The hydrogenation of some *cis*- and *trans*-octadecenoic acids to stearic acid by a rumen *Fusocillus* sp.

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1. The hydrogenation of a range of double-bond positional and configurational octadecenoic acid isomers (*cis* (Δ2 and Δ4 to Δ13) and *trans* (Δ2 and Δ5 to Δ13)) to stearic acid by a rumen *Fusocillus* sp. were examined.

2. The *cis* and *trans* Δ5 to Δ13 isomers were all hydrogenated to some extent by late-log-phase cultures added to suspensions of individual isomers and incubated for a further 3 h. Of the *cis*-isomers, Δ5 to Δ11 (79–73% conversion to stearic acid) were the preferred substrates. Δ12-*cis*- (30%) and Δ13-*cis*-isomers (5%) were poorly hydrogenated. Of the *trans*-isomers, Δ8, Δ9 and Δ10 were 45% converted to stearic acid, the other isomers were poorly hydrogenated. These results are in agreement with less extensive studies using sheep rumen micro-organisms.

3. When cultures were grown from small inocula in media containing individual isomers more extensive hydrogenation was found than with late-log-phase cultures. At 24 h, *cis* Δ2, Δ4 and Δ5 gave the highest conversions to stearic acid (90%) followed by the *cis* Δ6 to Δ12 and *trans* Δ8 to Δ10 isomers (approximately 75%), although at 6 and 12 h Δ9-*trans* gave higher yields of stearic acid than Δ9-*cis*, probably because the growth of the *cis* cultures showed a longer log-phase.

The hydrogenation of α-linolenic acid and linoleic acid by mixed populations of rumen micro-organisms and by pure cultures of rumen bacteria is well documented (Dawson & Kemp, 1970; Kemp *et al.* 1975). Normally the penultimate end-product, *trans*-11-octadecenoic acid, comprises at least 80% of the octadecenoic acids in the rumen, although under some conditions other isomers may be prominent (Leat *et al.* 1977; Body, 1976) and can be incorporated into milk and tissue lipids (Dawson & Kemp, 1970). The occurrence in the hydrogenation products of octadecenoic acid isomers, especially *cis*-9-octadecenoic acid (oleic acid) which is hydrogenated more rapidly to stearic acid than is *trans*-11-octadecenoic acid (Dawson & Kemp, 1970), gave rise to speculation concerning the preferred substrate for hydrogenation to stearic acid. There could be a common precursor of stearic acid, which might be oleic acid, or to which oleic is more readily isomerized than other isomers. Morris (1970) and his colleagues obtained evidence that oleic and elaidic acids were hydrogenated directly to stearic acid without the need for a common intermediate, and that the small amounts of the opposite isomer recovered from each incubation were due to fortuitous isomerization.

Without labelled octadecenoic acid substrates the search for a common intermediate or preferred substrate using mixed rumen organisms and even pure cultures grown in the usual media containing rumen fluid is not possible because of the high levels of endogenous octadecenoic acids relative to substrate levels. However, using a rumen *Fusocillus* sp. (Kemp *et al.* 1975) which hydrogenates several octadecenoic acid isomers to stearic acid and which grows in a fatty-acid-free defined medium, allowed a re-examination of the problem. It was thus possible to grow this bacterium with a range of *cis*- and *trans*-octadecenoic acids prepared synthetically by the methods of Gunstone & Ismail (1967*a*, *b*). Some preliminary results of this work have been reported (Kemp *et al.* 1977).

MATERIALS AND METHODS

Reagents were of analytical grade where available.

Cis-octadecenoic acids with double bonds in positions 2, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 and *trans*-octadecenoic acids with double bonds in positions 2, 5, 6, 7, 8, 9, 10, 11, 12 and 13 were synthesized by the procedures of Gunstone & Ismail (1967*a*, *b*) and stored at −70°. [1-^{14}C]oleic acid was purchased from Amersham International (Amersham, Bucks) and [1-^{14}C]elaidic acid was prepared from it by selenium isomerization (Gunstone & Ismail, 1967*b*) and purified by argentation thin-layer chromatography.

