large doses of phytanate to animals (21, 22).

If oxidation of phytanate in man proceeds, as it does in animals (10–12), primarily by way of α-hydroxyphytanate and pristanate as intermediates, we can conclude that the metabolic error in HAP lies in the alpha-hydroxylation step. On the basis of indirect studies and studies using model compounds it has been suggested that phytanate might be handled by omega oxidation or by a pathway involving CO_2 fixation (23–26). However, there is no direct evidence for such alternative pathways, certainly not for any major role for them. In view of the evidence available, we interpret the present results as indicating a primary metabolic error involving alpha-hydroxylation.

Stokke and coworkers, using model branched-chain compounds related in structure to phytanate, have presented evidence for alpha oxidation of them in animals and in man (26–30) and deficient alpha oxidation of them in HAP patients (27, 30). Until more is known about the substrate specificity of the enzymes involved in alpha oxidation of branched-chain acids, these indirect results should not be extrapolated to apply to phytanate. This is especially the case since the same group of investigators has shown that omega oxidation and alpha oxidation of the model compounds themselves

is apparently suppressed in HAP patients when their phytanate levels are high but less so when they are low (30, 31). Thus, it is not estalished that the apparent deficiency in alpha oxidation of the model substrate, only a minor fraction of which underwent alpha oxidation (most being degraded by omega oxidation), reflected a primary rather than a secondary effect.

The clinical features of HAP are quite distinctive as a syndrome, and most patients in whom the diagnosis has been made on clinical grounds have proved to have phytanic acid storage (1, 2, 5, 6, 32). Exceptions, however, have been noted. Kolodny and coworkers (33) reported a case with clinical features compatible with HAP but who had no phytanate in his plasma lipids. In collaboration with Dr. Herbert J. Kayden we have tested this patient's capacity to oxidize phytol-U-^{14}C and found it normal.[3] Repeated examination of plasma and two studies of liver biopsies failed to show any accumulation of phytanate. Another patient with the clinical features of HAP, studied in collaboration with Dr. I. A. M. Prior and Dr. W. S. Alexander in New Zealand, showed normal rates of oxidation of phytol-U-^{14}C and of intravenously administered phytanic acid-U-[14]C. In this case phytanic acid was reported in the plasma (8.1% of total fatty acids) about a year before the isotopic studies, but none at all was present at the time of the isotopic studies at later times. The patient subsequently came to postmortem and there was no phytanate accumulation in any of the tissues. A full report of this puzzling case is in preparation.[4] Evidently there may be more than one biochemical basis for the clinical picture of HAP. Furthermore, although the three cases studied here appear to share a common metabolic error, it is possible that phytanate accumulation may have a different biochemical basis in other cases.

In the present studies the patients showed a decreased ability to convert radioactive phytanic acid to urinary metabolites. Over 95% of the labeled metabolites were water soluble, representing degraded substrate rather than intact phytanate. Because the fraction of the administered dose excreted in the urine was so low, a significant part of the radioactivity in the urine could have arisen from radioimpurities in the injected phytanic acid.

HAP patients do appear to retain some ability to dispose of phytanic acid. This is evident, first, from the fact that they apparently reach a more or less steady-state level of plasma and tissue phytanate despite continued ingestion of phytanic acid and phytol in their regular diets over many years. Their plasma and tissue levels fall when the dietary load is reduced (20). In the

present studies with intravenously administered phytanic acid-U-^{14}C, in previous studies with orally administered phytol-U-[14]C (7, 9), and in experiments with fibroblast cultures (13, 14), a very low but still measurable amount of [14]CO_2 was produced; in all these studies, however, less than 5% of the administered substrate was converted to [14]CO_2. Some of this [14]CO_2 could possibly have arisen from radioimpurities that were not separated from methyl phytanate during the TLC and GLC purification. It is unlikely, though, that this could explain all of it since radiopurity was always at least 95%; the impurities would have had to be converted rapidly to [14]CO_2 and in very high yield. Excretion of lipid-soluble products in the feces does not appear to be a major pathway and no differences were noted between patients and controls. However, water-soluble degradation products (formed in the liver or during passage through the gut) were not measured. In rats with the bile duct cannulated, only 2% of an intravenously injected dose of phytanic acid appeared in the bile in 24 hr (34), including both lipid- and water-soluble products. Further studies are needed to fully assess the importance of biliary and/or intestinal secretion of phytanate or its metabolites in HAP patients.

ACKNOWLEDGMENTS

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