ALPHA-DECARBOXYLATION, AN IMPORTANT PATHWAY FOR DEGRADATION OF PHYTANIC ACID IN ANIMALS

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Patients suffering from Refsum's disease, an inherited disorder of the nervous system (Refsum, 1946), accumulate large stores of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in their blood and tissues (Klenk and Kahlke, 1963). There appears to be little or no endogenous synthesis of phytanic acid either in normal experimental animals (Avigan, Steinberg and Cammermeyer, 1966; Mize et al, 1966) or in patients with Refsum's disease (Steinberg et al, 1965, 1966a) and the metabolic error is presumed to lie in a relative inability to degrade phytanic acid. It has been suggested on the basis of indirect evidence that an error in omega-oxidation might be involved (Eldjarn, 1965), but there is as yet no direct information on the normal pathway for degradation of phytanic acid.

The results reported below show that the normal rat rapidly converts phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) to its α-decarboxylation product, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid).

METHODS

U-^{14}C-phytanic acid was prepared as described previously (Mize et al, 1966) from U-^{14}C-phytol isolated from algae grown in $^{14}CO_2$ and purified by preparative GLC of the methyl ester. Radiopurity, determined by GLC with collection and counting of fractions, was greater than 99% and the preparation was essentially free of labeled components of the same retention time as pristanic acid.

Deuterium-labeled phytanic acid was prepared by catalytic deuteration of phytenic acid (3,7,11,15-tetramethylhexadec-2-enoic acid). Analysis of the methylated product using an MS-9 high-resolution mass spectrometer (Associated Electronic Industries, Ltd., Manchester, England) showed a spectrum characteristic for methyl phytanate (Hansen et al, 1965), with the expected enrichment in higher masses. The ratio of m/e 327 to that of m/e 326 $[(M + 1)/M]$ was 2.09 for the deuterated preparation and 0.268 for reference phytanate. Assuming the incorporated deuterium to be evenly distributed between the two hydrogens added at the double bond of phytenic acid, these positions were each calculated to contain 47.6 atoms percent excess deuterium. In the spectrometer, cleavage between carbons 3 and 4 yields a fragment of m/e 101. The ratio of 102 to 101 was 1.79 in the deuterated sample, and 0.073 in the reference phytanate, giving a calculated enrichment of 46.2 atoms percent excess D in the 2 and 3 hydrogens, in good agreement with the above value for the deuterium content of the parent molecule ion, verifying that the labeling was limited to the carboxyl terminal part of the molecule. Pristanic acid was prepared from methyl phytanate by a normal Barbier-Wieland degradation.

Adult Osborne-Mendel rats (180-250 g), fed chow diets ad lib, were lightly anesthetized, injected intravenously with labeled phytanic acid complexed with human serum albumin, killed at different time intervals, and the livers were removed and treated with chloroform-methanol 2:1. The lipids extracted into the chloroform phase were saponified, the total fatty acid fraction was isolated, converted to methyl esters, and fractionated by TLC as previously described (Steinberg et al, 1966b). Small amounts of carrier methyl phytanate and methyl pristanate were added prior to TLC except in the experiment with deuterium-labeled phytanate. Methyl phytanate and methyl pristanate, which co-chromatograph in the TLC system used (benzene-hexane 2:1), were well resolved by subsequent GLC on a mixed liquid phase (4% ethylene glycol succinate plus 1.5% SE 30) on Gas Chrom Q (Applied Sciences Laboratories) at 185°-190° with N_2 carrier gas flowing at 80 cc/min. Fractions were collected into coiled

lengths of Teflon tubing cooled in a solid CO_2-ethanol mixture. The retention time of methyl pristanate was 19.2 min. and that of methyl phytanate 28.6 min. Radioassay was carried out in a Packard liquid scintillation spectrometer.

