Refsum's Disease: Nature of the Enzyme Defect

Abstract. *Two siblings with Refsum's disease, an inherited disorder of lipid metabolism, oxidized intravenously injected uniformly labeled phytanic acid-C^{14} at rates less than 5 percent of those found in normal subjects. The defect in oxidation of phytanic acid persisted in cultures of fibroblasts from the patients' skin. The rate of oxidation of the phytanic acid-C^{14} was less than 1 percent of that found in cultures of fibroblasts from normal skin. However, pristanic acid, previously shown to be the first product of phytanic acid degradation, was oxidized at a normal rate in the patients' cultures. These results indicate that the enzymatic defect in Refsum's disease is in the first step of the pathway for degradation of phytanic acid, that is, in the unusual alpha-oxidative process that leads to a shortening of phytanic acid by one carbon atom.*

Heredopathia atactica polyneuritiformis (HAP), first delineated as a clinical syndrome by Refsum (*1*), is genetically determined and transmitted in an autosomal recessive pattern (*1, 2*). The clinical features are primarily referable to dysfunction of the nervous system. The disease is one of the lipidoses, characterized by accumulation of a C_{20} branched-chain fatty acid, phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) (*3*). Our clinical studies showing that there is little or no endogenous biosynthesis of phytanic acid in these patients indicates that the stored phytanate has an exogenous origin (*4, 5*). One potential source is dietary phytol, which is readily converted to phytanic acid in animals, in normal man, and in patients with HAP (*4–8*). The feeding of high doses either of phytol or of phytanic acid itself to laboratory animals leads to marked accumulation of phytanic acid in blood and tissues (*7, 9*). Finally, clinical studies, in which the oxidation of uniformly labeled phytol-C^{14} in normal subjects was compared to that in patients with HAP, gave direct evidence that the underlying metabolic error lies in a relative inability of the patient to oxidize phytanic acid (*4, 5, 10, 12*).

In the rat and in the mouse a major pathway for phytanic acid degradation involves an initial alpha-oxidation to yield the (n−1) fatty acid, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) (*11*). Several further degradation products have been identified that represent products of beta-oxidation of pristanic acid (Fig. 1) (*12*). We have applied this new information on the pathway to localize further the metabolic block in HAP.

Uniformly labeled phytanic acid-C^{14}, prepared as described previously (*6*), was injected intravenously as a complex with serum albumin into two normal volunteers and into two patients with HAP. Respiratory $C^{14}O_2$ was collected and assayed as previously described (*5*). The clinical features in the patients have been reported in detail elsewhere (*10, 13*). The phytanic acid concentrations in their plasmas at the time of the studies were 53 and 61 mg/100 ml, respectively. The normal subjects converted 33.7 and 43.8 percent of the injected tracer dose to $C^{14}O_2$ in 24 hours, respectively (Table 1). The two patients with HAP oxidized only 2.0 and 0.6 percent, respectively, over the same time interval. Conversion of intravenously injected palmitic acid-1-C^{14} to $C^{14}O_2$ studied in one patient was within normal limits (35.3 percent in 24 hours) (*14*).

Skin specimens from the two HAP patients were obtained by dissection at the time of muscle biopsies and were used to initiate tissue cultures in a modified Eagle medium (*15*). Control cultures were started from the skins of two normal subjects and of two patients with unrelated disorders of lipid metabolism. The established cell lines consisted exclusively of fibroblast-like cells. In order to measure the capacity of the cultured cells to oxidize phytanic acid, we introduced a tracer amount of the labeled material complexed with human serum albumin, and allowed it to incubate with the culture for periods of 24 to 48 hours. The medium was then acidified, and $C^{14}O_2$ in the gas phase was trapped in alkali for radioassay (*5*). The production of $C^{14}O_2$ proceeded linearly for at least

