Letters in Applied Microbiology 1999, **29**, 193–196

Hydrogenation of polyunsaturated fatty acids by human colonic bacteria

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2245/99: received 24 June 1999 and accepted 16 July 1999

F.A.C. HOWARD AND C. HENDERSON. 1999. Emulsions of the fatty acids linoleic (C18 : 2 *n-6*), α-linolenic (C18 : 3 *n-3*) and arachidonic acid (C20 : 4 *n-6*) were incubated for 4 h under anaerobic conditions with human faecal suspensions. Linoleic acid was significantly decreased ($P < 0{\cdot}001$) and there was a significant rise ($P < 0{\cdot}05$) in its hydrogenation product, stearic acid. Linolenic acid was also significantly decreased ($P < 0{\cdot}01$), and significant increases in C18 : 3 cis-trans isomers ($P < 0{\cdot}01$) and linoleic acid ($P < 0{\cdot}05$) were seen. With each acid, there were non-significant increases in acids considered to be intermediates in biohydrogenation. The study provides evidence that bacteria from the human colon can hydrogenate C18 essential polyunsaturated fatty acids. However, with arachidonic acid there was no evidence of hydrogenation.

INTRODUCTION

The *n-3* and *n-6* polyunsaturated fatty acids (PUFA) are essential for effective cell membrane function. In humans, these acids must be acquired from external sources. Also, patients with acute inflammatory conditions are often advised to increase their intakes of *n-3* fatty acids. It is well established that the absorption of dietary fatty acids largely takes place in the small intestine following hydrolysis of dietary triacylglycerols by pancreatic lipase, which produces free fatty acids and 2-acylmonoglycerols by removing fatty acids from positions 1 and 2 in the triacylglycerol. The products of lipolysis are incorporated into mixed micelles from which they are transferred into epithelial cells of the intestinal mucosa. The efficiency of absorption is generally high, but lack of lipase and absence of bile acids can reduce absorption. The efficiency with which polyunsaturated acids are absorbed from the intestine is of considerable nutritional importance. When balance studies are used to estimate the uptake of long chain fatty acids in the human intestine, the results indicate that unsaturated fatty acids are much more effectively absorbed than are saturated fatty acids (Hashim and Babayan 1978; Nelson *et al*. 1996). In earlier work, Underwood *et al*. (1967) cautioned that such balance studies, in which daily dietary intakes of fat are compared with daily faecal excretion of fat, would be confounded by fatty acids in endogenous secretions into the gut and, possibly, by hydrogenation of unsaturated fatty acids by colonic bacteria. Certainly, any unabsorbed unsaturated fatty acids reaching the colon will be exposed to a population of anaerobic bacteria which produce hydrogen and create a reducing environment. It is well known that PUFA are subject to reduction to more saturated homologues by anaerobic bacteria in the rumen (Polan *et al*. 1964), and similar effects have been observed with bacteria isolated from rat intestine (Eyssen and Parmentier 1974). The aim of this study was to investigate whether essential PUFA are biohydrogenated by human colonic bacteria.

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MATERIALS AND METHODS

Incubation procedures

The experiments were conducted using conical flasks (100 ml). These had closures incorporating a vacuum tap so that the gas phase could be repeatedly evacuated and replaced with oxygen-free CO_2 to ensure anaerobic conditions.

The anaerobic phosphate buffer used as a diluent contained (g l^{-1}): K_2HPO_4, 0·45; KH_2PO_4, 0·45; $(NH_4)_2SO_4$, 0·90; NaCl, 0·90; $MgSO_4$, 0·09; $CaCl_2.2H_2O$, 0·09. After heating this solution to expel dissolved oxygen, $NaHCO_3$ 8·0 g l^{-1}

and L-cysteine 1·0 g l^{-1} were added, and oxygen excluded, using oxygen-free CO_2 as the gas phase.

Dried sugar-beet fibre, finely ground and sieved (mesh size 1·5 mm), served as an energy source for the colonic bacteria. The fibre was rehydrated in anaerobic buffer overnight by placing 0·4 g in each incubation flask along with 10 ml anaerobic buffer.

The colonic bacteria used were from a healthy adult female who was on an omnivorous diet and had not ingested antibiotics for at least 6 months. Freshly voided faecal samples were collected in sterile 'Stomacher' bags (Seward Medical, London, UK). Within 15 min of collection, 30 g of the faeces were mixed with 180 ml anaerobic phosphate buffer and the mixture blended under an atmosphere of oxygen-free CO_2.

Emulsions (4·9 g l^{-1}) of the PUFA (linoleic (C18 : 2 *n-6*), α-linolenic (C18 : 3 *n-3*) or arachidonic acid (C20 : 4 *n-6*)) were prepared by homogenizing the acid in phosphate buffer.

The faecal homogenates and fatty acid emulsions were prepared immediately before use and 10 ml of each were added to the flasks containing rehydrated sugar-beet fibre. Anaerobic conditions were established by replacing the gas phase with oxygen-free CO_2. The flasks were then incubated at 37 °C in a shaking water-bath (45 rev min^{-1}). Control incubations without added fatty acid emulsions, and unincubated (zero time) controls for each fatty acid, were also prepared. Microbial activity was arrested by adding 1 ml concentrated hydrochloric acid to each flask at zero time for the unincubated controls, or at 4 h in the incubated flasks.

Lipid extraction and analysis

The lipids in each flask were extracted by the method of Folch *et al.* (1957). Prior to extraction, 50 mg heptadecanoic acid (1% w/v solution in methanol) were added to each flask as internal standard. Aliquots (2 ml) of the chloroform phases to which 0·5 ml propyl gallate (2% w/v) solution had been added as antioxidant were evaporated to dryness under nitrogen, and fatty acid methyl esters (FAME) were prepared (Christie 1989).