RESULTS

As shown in Table I, ^{14}C-pristanic acid accounted for a very large fraction of the radioactive branched-chain fatty acids recovered from the livers of rats 2 minutes after intravenous injection of U-^{14}C-phytanic acid (41.5 and 35.4%). The ^{14}C in liver pristanic acid actually corresponded, respectively, to 5.5 and 10% of the total administered dose of phytanate radioactivity. Both the relative and the absolute amounts of ^{14}C-pristanate recovered in the liver decreased at longer time intervals but remained highly significant even at 135 minutes.

TABLE I

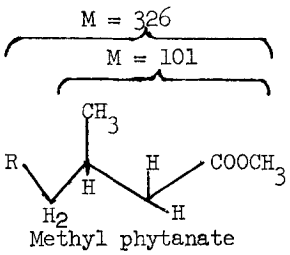
^{14}C-Pristanic Acid Recovered in Rat Liver after Intravenous Injection of ^{14}C-Phytanic Acid*

Experiment number	Time after injection	Percentage of dose recovered in total liver fatty acids	^{14}C-Pristanic acid as percentage of total ^{14}C-fatty acids
	(min)		
1	2	13.3	41.5
2a	2	28.4	35.4
2b	10	13.1	13.7
2c	30	9.2	5.7
3	135	8.5	5.2

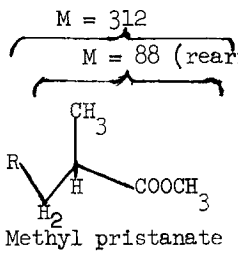
* After TLC of the methyl esters of the total fatty acids, the leading edge of the phytanate-pristanate zone was eluted and an aliquot was run on a GLC column as described under Methods. The radioactivity recovered in the pristanate zone is expressed as a percentage of the sum of the radioactivity in pristanate and phytanate. Previous studies have shown no significant conversion of ^{14}C-phytanate to normal straight-chain fatty acids (Mize et al, 1966). The radioactivity in the total GLC effluent preceding pristanate, that between pristanate and phytanate, and that following phytanate was less than 2% of that recovered with the branched-chain acids. Total recoveries of radioactivity injected into the GLC column were between 65 and 90%.

As shown in Table II, 5 minutes after intravenous injection of deuterated phytanic acid, it was possible to demonstrate the presence in the liver of highly enriched pristanic acid. The parent molecule was shown by precise mass measurement to have the empirical formula $C_{20}H_{40}O_2$. There was a prominent

mass 88 cleavage fragment (empirical formula from peak matching, $C_4H_8O_2$), highly characteristic for methyl esters of 2-methyl fatty acids (Ryhage and Stenhagen, 1960). The virtual absence of a peak at mass 74 indicated little or no contamination with straight-chain fatty acids.



Methyl phytanate



Methyl pristanate

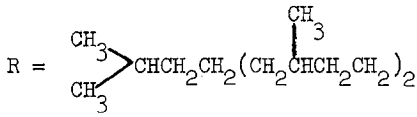


TABLE II

Deuterium-labeled Pristanic Acid Recovered from Rat Liver 5 Minutes after Intravenous Injection of Deuterium-labeled Phytanic Acid*

	Atoms percent excess in deuterated positions	
	Calculated** from (327/326) or (313/312)	Calculated** from (102/101) or (89/88)
Injected phytanate (2,3-dideutero)	47.6	46.2
Recovered pristanate (2-deutero)	30.6	29.2

* 10 mg of the sodium salt dissolved in 2 cc of 6% human serum albumin in saline.

** Calculations based on measured intensity ratios (327/326) and (102/101) in methyl phytanate and (313/312) and (89/88) in methyl pristanate. These ratios were corrected for the corresponding ratios in reference unlabeled phytanate and pristanate. $\Delta \frac{(M+1)}{M} = \frac{nC}{1-C}$, where n = no. of hydrogen positions among which excess deuterium is distributed; C x 100 = atoms percent excess.

Formation of pristanate from the administered 2,3-dideutero-phytanate would entail loss of the deuterium in the 2-position (i.e. of one-half the total) and the pristanate should then be labeled only in its 2-position. The data of Table II were calculated on the assumption that deuterium in phytanate was equally distributed between two hydrogens at C-2 and C-3 while all of that in pristanate was at C-2. The results are in accord with this, the enrichment in the mass 88 fragment of pristanate being equal to that in the parent molecule.