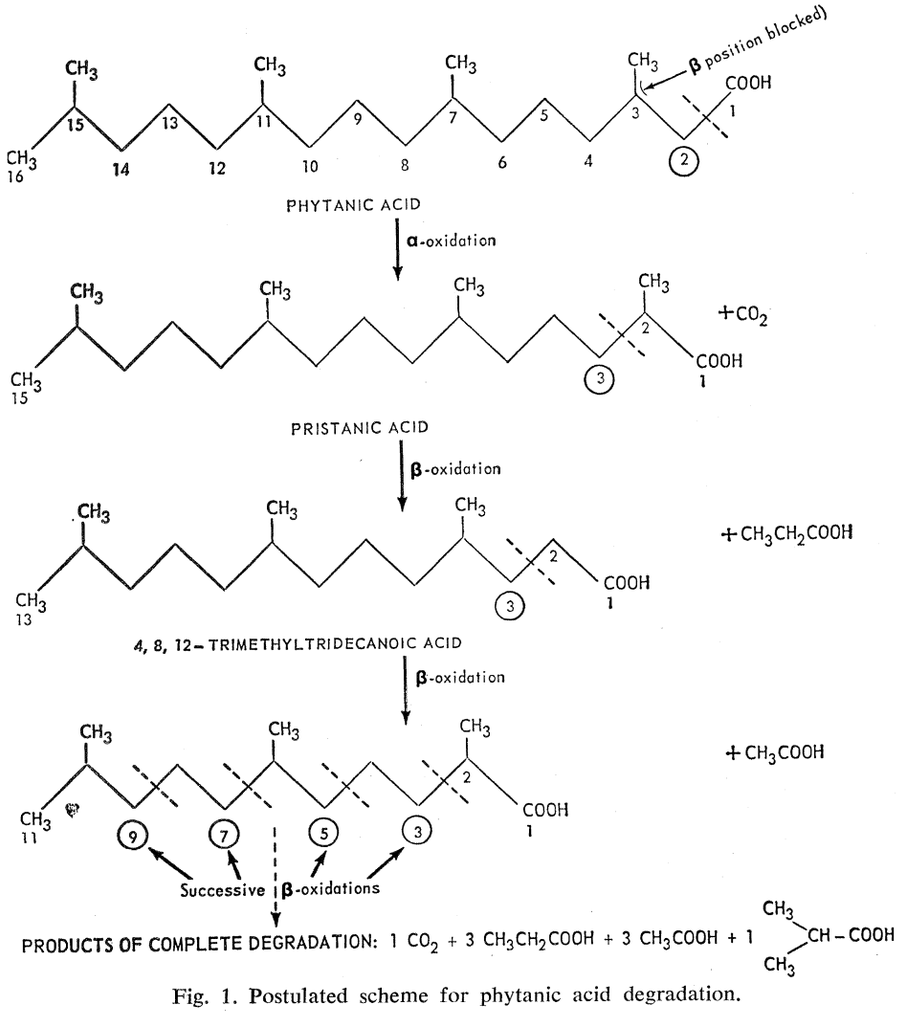

Fig. 1. Postulated scheme for phytanic acid degradation.

Table 1. Oxidation of uniformly labeled phytanic acid-C^{14} after its intravenous injection into two normal control subjects and into two patients with Refsum's disease (HAP).

Subjects	Percentage oxidized to $C^{14}O_2$ in		
	12 hr	24 hr	4 days
	Normal		
G.R.	38.1	43.8	55.2
G.McG.	30.8	33.7	36.8
	Refsum's disease		
K.S.	0.45	0.6	2.2
J.S.	1.8	2.0	2.4

48 hours. There was an obvious deficiency in phytanic acid oxidation in the cultures started from the two HAP patients, in which conversion of CO_2 was less than 1 percent of that in control cultures (Table 2). The low yield of $C^{14}O_2$ from phytanate is not attributable to dilution of the added tracer by unlabeled phytanate since analysis of the fatty acids in both control and HAP cultures revealed minimally detectable phytanic acid (less than 0.1 percent of total fatty acids). Thus, the results in the tissue culture experiments are free of a difficulty encountered in interpretation of results from clinical studies, where dilution of injected tracer by the large stores of unlabeled phytanic acid might contribute to a low yield of $C^{14}O_2$. That the low yield of $C^{14}O_2$ was not due to impaired uptake of phytanic acid from the medium was shown by determination of the total amount of phytanic acid-C^{14} in the lipids of the washed cells. In the control cultures, approximately 10 percent of the radioactivity added initially was found in the cell lipids, while 20 percent was found in the HAP cultures. The sum of radioactivity in $C^{14}O_2$ and in cell lipids was thus about the same in the two sets of cultures. After the cells had been incubated with phytanic acid-C^{14}, the cell lipids of the control cultures were shown by gas-liquid radiochromatography to contain a small but significant amount of radioactivity in pristanic acid, equal to 2 to 3 percent that in phytanic acid; in the HAP cultures no radioactivity was found in pristanate. In the control cultures radioactivity was also demonstrated in the normal straight-chain C_{16} and C_{18} fatty acids, indicating reutilization of carbons derived from breakdown of the phytanic acid; in the HAP cultures no radioactivity was found in any fatty acid other than phytanate, the labeled substrate added.

