The Occurrence of *cis*-Octadec-15-enoic Acid as a Major Biohydrogenation Product from Methyl Linolenate in Bovine Rumen Liquor

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The biohydrogenation products from methyl linolenate varied according to its concentration in the rumen. At low concentrations, over 60% of the biohydrogenated products was stearic acid, but when the concentration was raised above 100 mg/100 ml of rumen liquor, monoenoic acids, including *cis*-octadec-15-enoic acid, were the major products.

The microbial attack on dietary linolenic acid in rumen liquor, yielding a more saturated fatty acid, was originally observed by Reiser (1951), Reiser & Reddy (1956) and Shorland *et al.* (1955, 1957). Since then, these digestion processes have been thoroughly studied and frequently reviewed (Dawson & Kemp, 1970; Viviani, 1970).

Whether dietary-herbage galactolipids (Dawson *et al.*, 1974) or supplementary-grain triacylglycerols (Garton *et al.*, 1961; Hawke & Silcock, 1969) are incubated in rumen liquor, they are first hydrolysed before any biohydrogenation can occur. This probably also applies when other esterified forms of fatty acids, e.g. methyl esters, are used as substrates.

The extensive investigations of the biohydrogenation pathway of polyunsaturated fatty acids, which yields a range of geometric and positional isomers of octadecenoic acid, have been discussed by Dawson & Kemp (1970). The present communication reports the incubation of methyl linolenate (*in vitro*), at various concentrations, with bovine rumen liquor, and the effect of the concentration on the nature of the end products of biohydrogenation.

Materials and Methods

Rumen liquor

Approx. 500 ml portions of rumen liquor were taken from non-lactating Jersey cows, fitted with rumen fistulae. They had not been fed overnight, but up till then had been fed with fresh white clover with no restriction. Water was available *ad lib*. The liquor was strained through cheesecloth to remove excessive extraneous solid plant particles and transported to the laboratory in a glass vessel kept at 39°C.

Substrate

Methyl linolenate (1 g; Nu-Chek Preparations, Elysian, MN, U.S.A.) was suspended in 10 ml of aq. 1% (v/v) Tween 80 (Atlas Chemical Industries,

Wilmington, DE, U.S.A.) and stored at 4°C until required.

Incubation

The O₂ contents of duplicate portions (50 ml) of rumen liquor in 100 ml conical flasks were displaced with pure N₂ before substrates were added. The incubation period was 24 h with continuous shaking at 39°C under a blanket of pure N₂ to retain anaerobic conditions. Simultaneously, a third 50 ml portion of rumen liquor was incubated, without substrate, so that the background lipid concentration could be determined and any lipid changes during incubation could be monitored. All experiments were repeated with rumen liquor from another animal.

Extraction of lipids

After incubation, the mixtures were transferred to 250 ml separating funnels and acidified with 6 ml of 1 M-HCl. The lipids were recovered by extraction with 2 × 70 ml of diethyl ether followed by centrifugation (1600 g for 20 min). The total ethereal extract was washed twice with distilled water (120 ml), dried over anhydrous Na₂SO₄ and evaporated under reduced pressure at 40°C.

Analytical methods

To test for reproducibility of these incubation experiments, the individual lipid extracts were weighed and analysed by t.l.c. in hexane/diethyl ether/acetic acid (85:15:1, by vol.). The components were identified by comparing their chromatographic properties with those of authentic samples under identical conditions. The compositions of the duplicate lipid extracts (each obtained from 50 ml of rumen liquor) were very similar, so that they could be pooled to give the lipid content/100 ml of rumen liquor. This gave a range of total lipid extracts of 300-500 mg. These were fractionated on columns (32 cm × 2 cm¹ diam.) of silicic acid (Bio-Sil A; Bio-Rad

Laboratories, Richmond, CA, U.S.A.) eluted with chloroform (400ml) and methanol (500ml). Free fatty acids were removed with the first solvent, and the second eluted the residual material (sterols, phospholipids and pigments), so that together the total recoveries from the columns could be calculated. Throughout, all fractions were collected (10ml portions) and monitored by t.l.c. The free fatty acids recovered were methylated with diazomethane (Schlenk & Gellerman, 1960) and the resultant methyl esters were purified by further silicic acid column (15cm×2.0cm diam.) chromatography (Body *et al.*, 1970) before analysis by g.l.c.

