The hydrogenation of γ -linolenic acid by pure cultures of two rumen bacteria

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Two species of rumen bacteria that have been previously shown to partially hydrogenate α -linolenic acid have been examined for their ability to hydrogenate γ -linolenic acid. Free γ -linolenic acid is hydrogenated *in vitro* to stearic acid by a rumen *Fusocillus* sp. (N.C.I.B. 11026), but only to *cis,trans*-octadec-6,11-enoic acid by a *Butyrivibrio* sp. The sequential hydrogenations are preceded by a Δ^{12} -*cis*– Δ^{11} -*trans* isomerization identical with that observed in the hydrogenation of α -linolenic acid and linoleic acid.

The hydrogenation of α -linolenic acid (*cis,cis,cis*-octadec-9,12,15-enoic), the major fatty acid of grass lipids, and linoleic acid (*cis,cis*-octadec-9,12-enoic) by mixed rumen bacteria and pure cultures of rumen bacteria is well documented (Dawson & Kemp, 1970; Kemp *et al.*, 1975). Both fatty acids contain the *cis*-9,*cis*-12 double-bond system and the first step in the hydrogenation is the conjugation of these bonds by the action of a Δ^{12} -*cis*– Δ^{11} -*trans* isomerase (Kepler & Tove, 1969; Kepler *et al.*, 1970), followed by the hydrogenation of the *cis*-9 bond. The product from α -linolenic acid, *trans,cis*-octadec-11,15-enoic acid, is further hydrogenated to *trans*-octadec-11-enoic acid and stearic acid by mixed rumen bacteria, but no bacterium has been isolated capable of reducing the octadecadienoic acid further than an octadecenoic acid. Rumen bacteria able to hydrogenate the octadecadienoic acid may be assigned for convenience to one of two groups. Group A, which includes *Butyrivibrio fibrisolvens* strain A-38 (Kepler *et al.*, 1970) and the *Butyrivibrio* sp. S₂ (Hazlewood & Dawson, 1979) used in the present work, can reduce the *cis*-15 bond of *trans,cis*-octadec-11,15-enoic acid to yield *trans*-octadec-11-enoic acid, normally the major octadecenoic acid found in the rumen, but cannot reduce this or any other octadecenoic acid to stearic acid. Group B bacteria reduce the *trans*-11 bond of the octadecadienoic acid to *cis*-octadec-15-enoic acid, which they are unable to reduce to stearate, although they can reduce many *cis*- and *trans*-octadecenoic acids with double bonds nearer than C-12 to the carboxylic group (Kemp *et al.*, 1977). Group B contains *Fusocillus* sp. P_{2/2} used in the present experiments and the very similar T₃₄₄ (Kemp *et al.*, 1975), both isolated in our laboratories.

γ -Linolenic acid (*cis,cis,cis*-octadec-6,9,12-enoic) is present only in small amounts in the lipids of

animals and higher plants, but is found in appreciable quantities in the lipids of some fungi, algae and protozoa (Erwin, 1973) and in the seed oils of *Onagraceae* spp. and *Boraginaceae* spp. (Pryde, 1979). It is possible that γ -linolenic acid from any one of these sources could be ingested by ruminants and if not hydrogenated in the rumen might be incorporated into tissue lipids and milk. Like α -linolenic acid and linoleic acid, γ -linolenic acid has the *cis*-9,*cis*-12 double-bond system necessary for isomerization activity (Kepler *et al.*, 1970) and is hydrogenated to stearic acid *in vitro* by mixed rumen bacteria (P. Kemp, unpublished work). In the present paper we report the hydrogenation of γ -linolenic acid to stearic acid by Group B bacterium P_{2/2} and the isolation and characterization of all the intermediates in the sequence of hydrogenations.

Methods

Bacteria

Butyrivibrio S₂ (Hazlewood & Dawson, 1979) and *Fusocillus babrahamensis* N.C.I.B. 11026 (Kemp *et al.*, 1975) were stored at 3°C on agar (20 g · l⁻¹) slopes or deep frozen (–70°C) in the medium described below which was fortified with clarified rumen fluid (20%, v/v).

Growth of experimental cultures

The medium (medium 3) described by Hazlewood & Dawson (1979), which is low in fatty acids, was used with the following modifications (amounts are per litre in the complete medium): soluble starch (1g), deoic acid (10mg), palmitic acid (10mg) and γ -linolenic acid (Sigma) substrate in experimental tubes (10mg). Fatty acids were added dissolved in a small volume of methanol prior to autoclaving at 69 kPa (10 lbf/in²) (115°C) for 20 min. The medium

was reduced with a filter-sterilized solution, which gave in the final medium (per litre) NaHCO_3 (8g), cysteine hydrochloride (1g), glucose (0.5g), cellobiose (0.5g) and galactose (0.5g).

