Characterization of Bovine Rumen Liquor Isoprenoid Hydrocarbons with Reference to Dietary Phytol

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ABSTRACT

The isoprenoid hydrocarbons, *trans*-phyt-2-ene (82.2%), phyt-1-ene (6.5%), and phytane (11.3%), were detected in bovine rumen liquor by means of $AgNO_3$-impregnated thin layer chromatography and gas liquid chromatography with mass spectrometry and infrared spectrophotometry. The unsaturated hydrocarbons were identified by comparing their chromatographic, physical, and chemical properties with those of synthetic isoprenoid hydrocarbons prepared from dihydrophytol. The natural level of the original isoprenoid components within the rumen is comprised of phytol and dihydrophytol (of the ratio of 2:1). These represented at least 60% of the total free fatty alcohol content of the rumen liquor lipid extract and could, therefore, be the origin of the isoprenoid hydrocarbons observed.

INTRODUCTION

Isoprenoid compounds are widely distributed in nature. In the rumen, these all apparently originate from phytol (*trans*-3,7,11,15-tetramethyl-2-hexadecen-1-ol), an unsaturated isoprenoid alcohol which represents about 30% of the chlorophyll content of dietary green herbage for ruminant animals (1). Phytol can be readily hydrogenated to dihydrophytol (3,7,11,15-tetramethylhexadecan-1-ol) which is oxidized to produce phytanic (3,7,11,15-tetramethylhexadecanoic) acid by the action of microorganisms in rumen liquor (2). Phytanic acid is found in many ruminant tissues (3). More recently, phytol and dihydrophytol were found in milkfat (4) and as dihydrophytyl-phytanate (wax esters) in rumen liquor (5).

Other phytol metabolites, namely phytane (3,7,11,15-tetramethylhexadecane) and isomers of phytene (3,7,11,15-tetramethylhexadecene) hydrocarbons, have been found as minor constituents of milkfat (6) and butterfat (7). This communication reports the occurrence of these isoprenoid hydrocarbons in bovine rumen lipid extracts and discusses their relationship with the dietary phytol source.

MATERIALS AND METHODS

Rumen liquor was obtained from a Jersey cow fitted with a fistula and fed on fresh white clover ad libitum. This animal was fasted overnight before the rumen liquor was taken and strained through cheesecloth and the lipid constituents were extracted with diethyl ether as described before (5). From 100 ml of rumen liquor, a total lipid extract of 312.6 mg was recovered, dissolved in chloroform, and applied to an activated silicic acid column (18 g, Bio-Sil A, Bio-Rad Laboratories, Richmond, CA) for fractionation as outlined earlier (5). This produced the following main fractions of lipid components: fraction 1, hydrocarbons-carotenoids-wax esters-sterol esters (26.4 mg); fraction 2, free fatty acids-sterols-fatty alcohols (179.0 mg) and fraction 3, pigments-phospholipids (92.2 mg).

Isolation of the Hydrocarbons

Hydrocarbons (12.0 mg) were isolated from fraction 1 by preparative thin layer chromatography (TLC) after development with hexane-diethyl ether-acetic acid (85:15:1 v/v).

Isolation of the Free Fatty Alcohols

Fraction 2 was methylated with diazomethane (8) and the resultant FFA-methyl esters were removed by silicic acid (20 g)

TABLE II

Composition of the Hydrocarbon and Free Fatty Alcohol Constituents of Bovine Rumen Liquor

Components	mg/100 ml rumen liquor
Isoprenoid hydrocarbons	
Phytane	0.12
Phyt-1-ene	0.07
cis-Phyt-2-ene	-
trans-Phyt-2-ene	0.88
Other hydrocarbons	
C_{18}-C_{35}	10.93
Total	12.00
Isoprenoid fatty alcohols	
Phytol	12.72
Dihydrophytol	6.36
Other fatty alcohols	
C_{18}-C_{34}	12.72
Total	31.80

column chromatography with chloroform as eluant. Free fatty alcohols (31.8 mg) were isolated from the remaining components of fraction 2 by further fractionation using preparative TLC with benzene as the developing solvent system.

Preparation of Authentic Isoprenoid Hydrocarbons

A range of unsaturated hydrocarbons can be readily obtained by dehydrating the corresponding primary alcohol. In this case, dihydrophytol was prepared by hydrogenating phytol (K and K Laboratories Inc., Plainview, NY) in methanol over PtO_2 at 45 C for 6 hr at atmospheric pressure. Approximately 130 mg dihydrophytol was stirred and heated to 150 C with 0.5 ml 88% *ortho*-phosphoric acid (AR, British Drug Houses Ltd., Poole, England) for 1.5 hr. During this reaction, some charring was noticed so the final material was dissolved in diethyl ether and filtered while transferring it to a separating funnel. The ethereal extract was washed with distilled water until neutral, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure at 45 C. These dehydration products (120 mg) dissolved in chloroform were applied to an activated silicic acid column (20 g). The isoprenoid hydrocarbons (95.5 mg) were eluted with chloroform (70 ml) and the residual products (20.5 mg) with methanol (100 ml). This reaction produced a satisfactory quantity of unsaturated isoprenoid hydrocarbons that was further resolved by preparative TLC with 10% $AgNO_3$-impregnated purified (9) silica gel G (E. Merck, A.G. Darmstadt, Germany) plates with hexane-diethyl ether (95:5 v/v) as developing solvent system. By this procedure, three different unsaturated isoprenoid hydrocarbon isomers (A, B, and C) were isolated. The proportion of these components together with their chromatographic properties is shown in Table I.

