A PATHWAY FOR OXIDATIVE DEGRADATION OF PHYTANIC ACID IN MAMMALS

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is found in normal human and animal tissues, usually at very low concentrations (Avigan, 1966a; Kremer, 1965). In persons with Refsum's disease, however, high concentrations have been found in tissues (Klenk and Kahlke, 1963; Hansen, 1965), and these patients show a reduced capacity to metabolize this compound (Steinberg et al, 1966a). Elucidation of the pathway(s) for degradation of phytanic acid, in addition to its relevance for understanding of the metabolic error in Refsum's disease, may yield information useful in study of the degradation of other isoprenoid structures such as the side chains of Vitamins E and K.

We have previously reported that the formation of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) is an important initial step in the mouse and in the rat (Avigan et al, 1966b). The results reported below show that 4,8,12-trimethyltridecanoic acid, 2,6,10-trimethylundecanoic acid, and 4,8-dimethylnonanoic acid are also derived from phytanic acid, probably by successive β-oxidation steps of pristanic acid.

EXPERIMENTAL: The preparation of U-^{14}C-phytanic acid, the methods for extraction of total liver lipids, preparation of the methyl esters of fatty acids, and fractionation by TLC and GLC have been previously described (Steinberg et al, 1966b; Mize et al, 1966). 4,8-Dimethylnonanoic acid and 4,8,12-trimethyltridecanoic acid were synthesized from geraniol and farnesol, respectively. The alcohol was hydrogenated, oxidized with chromic anhydride in acetic acid, and the product converted to the acid chloride prior to reaction with a large excess of ethereal

diazomethane for 3 days. The resulting diazoketone was refluxed in an excess of methanol with freshly prepared silver oxide to yield the final product. The methyl ester of homotetrahydrogeranoic acid (4,8-dimethylnonanoic acid) was identified by its infrared spectrum, mass spectrum, and GLC retention time (1.7 relative to that of methyl tetrahydrogeranoate). The structure of 4,8,12-trimethyltridecanoic acid was confirmed by mass spectrometry (see below). Analyses on the LKB 9000 gas chromatograph-mass spectrometer were done using a 1.5 meter column packed with 1% SE-30 on Chromosorb P.

Identification and quantification of the branched-chain fatty acids in liver lipids, as methyl esters, was confirmed on two different liquid phases (15% EGS, and a mixture of 1.5% SE-30 and 4% EGS). Radioactivity associated with the various fatty acid methyl esters was determined in collected GLC fractions. When insufficient material was present to give a clearly defined mass peak, carrier reference standard was added before GLC of the mixture and collection of fractions. The GLC retention times of the methyl esters of 4,8-dimethylnonanoic and 4,8,12-trimethyltridecanoic acids were 0.12 and 0.53, respectively, relative to methyl palmitate on 17% EGS.

RESULTS: As shown in Table I, livers of mice fed diets containing either phytanic acid or phytol contained significant concentrations of phytanic acid, pristanic acid, and also 4,8,12-trimethyltridecanoic acid. These fatty acids accounted for 1 to 8% of the total fatty acids in liver after only 2 days on the diets. At the sensitivity level of the methods, these acids were not detectable in control livers.

The structure of 4,8,12-trimethyltridecanoic acid found in liver was verified by direct comparison of its mass spectrum with that of the synthetic compound, using the LKB 9000 gas chromatograph-mass spectrometer (Fig. 1). The base peak at m/e 87 is expected from preferred cleavage adjacent to the 4-methyl group; 2-methyl fatty acids, like pristanic acid, show a base peak at 88, while 3-methyl fatty acids, like phytanic acid, show a base peak at 101 or 74 (Ryhage and Stenhagen, 1960). The fact that peaks at 87 and 115, and those at 157 and 185,

Table I: Accumulation of Branched-chain Fatty Acids in Livers of Mice Fed Phytanic Acid or Phytol.*