The preparation and inoculation of the media were carried out by using the open-tube method under sterile O_2 -free CO_2 (Hungate, 1969; Latham & Sharpe, 1971). Cultures were incubated at 39°C and growth assessed by measuring culture turbidity with an EEL colorimeter (OR1 red filter, 13 mm light path).

Analysis of hydrogenation products

The extraction of the total lipids from cultures and the subsequent preparation and analysis of the fatty acid methyl ester products by argentation t.l.c. and g.l.c. were essentially as described by Kemp *et al.* (1975). Double-bond positions in octadecenoic acids were located by using periodate/permanganate oxidation (von Rudloff, 1956). Octadecadienoic acid methyl ester was subjected to partial reduction and the products were separated by AgNO_3 t.l.c. followed by oxidation of the octadecenoic acids (von Rudloff, 1956). Components on AgNO_3 thin-layer plates were located by viewing under u.v. light after spraying with a 0.2% (w/v) solution of 2,7-dichloro-fluorescein in ethanol. Peak areas from g.l.c. traces were obtained with a Hewlett-Packard 3380A integrator. Heptadecanoic acid was used as an internal standard for quantification purposes.

Results and discussion

When bacterium S_2 or $P_{2/2}$ was inoculated and grown for 20h in a medium containing γ -linolenic acid, the growth was the same as in the basal medium. From a 2% inoculum, maximum growth (absorbance values: S_2 , 1.1; $P_{2/2}$, 0.95) was found at 18–20h and half-maximum at 10–12h. Very good yields of products were obtained; recovery of products and unreacted substrate was 85–90% of the substrate used. With bacterium S_2 the only product (90% yield) was non-conjugated octadecadienoic acid. From bacterium $P_{2/2}$ cultures, almost equal amounts of stearic acid and a *trans*-octadecenoic acid were recovered and minor amounts (5%) of substrate. Hydrazine reduction (Privett & Nicholl, 1966) of the octadecadienoic acid from S_2 incubations yielded stearic acid and equal amounts of a *cis*- and a *trans*-octadecenoic acid, which gave on periodate/permanganate oxidation (von Rudloff, 1956) a dodecanoic acid and a six-carbon dicarboxylic acid from the *cis*-octadecenoic acid and an eleven-carbon dicarboxylic acid from the *trans*-octadecenoic acid. The structure of the parent acid must have been therefore *cis,trans*-octadec-6,11-enoic acid. The octadecenoic acid from the $P_{2/2}$ incubation was identified as

trans-octadec-11-enoic acid (under the isothermal g.l.c. conditions used, the detection of the expected heptonic acid fragment was difficult, since it was usually eluted in the solvent peak).

Since yields of early intermediates were poor when S_2 and $P_{2/2}$ were grown for periods shorter than 20h in media containing γ -linolenic acid, cultures grown for 20h in a medium without substrate were added to substrate suspended in a small volume (5% of the culture volume) of sterile medium and incubated for 2, 4, 8, 15, 30, 60, 120 and 240min. The products in the later tubes were exactly as those found in the experiments with bacteria grown for 20h in medium plus substrate. However, the early times (2, 4 and 8 min) gave high yields of a fatty acid which ran slowly both on g.l.c. and t.l.c. and had properties similar to those of the conjugated octadecatrienoic acid obtained from α -linolenic acid (Kemp & Dawson, 1968). At these short time intervals there was some variation in the relative proportions of the products between experiments. The results of two time courses were: octadecenoic acid, 2.3% and 15.6%; octadecadienoic acid, 10.5% and 25.6%; γ -linolenic acid, 53.1% and 25.0%; possible conjugated triene, 34.1% and 33.8%. Intermediate times gave mixtures of products that were between these extremes. The products were first characterized by their behaviour on AgNO_3 t.l.c. and g.l.c. They were, in order of appearance in the time course: a conjugated octadecatrienoic acid, a non-conjugated octadecadienoic acid that was not hydrogenated further by S_2 , and a *trans*-octadecenoic acid and stearic acid produced only by $P_{2/2}$. The structure of the *trans*-octadecenoic acid produced by $P_{2/2}$ was *trans*-octadec-11-enoic acid in the previous experiment. The S_2 octadecadienoic acid was also the same as that produced by S_2 grown in media containing γ -linolenic acid.

The conjugated octadecatrienoic acids were pooled for each organism and reduced with hydrazine (Privett & Nicholl, 1966). The reduction products were separated by AgNO_3 t.l.c., eluted and oxidized with periodate/permanganate (von Rudloff, 1956) and the fragments analysed by g.l.c. The results, which were identical for the two bacteria, are given in Table 1.

The only structure consistent with finding a *cis*-6, a *cis*-9 and a *trans*-11 octadecenoic acid in roughly equal proportions in the reduction products is *cis,cis,trans*-octadec-6,9,11-enoic acid, which arises presumably by the action of the $\Delta^{12}\text{-cis-}\Delta^{11}\text{-trans}$ isomerase (EC 5.2.1.5) described by Kepler *et al.* (1970). The dicarboxylic acid fragments recovered from the oxidation products of the two non-conjugated and the conjugated octadecadienoic acids in the reduction products are consistent with this conclusion.