Analysis of Hydrocarbons

The hydrocarbons were analyzed by gas liquid chromatography (GLC) using a dual flame Pye 104 instrument (Pye-Unicam Ltd., Cambridge, England) fitted with standard glass columns (1.5 m x 0.4 cm ID). Two different liquid phases, namely 10% EGSS-X on 60-80 mesh Gas-Chrom Z and 3% JXR on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA) solid supports, were operated at 120 and 160 C with nitrogen carrier gas flow-rates of 20 and 30 ml/min, respectively. To aid the identification of hydrocarbon mixtures, the chromatographic properties of these columns were calibrated with authentic standards and the 3% JXR column coupled

TABLE I

Composition and Chromatographic Properties of Isoprenoid Hydrocarbons Synthesized by the Dehydration of Dihydrophytol

Isoprenoid hydrocarbon	Composition (wt %)	$AgNO_3$-impregnated TLC R_f value[a]	GLC ECL[b] value on 3% JXR at 160 C	GLC ECL[b] value on 10% EGSS-X at 120 C
trans-Phyt-2-ene (A)	15.2	0.95	18.48	18.92
cis-Phyt-2-ene (B)	61.2	0.92	18.35	18.72
Phyt-1-ene (C)	23.6	0.86	17.85	18.18
Phytane	-	0.97	18.14	17.87

[a] R_f value of *n*-$C_{18:0}$ hydrocarbon = 1.00. TLC = thin layer chromatography.
[b] Equivalent chain length values with reference to *n*-hydrocarbons. GLC = gas liquid chromatography.

with a mass spectrometer (GLC-MS) (AEI MS30 Double Beam MS, AEI Scientific Apparatus Ltd., Manchester, England).

Analysis of Fatty Alcohols

The 3% JXR GLC column (as above) was used to analyze the fatty alcohol mixtures either in the free state or as their alcohol-acetate derivatives. Both isothermal conditions (180 C) or temperatures programmed over the 180 to 270 C range at 4 C per min were applied and carrier gas flow rates of 30 ml/min were uniform throughout. For the identification of the fatty alcohol components, their GLC properties were compared with those of authentic standards under identical conditions as outlined elsewhere (5,10).

RESULTS

The three isoprenoid hydrocarbon isomers A, B, and C (Table I) prepared by dehydrating dihydrophytol were initially inspected as films on KBr discs by infrared spectrophotometry (Infracord 137, Perkin-Elmer Ltd., Beaconsfield, Bucks, England). They all revealed standard isoprenoid coupled adsorption bands in the 1360-1370 cm^{-1} region, defined isoprene bands at 737 cm^{-1} and isopropyl bands at 1180 cm^{-1} (7). In particular, the spectrum of compound A also included the presence of a *trans*-double bond (968 cm^{-1}) while compound C differed showing a strong adsorption at 910 cm^{-1} indicating a distinct terminal vinyl group (11).

All these compounds had the same molecular ions at m/e 280 (M) by MS and some specific fragment ions of slightly variable strengths at m/e 252, 210, 196, 181, 140, 125, 111, 97, 70, and 57. Similarly, they all produced phytane [m/e 282 (M) and other fragment ions at m/e 267, 197, 127, and 57 which demonstrated the methyl-branched sites on the molecule (12)] as their only hydrogenation product. The above evidence together with the $AgNO_3$-impregnated TLC and GLC chromatographic properties (Table I) identified compound A as *trans*-phyt-2-ene, B as *cis*-phyt-2-ene and C as phyt-1-ene.

The isoprenoid hydrocarbons present in bovine rumen liquor and identified by GLC-MS showed a distribution pattern per 100 ml rumen liquor of phytane (0.12 mg), phyt-1-ene (0.07 mg), and *trans*-phyt-2-ene (0.88 mg). This represented 8.9% of the total hydrocarbon contents (Table II). The other hydrocarbons, including *n*-nonacosane (C_{29}) and *n*-hentriacontane (C_{31}) as the principal components, resembled those of the white clover neutral lipids (10) as expected.

In contrast, the composition of the fatty alcohol fraction differed remarkably from that of the hydrocarbons in respect to the quantity of isoprenoid components (Table II). Here at least 60% was represented by phytol (40%) and dihydrophytol (20%). The remaining 40% was spread over the C_{18} to C_{32} range with *n*-triacontanol (C_{30}) as the main contributor. This resembled the fatty alcohol distribution pattern of those associated with the neutral lipids of the dietary white clover (10).