Culture of bacterium. The *Fusocillus* sp., T344 (NCIB 11026; Kemp *et al.* 1975) was maintained on agar slopes (5 g/l) stored at 4° or in liquid medium stored at −70° and subcultured monthly. Before hydrogenation studies the bacterium was transferred and subcultured three times at daily intervals in the defined medium described by Roché *et al.* (1973) with the following modifications (g/l): starch 1·0, cellobiose 0·5, glucose 0·5, cysteine hydrochloride 1·0; resazurin was substituted for indigocarmine. The unreduced medium was autoclaved (6·9 kPa (10 psi) at 115°) for 20 min and the filter-sterilized reducing solution then added. The preparation and inoculation of the medium was by the open-tube method under sterile oxygen-free carbon dioxide (Hungate, 1969; Latham & Sharpe, 1971). Incubations were at 39° and growth was assessed from the optical density of the cultures, using an EEL colorimeter (Evans Electroselenium Ltd, Harlow, Essex) and OR 1 filter.

Rumen fluid. This was taken from a fistulated Clun Forest wether given 800 g good hay and 100 g crushed oats at 08.00 hours daily.

Substrate preparation. Free fatty acids (200 μg) in 0·05 ml methanol and 0·5 ml unreduced medium were placed in incubation tubes and plugged with cotton wool. After autoclaving (6·9 kPa (10 psi) at 115° for 20 min), 9 ml reduced medium was added and the tube closed with a butyl-rubber bung. Just before inoculation, the tubes were placed for 2 min in an ultrasonic bath at 50° (Fs 100, Decon Ultrasonics Ltd. Hove).

Analysis of products. The extraction of the cultures (medium and cells) and the analysis of the methyl esters of the fatty acids by gas–liquid chromatography (using heptadecanoic acid as internal standard) and thin-layer chromatography and the locating of double-bond positions by periodate permanganate oxidation (von Rudloff, 1956) were as described by Kemp *et al.* (1975).

RESULTS

Hydrogenation by late-log-phase cultures

Fusocillus sp. T344 cultures (10 ml) grown to near maximal optical density (16–18 h) were added to 200 μg substrate in 0·5 ml sterile O_2-free, but unreduced medium, and incubated for 3 h. Four culture tubes were pooled for each analysis and three or four tubes in repetitions (two). Agreement between experiments was good, both between the isomers in each series and between the *cis*- and *trans*-series. Recoveries of stearic acid and residual substrate were 85–90% of the substrate used.

Fig. 1 shows that the yields of stearic acid were similar from all the *cis* Δ5 to Δ11 isomers; in this and repetitions of this experiment the formation of stearic acid from the Δ5-*cis*-isomer was always slightly higher than any other isomer. In the experiments with *cis*-isomers (Fig. 1) the line joining the experimental points had a saw-tooth form in the Δ5 to Δ11 region and the curve joining the *cis* Δ5, Δ7, Δ9 and Δ11 points was slightly higher than that joining the *cis* Δ6 to Δ10 points. These results suggest an inverse relationship with melting point (Gunstone & Ismail, 1967*b*). A randomized block analysis of variance for *cis* Δ5 to Δ11 using data from three experiments showed that all differences between means were significant at the 5% level except for positions 6 and 7.