Table 1. Identification of the hydrazine reduction products of the conjugated octadecatrienoic acid

The conjugated methyl octadecatrienoate (~1 mg) was shaken with hydrazine (20 mg) in ethanol (0.5 ml) at 40°C. Samples (1 µl) were analysed by g.l.c. at intervals. When the yield of methyl octadecanoates was maximal (after 90 min), the reaction was stopped with 2M-HCl (0.5 ml). The products were extracted with hexane and separated by AgNO₃ t.l.c. with benzene/diethyl ether (99:1, v/v) as the solvent. Individual components were located by spraying the plate with 2,7-dichlorofluorescein and viewing under u.v. light, eluted with diethyl ether and cleaved at the double bond by periodate/permanganate oxidation. The dicarboxylic acid monomethyl ester products were methylated and identified by g.l.c. on 10% Peg A and 3% SE 30 at 197°C.

T.l.c. band	G.l.c. retention time relative to that of methyl palmitate on Peg A at 197°C	Characterization by:	Dicarboxylic acid recovered from IO ₄ ⁻ /MnO ₄ ⁻ oxidation	Identity of band (methyl ester)
1	1.85	Stearate	—	Stearate
2	2.06	<i>trans</i> 18:1	C ₁₁	<i>trans</i> -Octadec-11-enoate
3	3.34	Conjugated 18:2	C ₉	(<i>cis,trans</i> -Octadec-9,11-enoate)*
4	2.05	<i>cis</i> 18:1	C ₉	<i>cis</i> -Octadec-9-enoate
			C ₆ †	<i>cis</i> -Octadec-6-enoate
5	2.27 + 2.34 (doublet)	Non-conjugated 18:2‡	C ₆	(<i>cis,cis</i> -Octadec-6,9-enoate)*
6	3.8	Unchanged conjugated triene	—	(<i>cis,trans</i> -Octadec-6,11-enoate)*

* There was insufficient material for these components to be resubmitted to hydrazine reduction.

† The methyl ester of a C₁₂ monocarboxylic acid fragment was also detected, but the isothermal g.l.c. conditions used did not detect the methyl esters of the 7- and 9-monocarboxylic acids expected from the cleavage of a Δ⁹ and a Δ¹¹ bond. These fragments were eluted in the solvent peak.

‡ Two overlapping bands on t.l.c.

From these results it is clear that the hydrogenation of γ-linolenic acid, like that of α-linolenic acid and linoleic acid, must proceed via a conjugated intermediate produced by the Δ¹²-*cis*–Δ¹¹-*trans* isomerase. Although both S₂ and P_{2/2} can further reduce the *trans,cis*-octadec-11,15-enoic acid, the first reduction product from α-linolenic acid, albeit to different products (*trans*-octadec-11-enoic acid and *cis*-octadec-15-enoic acid respectively), S₂ is unable to reduce the *cis,trans*-octadec-6,11-enoic acid, the first reduction product from γ-linolenic acid. P_{2/2} can hydrogenate the *cis*-6 bond and then hydrogenate the product, *trans*-octadec-11-enoic acid, to stearic acid. *cis,trans*-Octadec-6,11-enoic acid isolated from incubations of S₂ with γ-linolenic acid was reincubated with 16h cultures of S₂ and P_{2/2}. No products were recovered from the S₂ incubation, but both stearic acid and *trans*-octadec-11-enoic acid were recovered from the P_{2/2} incubation.

Since the sequence of metabolic processes from γ-linolenic acid to stearic acid (Scheme 1) is similar to that which effects the conversion of α-linolenic acid to stearic acid, albeit requiring two bacteria (P. Kemp & D. J. Lander, unpublished work), it seems that this is the pathway by which γ-linolenic acid is hydrogenated in the rumen.

Garcia *et al.* (1976) examined the complete series of *cis,cis* methylene-interrupted octadecadienoic acids and found that only the 9,12- and 2,5-isomers were conjugated by the action of the isomerase (Kepler *et al.*, 1970). The product resulting from the

Scheme 1. C₁₈-fatty-acid intermediates in the sequence of reactions when γ-linolenic acid is hydrogenated by bacteria P_{2/2} or S₂

→, Reactions by P_{2/2} or S₂; ---→, reaction by P_{2/2} only.

isomerization of the Δ^{2,5} acid was slowly hydrogenated to a monoene (Garcia *et al.*, 1976). The reason why the Δ_{9,12} double-bond system in polyunsaturated fatty acids requires to be conjugated

prior to hydrogenation is not clear, since evidence with isolated rumen bacteria (P. Kemp, unpublished work) indicates that hydrogenation of some methylene-interrupted diene systems in C₁₈ fatty acids can proceed without prior conjugation.

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