DISCUSSION

The microbial action on phytol in rumen liquor has been studied extensively by Patton and Benson (2). Their conclusive observation that dihydrophytol is the biohydrogenation product from the chlorophyll-phytol moiety would also apply with this work and these isoprenoid fatty alcohols are the major contributions to the entire free fatty alcohol contents of the rumen liquor under investigation. No doubt these isoprenoid fatty alcohols (ca. 19.0 mg/100 ml rumen liquor) are also the source of the isoprenoid hydrocarbons (ca. 1.0 mg/100 ml rumen liquor) now found in the rumen.

Phytol can be readily dehydrated catalytically (13) or biologically (14) under mild conditions to yield phytadiene isomers. One of the relevant phytadiene isomers, neophytadiene (3-methylene-7,11,15-trimethylhexadec-1-ene), has been reported by Urbach and Stark (7) to be present as part of the isoprenoid hydrocarbons in both butterfat and ryegrass lipid extracts. They proposed that any dietary ryegrass neophytadiene would be biohydrogenated to form phyt-1-ene as the isoprenoid hydrocarbon constituent of rumen liquor. However, in the present study, the actual findings differed markedly. Initially, the dietary white clover source used provided (10) no isoprenoid hydrocarbon of the same nature as neophytadiene. Secondly, since *trans*-phyt-2-ene was the major isoprenoid hydrocarbon present in rumen liquor, this also suggests that another source of isoprenoid hydrocarbons must be considered.

The ability of the microorganisms within the rumen liquor to produce methane from dietary carbohydrate is well known (15). These conditions would be adequate to allow phytol, with the natural *trans*-2 configuration, to freely exchange its primary hydroxyl-group with hydrogen to form *trans*-phyt-2-ene as a main end-product. Even the proximity of the labile double bond near the terminal hydroxyl-group could also permit the formation of the observed

phyt-1-ene (vinyl-structure) isoprenoid hydrocarbon when hydroxyl-proton interchange takes place.

On the other hand, phyt-1-ene could be alternatively produced as part of the migration process formed during the biohydrogenation action on *trans*-phyt-2-ene to yield phytane as the end-product. Similar isomerization and migration of double bonds during the parallel biohydrogenation of polyunsaturated fatty acids by rumen microorganisms has been established and the distribution pattern of their various products has been recently reinvestigated (16).

The chemical, physical, and chromatographic properties of the unsaturated isoprenoid hydrocarbons are governed by their molecular structure. The steric hindrance imposed on the unsaturated site of the phyt-2-ene isomers reduces the formation of $AgNO_3$-complexes to prevent distinct resolution by TLC between the different structural configurations (i.e., *cis*- or *trans*-isomers) and phytane. However, combinations of $AgNO_3$-impregnated TLC, GLC-MS, and infrared spectrophotometry did resolve the isoprenoid hydrocarbons present in bovine rumen liquor. These results also indicate that the monounsaturated isoprenoid hydrocarbons shown in various ruminant products (6,7) are probably derived from the initial microorganism reaction on dietary phytol and intermediates in the rumen.

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REFERENCES

1. Hansen, R.P., J. Dairy Res. 33:333 (1966).
2. Patton, S., and A.A. Benson, Biochim. Biophys. Acta 125:22 (1966).
3. Lough, A.K., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 14, Part 1, Edited by R.T. Holman, Pergamon Press, Oxford, England, 1973, pp. 1-48.
4. Flanagan, V.P., A. Ferretti, D.P. Schwartz, and J.M. Ruth, J. Lipid Res. 16:97 (1975).
5. Body, D.R., Biochim. Biophys. Acta 380:45 (1975).
6. Flanagan, V.P., and A. Ferretti, J. Lipid Res. 14:306 (1973).
7. Urbach, G., and W. Stark, J. Agr. Food Chem. 23:20 (1975).
8. Schlenk, H., and J.L. Gellerman, Anal. Chem. 32:1412 (1960).
9. Body, D.R., and G.M. Gray, Chem. Phys. Lipids 1:254 (1967).
10. Body, D.R., Phytochemistry 13:1527 (1974).
11. Chapman, D., in "The Structure of Lipids," Methuen and Co. Ltd., London, England, 1965, pp. 58-59.
12. Gelpi, E., and J. Oro, JAOCS 45:144 (1968).
13. Johnstone, R.A.W., and P.M. Quan, J. Chem. Soc. 5706 (1963).
14. Blumer, M., J.C. Robertson, J.E. Gordon, and J. Sass, Biochemistry 8:4067 (1969).
15. Czerkawski, J.W., Proc. Nutr. Soc. 31:141 (1972).
16. Body, D.R., Biochem. J. 157:741 (1976).

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