THE FORMATION OF ALPHA-HYDROXY PHYTANIC ACID FROM PHYTANIC ACID IN MAMMALIAN TISSUES

Su-Chen Tsai, J. H. Herndon, Jr., B. W. Uhlendorf, H. M. Fales, C. E. Mize

Laboratory of Metabolism, National Heart Institute and
Laboratory of Viral Immunology, Division of Biologics Standards
Bethesda, Maryland 20014

Received July 13, 1967

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is rapidly oxidized in animals (Mize et al., 1966a) and in man (Steinberg et al., 1967a) following intravenous injection. It has been shown that a major pathway for degradation of this branched-chain fatty acid involves an initial α-oxidation step to yield pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) (Avigan et al., 1966), followed by successive β-oxidations to yield lower branched-chain homologs (Mize et al., 1966b). Clinical studies indicated that a defect in this pathway is responsible for the large accumulation of phytanic acid in heredopathia atactica polyneuritiformis (Steinberg et al., 1967b), an inborn error of metabolism involving primarily the nervous system (Refsum, 1946). The enzymatic error has been localized between phytanic acid and pristanic acid (Steinberg et al., 1967a), but further definition requires elucidation of the mechanism of this one-carbon degradation. We now wish to report evidence for the formation of alpha-hydroxy phytanic acid from phytanic acid in three systems: rat liver homogenate, tissue culture of human skin fibroblasts, and mouse liver in vivo.

METHODS. Phytanic acid (Aldrich Chemical Co., Milwaukee, Wisc.) that had been prepared chemically from naturally-occurring phytol was used as starting material for synthesis of alpha-hydroxy-phytanic acid. The acyl chloride of phytanic acid was treated with excess bromine at 100°. The resulting α-bromo

acyl chloride was hydrolyzed with dilute alkali in dioxane to yield α-hydroxy phytanic acid, which was recovered from acidified medium by partitioning into ether. The methyl ester of the final product ran as a single component by gas liquid chromatography (GLC), but was resolved by thin layer chromatography (TLC) into two components, each of which yielded the mass spectrum characteristic of the expected alpha-hydroxy acid. These components are tentatively assumed to be the isomers representing different configuration of the hydroxyl group. The configuration of the 3-methyl group may also be involved, since the chemical conversion of phytol to phytanic acid is not stereospecific. The final material was purified by preparative GLC prior to use. Uniformly-labeled phytanic acid-^{14}C was prepared from phytol-U-^{14}C as described previously (Mize et al., 1966a).

Liver homogenates were prepared from normal adult Sprague-Dawley rats fed Purina chow until sacrifice. The liver was homogenized with a Ten-Broeck glass homogenizer in 0.1 M potassium phosphate buffer, pH 7.4, (2 ml/gm) containing 3% bovine serum albumin, 30 mM nicotinamide, 4 mM $MgCl_2$, and 5 mM potassium ascorbate. The whole homogenate was centrifuged at 700 Xg for 8 minutes and the supernatant material was used directly for incubation. Each flask contained 2.4 μmol sodium fumarate, 3.8 μmol NAD, 20 mμmol phytanic acid-U-^{14}C (4.3 μc/μmol), and homogenate (90 mg total protein) in a total volume of 3.2 ml. The stoppered flasks were incubated at 37° with gentle shaking, and the experiments were terminated by adding sulfuric acid through the stopper by syringe to 0.4 mM final concentration. $^{14}CO_2$ was collected in a center well on filter paper wet with 0.5 ml Hyamine-10X hydroxide (Packard Instrument Co., Chicago, Ill.) that was subsequently assayed for radioactivity in 0.5% diphenyloxazole in toluene (Eisenberg, 1958). The acidified incubation medium was treated with an equal volume of 10% KOH in 85% EtOH, heated 45 minutes over a steam bath, and the total fatty acid fraction isolated and converted to methyl esters as previously described (Steinberg et al., 1966). Radioactivity in individual methyl esters was quantitated by a modification of a method employing the collected effluent from a GLC column (Karmen et al., 1962).