Addition to Diet (by weight)	Concentration in Liver (mg/g wet wt.) 4,8,12-Trimethyl-tridecanoic acid	Pristanic acid	Phytanic acid
None	N.D.	N.D.	N.D.
Phytol, 2%	1.0	1.4	1.7
Phytanic acid, 2%	0.3	0.3	0.8

* Weanling mice (NIH G.P.strain) were fed the respective diets for 40 hrs; livers from 2-3 animals in each group were pooled for GLC analysis. N.D. = not detectable.

were larger than those at 101 and 171, respectively, indicates a methyl branching at C_4 and C_8. The prominence of m/e 213 was unexpected but clearly similar in the two samples. The remaining features of the spectra are remarkably parallel, leaving little doubt as to the identity of the basic structures. These data do not, however, provide evidence regarding configuration at the two asymmetric centers.

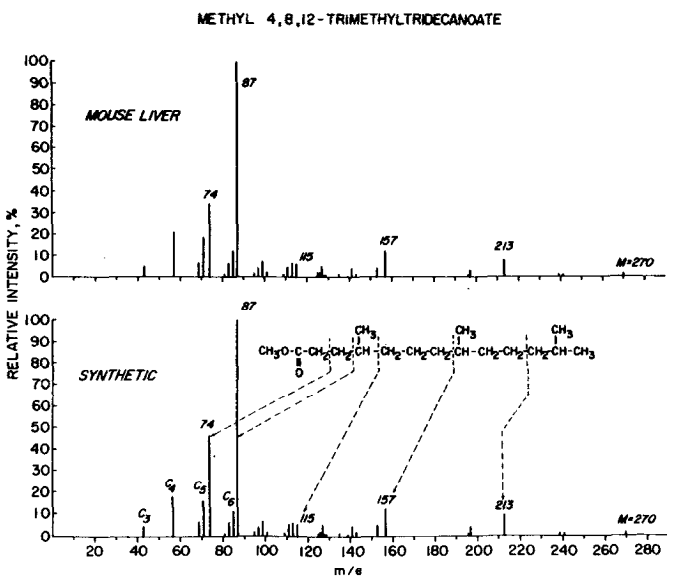

Figure 1:

Mass spectrum of synthetic methyl 4,8,12-trimethyltridecanoate (below) and that of the component of identical GLC retention time in the liver of a mouse fed phytanic acid for 40 hrs (above). The m/e 74 results from rearrangement addition of a proton to the indicated fragment (McLafferty, 1957).

Conversion of intravenously injected U-^{14}C-phytanic acid to 4,8-dimethylnonanoic, 4,8,12-trimethyltridecanoic, and pristanic acids, is shown by the data of Table II.

Table II: Radioactivity in Branched-chain Fatty Acids of Mouse Liver after Intravenous Injection of U-^{14}C-Phytanic Acid.*

Time after Injection (min)	Radioactivity Incorporated (% of Total Liver Fatty Acid Radioactivity)		
	4,8-Dimethylnonanoic acid	4,8,12-Trimethyltridecanoic acid	Pristanic acid
5	0.3	13.4	43.3
10	0.7	14.3	11.6
30	1.0	30.2	1.0

* Adult mice (avg.wt. 25 g) were fed 2% phytanic acid in the diet for 60 hrs, then injected intravenously with 3.1 μc of U-^{14}C-phytanic acid complexed with bovine serum albumin.

The peaks of radioactivity were sharp, the fractions collected before and after being near background. In the case of the last two compounds, a well-defined peak was seen on the mass record, agreeing in retention time with that of synthetic reference compound. In the case of the 4,8-dimethylnonanoic acid, no definite mass peak was recorded, but the peak of radioactivity emerged coincident with the point of elution of the synthetic reference sample.

Conversion of U-^{14}C-phytanic acid to 4,8,12-trimethyltridecanoic acid was also demonstrated in the rat. A Sprague-Dawley rat fed on standard laboratory chow was given 10 μc of U-^{14}C-phytanic acid intravenously and killed after 2 min. Approximately 40% of the total fatty acid ester radioactivity in the liver coincided with added synthetic carrier methyl 4,8,12-trimethyltridecanoate on a 1.5% SE-30, 4% EGS column. The collected material was rechromatographed on a 17% EGS column. Over 95% of the injected radioactivity was again collected with carrier methyl 4,8,11-trimethyltridecanoate.