The methylated free fatty acids were further resolved by preparative t.l.c. on purified 10% AgNO₃-impregnated (Body & Gray, 1967) silica gel G (E. Merck, A.G., Darmstadt, Germany), with benzene/hexane (1:1, v/v) as solvent. The corresponding methyl ester bands, according to their degree of unsaturation and their structural configuration (i.e. *cis*- or *trans*-isomers), were isolated, extracted (Privett & Nickell, 1966) and analysed by g.l.c.

G.l.c. analyses of the fatty acid methyl esters were carried out on both polar (10% EGSS-X) and non-polar (3% ApL) columns. All technical conditions and identification aids have been outlined elsewhere (Body, 1974, 1975).

Determination of the position of the double bonds

A modified KMnO₄/acetic acid oxidation method as described by Body & Shorland (1965) was used to determine the positions of the double bonds in the unsaturated fatty acids formed during the incubation of methyl linolenate in bovine rumen liquor. The dibasic acid dimethyl esters so formed were analysed by g.l.c. by using a 10% EGSS-X liquid phase operated at 180°C.

Results

No methyl linolenate was detected in the lipid extracts of the rumen liquor, indicating that it had been completely hydrolysed over the 24h period used. The lipid extracts (containing 300–500mg of lipid/100ml of rumen liquor) were further fractionated by silicic acid column chromatography. The yields throughout were high (96–98%), and the following range of general constituents in the rumen lipid extracts were obtained: hydrocarbons, carotenoids and wax esters, 9–14%; free sterols and fatty alcohols, 14–19%; phospholipids and pigments 8–12%; free fatty acids, 54–69%. These values vary depending on the quantity of substrate applied, but they each include complete recovery of the products over the 24h incubation period.

When the free fatty acid fractions were analysed by g.l.c. at zero time and after incubation, no linolenic acid was detected. This is direct evidence that the

initial stage of biohydrogenation was completed. The background fatty acid composition of the rumen liquor (obtained from animals starved overnight) had high concentrations (60–62%) of stearic acid and low concentrations (8–13%) of unsaturated fatty acids (mainly *trans*-monoenoic fatty acid isomers). This shows that the natural biological reaction on the original dietary polyunsaturated fatty acids had been completed and would not therefore interfere to any extent with the reactions of added substrates. Hence these background concentrations could be subtracted from those obtained on incubation of the methyl linolenate to give the actual concentrations of the biohydrogenated products of the administered substrates (see Table 1).

In both Expts. 1 and 2, when the substrate concentrations were 107 and 113mg/100ml of rumen liquor respectively and allowing for variation since two different rumen sources were used, the distribution of the biohydrogenated products was similar. When 43mg of substrate/100ml of rumen liquor was applied (Expt. 1), 63.2% of the incubated linolenic acid was completely biohydrogenated to stearic acid, with 29.0% still in the form of monoenoic fatty acids. However, when the concentration of the substrate was raised to 107mg/100ml (Expt. 1) and 113mg/100ml (Expt. 2), over the same period of time (24h), only 2.7–10.2% of linolenic acid was converted into stearic acid. The major hydrogenated products were still in the monoenoic fatty acid form (77.3–82.3%). However, a considerable proportion of these monoenoic fatty acid isomers had an uncommon structure.

After incubation of the substrate, the biohydrogenated fatty acid methyl ester products were resolved by t.l.c. on AgNO₃-impregnated silica gel. As the substrate concentration in the rumen liquor was increased, the overall proportion of *trans*-isomers in the free monoenoic fatty acid group of products was decreased and the concentration of *cis*-

Table 1. Percentage of methyl linolenate converted into biohydrogenated products in bovine rumen liquor

Experimental and analytical details are presented in the text. The unspecified C_{18:1} fatty acids are predominantly made up of *trans*-isomers (see Table 2).