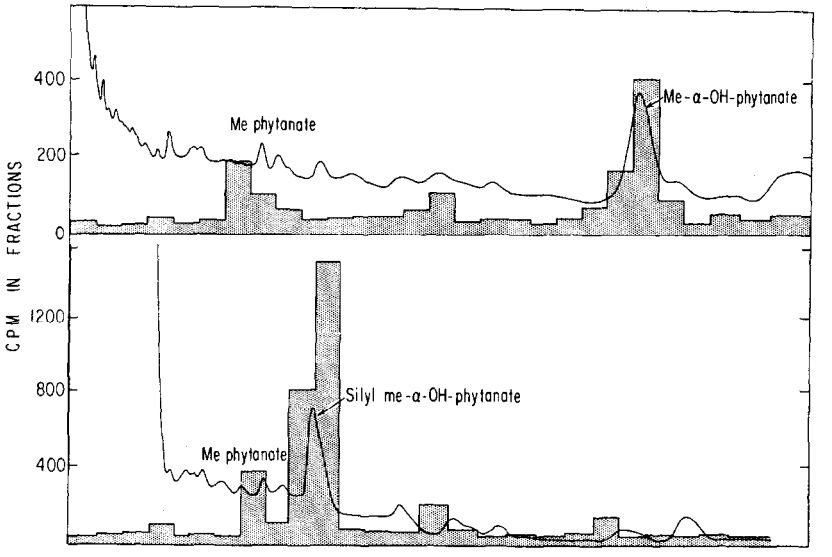
Skin fibroblast cultures were derived from skin biopsies of normal volunteers and of patients with disorders not known to affect metabolism of branched-chain fatty acids, as described previously (Steinberg et al., 1967a), and were used before their twentieth passage in culture. Phytanic acid-U-^{14}C (1-2 μc of > 99% radiopurity, specific activity > 90 μc/μmol) was complexed with albumin and incubated with the cells ($\geq$ 8 x 10^6) at 36.5° for 48 hours. The cells were trypsinized after incubation, washed free of residual medium, ruptured by sonication, and finally treated with 25 vols chloroform-methanol 2:1. Fatty acids were isolated as described elsewhere (Steinberg et al., 1966).

Weanling white mice were fed 2% phytanic acid by weight in the diet for 7 days. At the time of sacrifice, the livers were minced and treated with chloroform-methanol 2:1, and total fatty acids were similarly isolated.

RESULTS. In vitro experiments utilizing rat liver homogenate demonstrated the formation of labeled α-hydroxy phytanic acid from phytanic acid-U-C^{14}. The methylated fatty acids obtained after incubation were fractionated by GLC on a mixed column of 7% EGS-1.5% SE-30 on Chromsorb W. A small but consistent amount of the total homogenate fatty acid radioactivity was associated with methyl trimethyltridecanoate (3.4%), methyl pristanate (1.2%), and an unidentified polar material (2.8%) with a retention volume (R_v) 3.3 that of methyl palmitate. Carrier methyl α-hydroxy phytanate was added to the total fatty acids and the mixture silylated (Klebe et al., 1966). Radioactivity was no longer found at the previous location, while a new peak of radioactivity (3.2%) appeared coincident with the mass peak of the silyl ether of α-hydroxy phytanate (R_v 2.5 that of methyl palmitate).

The total methylated fatty acids of the liver homogenate after incubation with phytanic acid-U-^{14}C were also subjected to TLC after addition of carrier alpha-hydroxy phytanate. The zone corresponding to α-hydroxy-phytanate (R_f 0.22 to 0.30 in ethyl acetate-hexane 1:9) was eluted and rechromatographed by GLC on 17% EGS on Chromsorb W. Forty-seven percent of the radioactivity in this TLC zone co-chromatographed with α-hydroxyphytanate (R_v 5.3 that of palmitate).

When the hydroxy-acid TLC zone was silylated and similarly rechromatographed by GLC, 52% of the radioactivity was associated with the silyl ether of α-hydroxy phytanate (R_v 2.2 of palmitate). These results are shown in Figure 1.



Legend: Gas-liquid radiochromatograms of the TLC zone corresponding to methyl alpha-hydroxy phytanate (top), or of the same TLC zone subjected to silylation (bottom), eluted from a thin layer plate on which were fractionated the methylated fatty acids derived from rat liver homogenate incubated with phytanic acid-U-C^{14} for 2 hours (see text for details).

Oxidation of phytanic acid-U-^{14}C to $^{14}CO_2$ by liver homogenate proceeded at a linear rate for 90 minutes after a lag period of approximately 30 minutes, with conversion of 29% of the added radioactivity to $^{14}CO_2$ during this time. The disappearance of phytanic acid-^{14}C during the first hour of incubation was accompanied by the linear formation of α-hydroxy-phytanic acid-^{14}C over the same period.

Labeled α-hydroxy phytanic acid was also identified in the lipids of skin fibroblasts incubated with phytanic acid-U-^{14}C. The methylated fatty acids of the fibroblasts were analyzed for radioactivity after fractionation by GLC on polar (17% EGS on Chromsorb W) or non-polar (3% OV-1 on Gas-Chrom Q) liquid phases. On both columns, 0.3 to 1.3% of the collected radioactivity appeared as a well-defined peak that co-chromatographed with authentic α-hydroxy phytanate.

Previous studies have shown that similarly cultured fibroblasts also degrade labeled phytanate to pristanate (Steinberg et al., 1967a), and more recently the conversion of phytanate to trimethyltridecanoate by fibroblasts has been demonstrated. Radioactivity in these two compounds was present at levels two to four times that of α-hydroxy phytanate in the present experiments.

Data from direct GLC analysis of the fibroblast fatty acids were supplemented by performing a preliminary fractionation of the methylated fatty acids by TLC on silica gel-G. The plates were developed in benzene:hexane 2:1 and the zone corresponding to authentic methyl α-hydroxy phytanate was eluted. GLC analysis of the material from this zone on a 17% EGS column showed that 76% of the radioactivity co-chromatographed with methyl α-hydroxyphytanate.