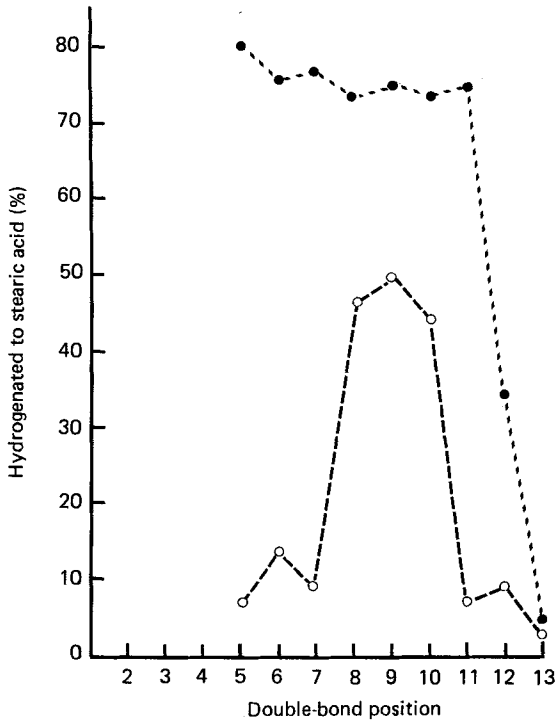

Fig. 1. Hydrogenation of *cis*- (●) and *trans*- (○) octadecenoic acids to stearic acid after 3 h incubation of each isomer with late-log-phase cultures of *Fusocillus* sp. T344.

The differences were not noticeable in one experiment extended to 6 h incubation but which showed similar conversions to stearic acid.

Of the *trans* series only the Δ8, Δ9 and Δ10 isomers were extensively hydrogenated, and then to a less extent than their *cis* counterparts (Fig. 1). Yields of stearic acid from the Δ9-*trans*-isomer were only slightly better than from the Δ8-*trans*- or Δ10-*trans*-isomers, each of which gave better yields than the Δ9-*trans*-isomer on one occasion (of five experiments).

Hydrogenations in growing cultures

Tubes (10 ml) of media containing 200 μg octadecenoic acid isomer were inoculated with 0·25 ml of a fresh culture of T344, closed with a butyl-rubber bung, mixed by inverting once and incubated for 0, 6, 12, 18, 24 and 48 h. Four to six tubes were pooled for each analysis. There was minimal variation (±10%) in growth at any time, as assessed by optical density, between cultures containing individual *trans*-isomers. In the tubes containing *cis*-acids, optical densities were 25% lower than in *trans* tubes at 6 h and marginally (approximately 5–10%) less at 9 h, but no difference was found at later times. Omitting starch, which is not fermented by T344, resulted in erratic growth and hydrogenation. Increasing the substrate concentration to 40 μg/ml in the presence of starch (1 mg/ml) resulted in total inhibition of growth with both *cis*- and *trans*-acids although late-log-phase cultures grown in the absence of starch were able to tolerate higher concentrations of substrate (50–60 μg/ml) before hydrogenation was inhibited. Presumably the higher number of cells provided more surfaces for the sequestration of the octadecenoic acid which reduced surface concentration and inhibitory effects.