In preliminary studies, mice were fed diets containing 2% phytanic acid by weight for 2 days. The liver lipids showed the expected accumulation of phytanic acid itself (11.4% of total fatty acids), but also accumulation of another fatty acid (7.6% of total fatty acids) with TLC and GLC properties identical with those of pristanic acid. The mass spectrum of the methyl ester of this material was found to be identical to that of methyl pristanate.

DISCUSSION

The metabolic product derived from ^{14}C-phytanic acid had chromatographic properties, both in TLC and GLC, identical with those of authentic pristanic acid. The mass spectrometric data on the product derived from deuterium-labeled phytanic acid establish its structure unequivocally as that of pristanic acid. The combined application of stable isotopes and high resolution spectrometry was particularly valuable here, and should be in similar problems when only microgram amounts of product are available. The ability to confirm simultaneously both structure and origin from labeled precursor using only microgram quantities of product makes the approach uniquely powerful. Since there was so little dilution of the deuterium, virtually all of the pristanate isolated must have been derived directly from the administered phytanate. Either there was little or no endogenous pristanate present in the liver or the endogenous pool must have had a short half-life. We have demonstrated trace amounts of pristanic acid in normal rat liver (< 3 μg/g wet wt.). Pristanic acid has previously been found as a trace component in butterfat (Hansen and Morrison, 1964) and in sheep fat (Hansen, 1965a), which is of particular interest here since ruminant plasma and depot fat contain significant concentrations of phytanic acid (Lough, 1964; Hansen, 1965b).

The high yield of ^{14}C-pristanic acid in the rat studies and the net accumulation of pristanic acid in mice fed large doses of phytanic acid establish that α-decarboxylation is a major pathway for degradation of phytanic acid. Phytanic acid itself, because of the β-methyl substitution, is not susceptible to the usual β-oxidation. After α-decarboxylation, however,

the β-position, in the pristanic acid, is not blocked. Classical β-oxidation of pristanic acid would be expected to yield propionic acid. Successive β-oxidations could continue down the chain yielding, overall, 3 moles of propionic acid, 3 moles of acetic acid and 1 mole of isobutyric acid. Preliminary results in this laboratory show incorporation of a large fraction of the radioactivity of intravenously injected ^{14}C-phytanic acid into glucose, results compatible with the proposed further pathway of metabolism of pristanic acid and of the fragments formed from it. Although α-decarboxylation seems to be a major metabolic process for this compound in the rat and in the mouse, other reactions such as ω-oxidation (Eldjarn, 1965) may also be operative. The relative importance of alternative pathways in these and other species remains to be evaluated.

One-carbon degradation of long-chain fatty acids has been demonstrated in plants (Martin and Stumpf, 1959; Hitchcock and James, 1964). In animals there is evidence for its occurrence in brain (Mead and Levis, 1963; Davies et al, 1966). α-hydroxy acids are believed to be intermediates in the α-decarboxylation process in brain and may be involved in the one-carbon degradation of phytanic acid shown here. High concentrations of α-hydroxy acids are found only in nerve tissue (Kishimoto and Radin, 1963), suggesting that α-hydroxylation reactions may be peculiarly important in this tissue. This, coupled with the fact that the major manifestations of Refsum's disease are in the nervous system, suggests the possibility that the metabolic error in the disease lies in a relative inability to carry out α-hydroxylation reactions. The accumulation of phytanic acid and the deranged nerve cell metabolism might thus be independently explained on this basis.

Long-chain α-hydroxy fatty acids have been found outside the nervous system although only in low concentrations (Kishimoto and Radin, 1963). The β-methyl substitution of phytanic acid and its highly branched structure may make it a peculiarly suitable substrate for α-decarboxylation. However, the present results suggest the value of further studies on the importance of

α-decarboxylation in metabolism of fatty acids of various structures.

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