Substrate concn. (mg/100ml) ...	Products (%)		
	Expt. 1		Expt. 2
	43	107	
C ₁₈ fatty acid			
C _{18:0}	63.2	2.7	10.2
C _{18:1}	27.3	38.4	40.1
C _{18:1} (<i>cis</i> -15 and <i>trans</i> -16)	1.7	43.9	37.2
C _{18:2} (<i>trans</i> -11, <i>cis</i> -15)	7.8	15.0	12.5

Table 2. *Composition of the free octadecenoic acids in bovine rumen liquor*

Experimental and analytical details are described in the text. The quantitative distribution is expressed as mg/100 ml of rumen liquor. The values in parentheses are the unsaturated sites on the molecule. tr, Trace, less than 0.1 mg.

Substrate concn. (mg/100ml) ...		Distribution (mg/100ml)					
		0		43		107	
		<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>
$C_{18:1}$ isomers ...							
(5)					tr		
(7)			tr	0.1	tr		
(8)		0.1	0.1	0.4	0.1	0.7	
(9)		0.4	0.6	0.6	0.2	0.9	
(10)		1.8	0.4	1.8	0.2	3.1	
(11)		9.4	1.0	6.6	0.6	8.6	
(12)		1.5	0.4	1.7	0.3	5.6	0.3
(13)		3.2	0.1	5.8	0.4	13.4	1.0
(14)		2.8	0.1	5.4	0.8	14.3	3.0
(15)		2.8		5.4	4.3	16.3	35.0
(16)		2.7		4.3	0.5	7.3	1.6
		27.4		39.5		111.1	
Ratio <i>trans/cis</i>		90:10		81:19		63:37	

isomers was reciprocally increased (Table 2). Further details on the distribution of the individual positional isomers of the monoenoic fatty acid formed in Expt. 1 (calculated from their oxidation products, determined by g.l.c.) are also shown in Table 2. When the substrate concentration applied was 43 mg/100 ml of rumen liquor, the production of *trans*-isomers increased from 24.7 mg/100 ml (zero time) to 32.1 mg/100 ml and that of the *cis*-isomers only from 2.7 mg/100 ml to 7.4 mg/100 ml. However, when the substrate concentration exceeded 100 mg/100 ml, even though the *trans*-isomer concentration was increased three-fold, the production of *cis*-isomer was also increased so that *cis*-octadec-15-enoic acid was the principal component of either geometrical structure.

The methyl ester derivatives of the dienoic acid series produced from the incubated substrate at either concentration and isolated by t.l.c. on AgNO₃-impregnated silica gel had similar chromatographic properties under these conditions to those dienoates containing at least one *trans*-double bond. Further, both groups of dienoates produced C₁₁ dibasic acid as the major oxidation product (60.3 and 51.3 % when the substrate concentration was 43 mg/100 ml and 107 mg/100 ml respectively). The spread of the residual oxidation products represented a wide range of minor dienoic acids accumulated during the biohydrogenation process, at both substrate concentrations. However, the principal corresponding dienoate isomers had similar g.l.c. properties on both polar (10% EGSS-X) and non-polar (3% ApL) liquid phases to those of authentic methyl *trans,cis*-octadeca-11,15-dienoate. Hence, considering the

above evidence, it seems likely that these dienoates are identical. This isomer has been described (Dawson & Kemp, 1970) as a typical dienoic fatty acid intermediate formed during the biohydrogenation of linolenic acid in rumen liquor.