GLC of the methyl esters of total liver fatty acids from mice fed phytanic acid also showed a small component, absent from control livers, with a retention time shorter than that of 14:0. On the LKB 9000 with a 1% SE-column, its spectrum

showed a parent ion at m/e 242. The base peak was at m/e 88, indicating a 2-methyl substitution; the intensity of m/e 143 was much smaller than that of 129 or 157, indicating a branching at C_6 (Ryhage and Stenhagen, 1960). There was also a significant peak at (M-90), which also appears in the spectrum of methyl pristanate (Hansen and Morrison, 1964), and which we ascribe to the successive loss of m/e 88 and loss of 2 hydrogens to yield: $R - C(CH_3) = CH-CH = CH_2$. There was a relatively large peak at m/e 101, as in the mass spectrum of pristanic acid. These mass spectrometric findings, coupled with the appearance of the compound only after feeding of phytanic acid, justify its tentative identification as 2,6,10-trimethylundecanoic acid, structurally analogous to pristanic acid and differing from it by one isoprene unit.

DISCUSSION: The present data confirm and extend the previously reported finding of the importance of α-oxidation in the breakdown of phytanic acid. The accumulation of 4,8,12-trimethyltridecanoic acid in the livers of phytanic acid-fed mice is unambiguously demonstrated by its characteristic mass spectrum which was identical with that of the synthetic compound. This, coupled with the isotopic studies showing its rapid formation from labeled phytanic acid, establishes that 4,8,12-trimethyltridecanoic acid is also on a major degradative pathway. Combined GLC-mass spectrometry also showed the presence in the livers of phytanic acid-fed mice of a minor component with GLC retention time and mass spectrum compatible with its identification as 2,6,10-trimethylundecanoic acid. Finally, it was shown that labeled phytanic acid is converted to a compound cochromatographing with synthetic 4,8-dimethylnonanoic acid and that the relative amount of radioactivity in this component rose with time as that in pristanic acid fell. These results are compatible with the pathway of degradation proposed below:

Phytanic acid ---→ Pristanic acid ---→ 4,8,12-trimethyltridecanoic acid ---→ 2,6,10-trimethylundecanoic acid ---→ 4,8-dimethylnonanoic acid ---→ [2,6-dimethylheptanoic acid] ---→ [isocaproic acid ---→ isobutyric acid]

The first step, the α-oxidation of phytanic acid, is in some respects unique, because β-oxidation is blocked by the 3-methyl group. After pristanic acid is formed, however, β-positions along the chain become accessible. It appears that a repeated sequence of β-oxidations, which may occur by mechanisms closely related to those for β-oxidation of straight-chain fatty acids, completes the degradation of the molecule. The proposed pathway for the further oxidation of pristanic acid is consonant with the early results of Kay and Raper (1924) on degradation of β-phenyl-α-methylpropionic acid and related compounds, and with the hypothetical scheme proposed by Simon et al (1956) for degradation of the side-chain of Vitamin E after ω-oxidation. No direct evidence is available regarding the nature of the small fragments split off in the degradation of pristanic acid. The simplest hypothesis is that two- and three-carbon fragments are alternately removed as acetyl-CoA and propionyl-CoA, respectively. The fact that significant amounts of radioactivity are found in glucose in the livers of rats shortly after intravenous injection of U-^{14}C-phytanic acid favors this possibility (Avigan et al, 1966b). While the present results strongly suggest that the pathway described for phytanic acid oxidation is the major one, alternative routes involving ω-oxidation (Eldjarn, 1965) or CO_2 fixation, as in degradation of farnesoic acid in bacteria (Seubert and Remberger, 1963), cannot be ruled out (Eldjarn, Try and Stokke, 1966).

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