Good growth, but variable hydrogenation, was obtained with oleic acid at up to

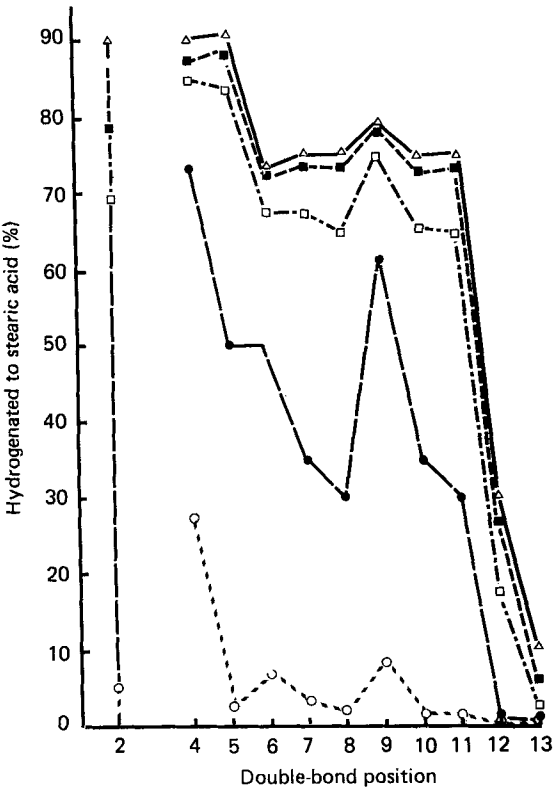

Fig. 2. Hydrogenation of *cis*-octadecenoic acids to stearic acid by growing cultures of *Fusocillus* sp. T344, 6 h (○), 12 h (●), 18 h (□), 24 h (■) and 48 h (△) after inoculation.

100 μg/ml in the presence of larger amounts of starch (10 mg/ml) or powdered dried grass (10 mg/ml). With powdered grass the hydrogenation was complicated by products of hydrogenation of the additional linoleic and linolenic acid.

The results of hydrogenation by growing cultures are shown in Fig. 2 (*cis*-isomers) and Fig. 3 (*trans*-isomers). At 18, 24 and 48 h there were similarities between the hydrogenation values in Figs. 2 and 3 with those in Fig. 1 at positions $\Delta 7$ to $\Delta 11$, except that in Figs. 2 and 3 there was no difference in the extent of hydrogenation between *cis*- and *trans*-isomers. When residual octadecenoic acids were examined by argentation thin-layer chromatography and by oxidation the major bond position was always that of the substrate, but generally there was a small amount of the adjacent positional isomers and of the isomer of the substrate with the opposite configuration, suggesting isomerization. *Cis* → *trans* isomerization was more extensive than *trans* → *cis*. Preparation of the media and incubation of non-inoculated media for 24 h did cause some *cis* → *trans* but no positional isomerization.

Hydrogenation by rumen micro-organisms

For comparative purposes, 0·5 mg and 1 μCi [1-^{14}C]oleic acid or elaidic acid were incubated with 10-ml portions of rumen fluid strained through four layers of muslin. Incubations were at 39°, and the conversions of substrate to stearic acid at 15, 30 and 60 min were 35, 50 and 62% with oleic acid and 3, 19 and 33% respectively with elaidic acid as substrate. Small amounts of *trans*-acid were detected in the residual *cis*-substrate and *cis*-acid in the *trans*-substrate but no further identification was made.

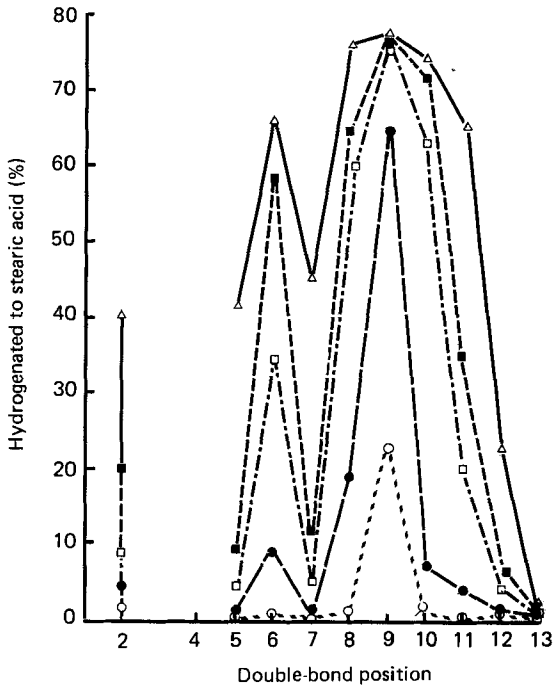

Fig. 3. Hydrogenation of *trans*-octadecenoic acids to stearic acid by growing cultures of *Fusocillus* sp. T344, 6 h (○), 12 h (●), 18 h (□), 24 h (■) and 48 h (△) after inoculation.