Discussion

It was demonstrated by Noble *et al.* (1974) that the yield of biohydrogenation products in rumen liquor was higher with esterified rather than free fatty acids as substrate. Whether the esterification is as triacylglycerol (Hawke & Silcock, 1969), galactolipid (Dawson *et al.*, 1974) or, as in the present investigation, methyl ester, they have to be hydrolysed first before the microbial biohydrogenation process can commence. The advantage of using methyl linolenate as the substrate followed by the efficient extraction process with acidified diethyl ether was to simplify the analytical procedures. The unchanged substrate can be readily removed by either t.l.c. or silicic acid column-chromatographic techniques to leave available the fully recoverable source of biologically treated free fatty acid products.

It has been well established (Dawson & Kemp, 1970) that the general biohydrogenation pattern of linolenic acid by rumen micro-organisms passes through various intermediate stages. It is first isomerized to *cis,trans,cis*-octadeca-9,11,15-trienoic acid before hydrogenation commences. Then *trans,cis*-octadeca-11,15-dienoic acid is produced followed by *trans*-octadec-11-enoic acid. This monoenoic fatty acid is considered to be the final unsaturated com-

ponent before the completion of the conversion of linolenic acid into stearic acid. This last step also applies to the total biohydrogenation pathway changing linoleic acid to stearic acid. However, other factors can influence the limit of the biohydrogenation of unsaturated fatty acids in rumen liquor and these have been extensively examined by Harfoot *et al.* (1973). They concluded that when the linoleic acid concentration exceeded 100mg/100ml of rumen liquor, its biohydrogenation to stearic acid stopped at the monoenoic fatty acid stage, with *trans*-octadec-11-enoic acid as the main product.

Similar observations were made with methyl linolenate as substrate. When the substrate concentration was less than 50mg/100ml of rumen liquor, the principal biohydrogenation end product was stearic acid, with *trans*-monoenoic fatty acids, over the range *trans*-11 to *trans*-16, as the predominant intermediates. However, when the substrate concentration was raised above 100mg/100ml of rumen liquor, approx. 80% of the hydrogenated product was in the monoenoic fatty acid form. The unusual principal monoenoic isomer was *cis*-octadec-15-enoic acid.

Even in the control incubation when *trans*-octadec-11-enoic acid was the main component, the concentrations of the other isomers in the range *trans*-13 to *trans*-16 were significant. After exogenous substrate (43mg/100ml and 107mg/100ml) had been administered to the rumen liquor, the amount of fatty acid in the *trans*-13 to *trans*-16 range increased and this became comparable with the amount of *cis*-octadec-15-enoic acid produced. This may suggest that isomerization about the unsaturated C-15 position is necessary (as is that about the original C-12 position) to produce *trans*-11 isomers before any further biohydrogenation can take place.

In the recognized general biohydrogenation process, the unsaturated sites on the isomerized octadeca-9,11,15-trienoic acid are preferentially hydrogenated in the order C-9 > C-15 > C-11. However, this order of preference was changed, when the concentration of methyl linolenate was increased above 100mg/100ml of rumen liquor, to C-9 > C-11 > C-15, even though the configuration of the dienoic acid intermediate was similar in both cases. The biohydrogenation process also seemed to stop at the C-15 unsaturated stage because there was a very low yield of stearic acid under these conditions.

To some extent, these considerations have been supported by other observations by Noble *et al.* (1974). They showed, when incubating trilinolein at concentrations less than 100mg/100ml of rumen

liquor, that the main monoenoic fatty acid formed was *trans*-octadec-11-enoic acid. However, by increasing the trilinolein concentration above 100mg/100ml of rumen liquor, the monoenoic fatty acid produced was changed to *cis*-octadec-9-enoic as the main isomer. These findings suggest that the order of preference of biohydrogenation of the unsaturated sites of the released linoleic acid after isomerization also changed from C-9 > C-11 to C-11 > C-9 as the substrate concentration increased. It certainly appears that the final overall distribution pattern of the monoenoic fatty acids produced by biohydrogenation of either linoleic acid (Harfoot *et al.*, 1973) or linolenic acid is strongly influenced by the concentration of the substrate supplied to the microorganisms in the rumen.

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