Pristanic acid-C^{14} was prepared from uniformly labeled phytanic acid-C^{14} by Barbier-Wieland degradation, and its oxidation was studied in tissue cultures as described above. There was no significant difference between control cultures and HAP cultures in the rate of oxidation of this intermediate (Table 2). The rate of oxidation of palmitic acid in one of the HAP cultures (J.S.) was fully as great as that in control cultures. In the other (K.S.) it appeared to be somewhat lower than in control cultures but still 20 times greater than the rate of oxidation of phytanic acid in this HAP culture.

These clinical and tissue culture studies provide evidence for a specific metabolic block in phytanic acid degradation in Refsum's disease. Interpretation of the results with labeled pristanic acid requires consideration of the pathway for phytanic acid degradation recently shown to be a major pathway in normal animals (*11, 12*). Degradation begins with the alpha-oxidation of phytanic acid and its conversion to pristanic acid, and there follows a series of successive beta-oxidation steps yielding various intermediates (Fig. 1). Although it remains to be established that this is a major pathway in human tissues, the conversion of phytanic acid to pristanic acid demonstrated in these tissue culture studies supports this interpretation. Furthermore, pristanic acid has been found in trace amounts in several normal human tissues (*16*).

A defect in omega-oxidation has been proposed by Eldjarn to account for the accumulation of phytanic acid in Refsum's disease (*17*). However, subsequent studies failed to reveal a defect in omega-oxidation of a number of substrates (*18, 19*). A pathway involving fixation of CO_2 has also been proposed but subsequently withdrawn (*19, 20*). In the absence of definitive evidence for significant phytanic acid degradation by any other pathways, we tentatively assume that the pathway in Fig. 1 is a major pathway in man as well as in laboratory animals. In this case, the fact that phytanic acid oxidation was all but completely blocked, whereas pristanic acid was oxidized at a normal rate by the patients' tissue cultures, localizes the metabolic error in Refsum's disease to the first step in the pathway—the conversion of phytanic acid to pristanic acid.

One-carbon degradation of long-chain fatty acids has been demonstrated in plants (*21*). In animals, however, it is well documented only in nerve tissue (*22*). Shortly after our report on the alpha-oxidation pathway for phytanic acid degradation (*11*), Eldjarn *et al.* published on the fate of orally administered 3,6-dimethyloctanoic acid-8-C^{14} in man. Most of the radioactivity was recovered in the urine, in products resulting from oxidation of the omega end of the molecule, and the yield of these was apparently the same in normal subjects and in patients with Refsum's disease. In controls a small fraction (1.5 percent) was found to have undergone alpha-oxidation, but none was found in the urine of the patients, a result compatible with the block demonstrated in our studies.

As previously discussed (*10, 23*),

Table 2. Oxidation of labeled substrates added to skin fibroblast cultures from control subjects and from patients with Refsum's disease (HAP). The reference control cells included two lines started from skin of normal controls and two lines started from skin of patients with lipidoses unrelated to Refsum's disease, one probable Niemann-Pick disease, and one undiagnosed lipidosis. Since the data on the oxidation of palmitic acid were obtained in part from incubations with palmitic acid-1-C^{14} and in part from incubations with palmitic acid-9,10-H^3, and since cultures from patient K.S. gave lower values than those from patient J.S., the individual values are presented. If data are pooled, the means for controls and patients are not significantly different.

Percentage oxidized by 10^7 cells in 48 hours		
Phytanic acid-C^{14}	Pristanic acid-C^{14}	Palmitic acid
	Control subjects	
		6.5
6.1 ± 1.1* (N = 6)	8.2 ± 1.0 (N = 4)	6.5
		8.6
		8.8
		9.7†
	Refsum's disease patients	
0.058 ± 0.019 (N = 6)	8.1 ± 2.3 (N = 6)	1.6 (K.S.)
		2.0† (K.S.)
		5.0 (J.S.)
		13.8† (J.S.)