DISCUSSION

Our results give no support for a common intermediate in the hydrogenation of octadecenoic acids. There is some isomerization, but this appears to be restricted to producing isomers which are the immediate neighbours of the substrate. *Cis/trans* isomerization may not all arise from enzymic activity. Although oleic acid is a good substrate for the hydrogenation in growing cultures the *cis* Δ2, Δ4 and Δ5 isomers gave higher yields of stearate at 24 h. At earlier times there were different specificities, Δ4-*cis* and Δ9-*trans* both being preferred to Δ9-*cis*. Some lag in the hydrogenation of *cis*-acids might be expected in growing cultures because of the longer log phase of growth, which may be caused by the *cis*-acids being more inhibitory to growth (Nieman, 1954). This effect would, of course, decrease as growth and hydrogenation progressed.

Results with the Δ5 to Δ13 isomers and late-log-phase cultures (Fig. 1) show a broad similarity to the values in Figs. 2 and 3. The results with rumen fluid using the Δ9-*cis* and Δ9-*trans*-isomers agree with the values in Fig. 1 and with published observations (Dawson & Kemp, 1970; Morris, 1970).

The lessening of the inhibitory effects of substrates on growth brought about by using starch, which does not support growth, or powdered grass, is presumably due to the fatty acids being adsorbed and thus their toxic action minimized. It reflects the situation in the rumen, where the enormous surface area provided by the food particles permits levels of free fatty acids 50–100 times greater than those used in our experiments. Hydrogenation by late-log-phase cultures was not so easily inhibited, possibly because the larger number of bacteria reduces the effective surface concentration. It is possible that the slightly higher rates of hydrogenation of some odd-double-bond-position *cis*-isomers relative to the

even-number-bond positions adjacent to them is due to better emulsification of the substrate, which would give initial higher surface concentration of substrate.

Our results were obtained using a single bacterial species, but we have isolated two others (P2/2 *Fusocillus babrahamensis*, NCIB 10838 (Kemp *et al.* 1975) and R8/5, an unidentified gram -ve rod (Hazlewood *et al.* 1976)) with very similar hydrogenation characteristics which suggest that a similar specificity for octadecenoic acid isomers would operate in the rumen. The ability to remove a wide range of potentially toxic fatty acids would have survival value, not only for the rumen ecosystem in general but also for the host. We have no result which would relate rates of hydrogenation with the toxicity of individual isomers, but linolenic and linoleic acids, which are more highly bacteriostatic than oleic acid, are hydrogenated faster than oleic acid by rumen micro-organisms (Dawson & Kemp, 1970). The poor hydrogenation of the Δ13 isomer suggests that acids with longer paraffin chains like erucic acid (*cis*-13-docosaenoic) would escape hydrogenation and be incorporated into ruminant tissues and milk.

It was unfortunate that we were unable to examine the whole range of isomers, especially those at the carboxyl end of the paraffin chain, i.e. *cis* Δ3 and *trans* Δ3 and Δ4. At the methyl end we know (Kemp *et al.* 1975) that the Δ15 isomer is not hydrogenated either by pure cultures or by mixed rumen micro-organisms, and mixtures of *cis*- and *trans*-isomers with double bonds in the C14–16 position have been similarly resistant (P. Kemp, unpublished results).

The reasons for *trans*-11-octadecenoic acid being the major octadecenoic acid found in the rumen appear to be that it is the penultimate end-product in the hydrogenation of both linoleic and α-linolenic acids and that it is slowly hydrogenated compared with oleic acid, which would be introduced in the diet. The higher levels of 11-*trans* relative to other *trans*-isomers in ruminant tissues reflect the relative amounts in the rumen. At least one rumen fatty acid auxotroph (Hazlewood *et al.* 1979) prefers *trans*-11-octadecenoic acid, which it produces by the hydrogenation of linolenic and linoleic acids, to other *trans*-isomers, but probably only utilizes this acid if the levels of palmitic acid become growth limiting.

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