Livers of mice fed large amounts of phytol or phytanic acid in the diet have been shown previously to accumulate phytanic acid and several of its metabolites that result from the sequence initiated by α-oxidation. In the course of these investigations, an unidentified GLC peak in the fatty acid fraction representing 2% of total fatty acids was noted but not identified. This component had a retention time of 3.3 relative to that of methyl palmitate (15% EGS-3% SE-30 mixed column). The unknown acid was collected as the methyl ester and analyzed on an LKB-9000 GLC-Mass Spectrometer. The molecular ion was found at m/e 342, and the base peak at m/e 90, with prominent fragmentation peaks at m/e 283, 117 and 57. The spectrum was essentially identical with that of synthetic methyl alpha-hydroxy phytanate.

DISCUSSION. The data show that phytanic acid is converted to α-hydroxy phytanic acid in tissues from the three species studied. In the studies in rat liver homogenates and skin fibroblast cultures, only tracer amounts of phytanic acid were added and identification rests on the identical chromatographic properties of the labeled compound isolated and those of the synthetic α-hydroxy phytanic acid. Other metabolites of phytanic acid have been shown to accumulate uniquely

in mice fed phytanic acid (Mize et al., 1966b), and in the present studies, we have now also shown that a small but significant accumulation of α-hydroxy phytanic acid occurs. The mass spectrum identifies the compound unambiguously. While we cannot rule out the possibility that the α-hydroxy phytanic acid is a side product, it seems reasonable to conclude that it is an intermediate in the conversion of phytanic acid to pristanic acid. The time course of the appearance of labeled α-hydroxy phytanic acid in the liver homogenate studies is compatible with this interpretation, as are preliminary data that indicate unlabeled α-hydroxyphytanate added to the incubation medium reduces both the production of $^{14}CO_2$ and the incorporation of radioactivity into both pristanate and trimethyltridecanoate from phytanate-^{14}C. A direct analogy is to be found in one-carbon degradation of straight-chain fatty acids both in mammalian brain (Hajra and Radin, 1963) and in plants (Hitchcock and James, 1966). In both systems, formation of α-hydroxy acids has been demonstrated, and they are believed to be intermediates in α-oxidation. In plants, the subsequent steps are thought to involve formation of the corresponding alpha-keto acid or the (n-1) aldehyde (Martin and Stumpf, 1959), and in brain the alpha-keto acid is believed to be an intermediate (Levis and Mead, 1964), but evidence on this point is limited.

While the mechanism of phytanic acid oxidation described here resembles the α-oxidation of straight-chain fatty acids in brain, it remains to be determined whether these enzyme systems are identical or closely related. We have suggested previously that the metabolic defect in heredopathia atactica polyneuritiformis might involve an error in an alpha-hydroxylating system (Avigan et al., 1966), and it should now be possible to test this hypothesis directly. A question of great importance is whether the alpha-hydroxylating system responsible for oxidizing phytanic acid plays an additional role in the metabolism of other substrates, particularly in the nervous system.

REFERENCES

Avigan, J., Steinberg, D., Gutman, A., Mize, C.E. and Milne, G.W.A. Biochem. Biophys. Res. Comm. 24:838 (1966).

Eisenberg, F., in Liquid Scintillation Counting (Bell and Hayes, Eds.) p. 123, Pergamon Press, New York (1958).
Hajra, A.K. and Radin, N.S. J. Lipid Res. 4:270 (1963).
Hitchcock, C. and James, A.T. Biochim. Biophys. Acta 116:413 (1966).
Karmen, A., Giuffrida, L. and Bowman, R.L. J. Lipid Res. 3:44 (1962).
Klebe, J.F., Finkbeiner, H. and White, D.M. J. Am. Chem. Soc. 88:3390 (1966).
Levis, G.M. and Mead, J.F. J. Biol. Chem. 239:77 (1964).
Martin, R.O. and Stumpf, P.K. J. Biol. Chem. 234:2548 (1959).
Mize, C.E., Avigan, J., Baxter, J.H., Fales, H. and Steinberg, D. J. Lipid Res. 7:692 (1966a).
Mize, C.E., Steinberg, D., Avigan, J. and Fales, H.M. Biochem. Biophys. Res. Comm. 25:359 (1966b).
Refsum, S. Acta psychiat. scand. suppl. 38:9 (1946).
Steinberg, D., Avigan, J., Mize, C.E., Baxter, J.H., Cammermeyer, J., Fales, H.M. and Highet, P.F. J. Lipid Res. 7:684 (1966).
Steinberg, D., Herndon, J.H., Jr., Uhlendorf, B.W., Mize, C.E., Avigan, J., and Milne, G.W.A. Science 156:1740 (1967a).
Steinberg, D., Herndon, J.H., Jr., Uhlendorf, B.W., Avigan, J., Mize, C.E. and Fales, H.M. J. Clin. Invest. 46:1120 (1967b).