* Mean ± standard error; difference in phytanate oxidation significant, $P < .001$; difference in pristanate oxidation not significant. N, number of observations. † Based on release of radioactivity from palmitic acid-9,10-H^3 to water in the medium.

there is as yet no conclusive evidence that the clinical manifestations in Refsum's disease are directly attributable to the accumulation of phytanic acid, although a causative role has not been ruled out. Our findings tend to strengthen the likelihood that the primary lesion affects an enzyme system that plays an important role in nerve cell function and that deletion or alteration of it leads to the nerve dysfunction. Thus, the accumulation of phytanic acid may, in a sense, be incidental and not of pathogenetic importance. The precise functional significance of the one-carbon degradation system in nerve tissue and of the alpha-hydroxy acids presumed to be formed by this mechanism is not known. We suggest that direct examination of nerve tissue for its alpha-oxidative capacity in other diseases of the nervous system may be fruitful.

DANIEL STEINBERG
JAMES H. HERNDON, JR.
B. WILLIAM UHLENDORF
CHARLES E. MIZE
JOEL AVIGAN, G. W. A. MILNE
Laboratory of Metabolism, National Heart Institute, and *Laboratory of Viral Immunology, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland*

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Hereditary Renal Disease in a Mutant Strain of Rats

Abstract. *Disease of the kidney developed in breeding stock of Gunn rats. The renal lesion is the result of a new mutation. The genetic defect is inherited as an autosomal dominant trait and is apparently lethal in the homozygous condition. The abnormality manifests itself as a congenital hydronephrosis with related cystic changes in the kidney.*

Inherited congenital hydronephrosis is a well-known clinical entity in man (*1*). This renal lesion has also been observed in laboratory animals (*2*). However, as far as we are aware, no colony of animals characterized by such pathology has been described. We now report the discovery of a new mutant strain of rats carrying a genetic disorder of the kidneys. The disorder was noticed in a colony of rats maintained by one of us (B.B.L.) at the University of Tennessee Memorial Research Center. Since the availability of a strain of rats with such a genetically controlled disorder would provide an experimental model for studies of renal pathophysiology and embryology, a colony was developed, and the mode of inheritance of the mutant was studied.

The genetic abnormality was found among the breeding stock of our colony of Gunn rats, which is a mutant of the Wistar strain of albino rats (*3*); it is characterized by the presence of jaundice secondary to the absence of bilirubin glucuronyl transferase activity in the liver (*4, 5*). Our colony of rats is derived from inbred Gunn rats (supplied in 1965 by Dr. R. L. Swarm of the University of Cincinnati). When the renal lesion was observed, a series of crosses was started to determine whether this anomaly was due to a new mutation or whether it was due to another expression of the gene responsible for the jaundice. For this purpose, four homozygous jaundiced male rats with renal lesions, detected by pyelography, were crossed to female animals heterozygous for the jaundice trait obtained from another colony of Gunn rats (provided by Dr. P. E. Zollman of the Mayo Clinic). These heterozygous female Gunn rats had normal kidneys as judged by pyelography and later by autopsy. The descendants from the original four pairs of animals were studied with pyelography and histological studies were frequently made after death. New crosses were made between siblings, as were backcrosses between the third and second generation, to analyze the inheritance of both traits (Fig. 1).

The occurrence of the kidney lesion was studied by macroscopic examination, histologic observation, and by pyelography. Urograms were carried out by injecting the contrast media (*6*) at a dose of 0.30 ml per 100 g of body weight. Films were taken at convenient intervals after the injection. A total of 976 kidneys was examined macroscopically, and 100 were examined microscopically; roentgenograms were obtained in 200 animals. The age of the rats ranged from 1 week to 1 year.

Three types of macroscopic lesions were observed (Fig. 2). Hydronephrosis was the most common abnormality (85 percent of the rats with kidney alterations). In rats exhibiting limited or moderate degrees of hydronephrosis, the kidneys appeared normal in size, with a smooth regular surface. In other animals, however, advanced hydronephrosis was found, and the kidney appeared as a translucent hydronephrotic sac with an irregular surface. On cut section, a multilocular cavity, which was separated into smaller subdivisions by incomplete trabeculas, was seen in the medulla and cortex. The hydronephrosis was unilateral in 60 percent and bilateral in 40 percent of the rats examined. In both cases, the enlargement of the renal pelvis was accompanied by dilatation of the ureter to variable degree with or without ureteral strictures. Despite the partial obliteration, the ureter was still patent, as demonstrated by pyelography and at autopsy. The ureters were normal in 25 percent of the