The FAME were separated by gas liquid chromatography using a Phillips 4300 Gas Liquid Chromatograph (Phillips Scientific, Cambridge, UK). The column was open tubular fused silica (25 m × 0·32 mm i.d.), wall-coated with CP-TM-SIL88. The carrier gas was helium with split injection, and detection was by flame ionization (detector temperature 200 °C). The initial column temperature was 150 °C for 2 min, increasing at 5 °C per min for 2 min to 160 °C, holding for 5 min, then increasing at 5 °C per min for 4 min to 180 °C and holding for 3 min.

The data were collected using the JCL 6000 Chromatography Data System (Jones Chromatography Ltd, Hengoed, UK). The FAME were identified by comparison of retention times with standard FAME mixtures (Sigma), and FAME from carbon chain length 16 upwards were quantified by comparison of their peak areas with those of the internal marker, heptadecanoic acid.

The individual FAME contents of each assay sample were expressed as gram FAME per 100 grams total FAME. The average and standard deviation values of replicate ($n = 4$) samples from the different treatments were determined and these were compared using the Student's *t*-test (two-tailed test).

RESULTS AND DISCUSSION

Table 1 shows the effect of incubating faecal preparations without addition of exogenous fatty acids. Almost 60% of the long chain fatty acids were the saturated acids, palmitic and stearic. The major unsaturated acid was linoleic acid with lesser amounts of oleic acid. No significant changes in the FAME of the faecal samples were observed following incubation, indicating, possibly, that the unsaturated acids present were incorporated into membrane structures in faecal bacteria where they were shielded from hydrogenation processes. The protection of the double bonds of linoleic acid by its incorporation into the membrane lipids of rumen bacteria and protozoa was observed by Demeyer *et al.* (1978).

When linoleic acid was included in the incubation mixtures

Table 1 Fatty acids (FAME) in control (unincubated) and incubated (4 h at 37 °C) faecal homogenates with no added fatty acid emulsion

Fatty acid (methyl esters)	No added fatty acid Control* (g 100 g^{-1})	Incubated* (g 100 g^{-1})
C16:0	29·15 ± 3·77	26·66 ± 2·88
C18:0	28·08 ± 10·87	21·46 ± 9·36
Unidentified	tr	tr
C18:1trans	tr	2·52 ± 5·04
C18:1cis	13·95 ± 4·95	11·96 ± 9·64
C18:2trans,trans	tr	tr
C18:2cis,trans	tr	3·50 ± 6·99
C18:2cis,cis	28·82 ± 17·19	25·78 ± 19·25
C18:3all trans	tr	tr
C18:3cis/trans†	tr	tr
C18:3all cis	tr	tr
C20:3cis/trans†	tr	tr
C20:4all cis	tr	tr
C22:0	tr	tr

* Mean ($n = 4$) ± S.D.
tr, Below minimum level for quantification.
† Includes cis,cis,trans and cis,trans,trans.

(Table 2), there was a highly significant decrease ($P < 0{\cdot}001$) in that acid and a significant rise ($P < 0{\cdot}05$) in its hydrogenation product, stearic acid. Increases were also noted in C18 : 2 cis,trans, C18 : 2 trans,trans, C18 : 1 cis and C18 : 1 trans isomers, which are intermediates in bacterial hydrogenation processes in the rumen (Harfoot 1981). However, because of the high variability in the values for these acids, the data did not reach significance.

In the incubations which included added linolenic acid (Table 3), there was a highly significant decrease ($P < 0{\cdot}01$) in linolenic acid, and a significant rise in C18 : 3 cis,trans isomers ($P < 0{\cdot}01$) and in linoleic acid ($P < 0{\cdot}05$). Increases were also noted in C18 : 3 all trans, C18 : 2 cis,trans, C18 : 1 cis and stearic acids, all indicative of hydrogenation processes, but again, due to high variability, these results did not reach significance. In this case, there was a highly significant increase in an acid tentatively identified from its retention time as a cis-trans isomer of eicosatrienoic acid. This may be a product of chain elongation from linolenic acid, but further study is required to confirm its identity.

When arachidonic acid was included in the incubation mixtures, the decrease in that acid was not significant and there were no indications of hydrogenation products formed (data not shown).

The biohydrogenation of unsaturated fatty acids by the anaerobic bacteria of the rumen in cattle and sheep has been extensively studied (Polan *et al.* 1964), and similar activities in the rat large intestine were reported by Eyssen and Parmentier (1974). In a study of rumen bacteria by Fujimoto *et al.* (1993), *Butyrivibrio fibrisolvens* was identified as having the greatest hydrogenating capacity. As this species has been isolated from human faecal material (Montgomery 1988; Rumney *et al.* 1995), the biohydrogenation of PUFA may be possible in the human intestine. Pearson (1973) reported that a wide range of anaerobic and facultatively anaerobic bacteria isolated from the human colon could convert oleic acid to 10-hydroxystearic acid, but did not report any evidence for hydrogenation of oleic or linoleic acid.

The mechanisms proposed for the biohydrogenation of linolenic and linoleic acid in the rumen include isomerization of cis to trans bonds and subsequent reduction of the isomers to more saturated homologues. Depending on the sequence of these reactions, a variety of intermediates and end-products is possible (Harfoot 1981; Fujimoto *et al.* 1993). While the present study shows consistent evidence of reduction of linoleic and linolenic acid, the hydrogenation products showed considerable variability. This may reflect variations in the sequence of the reactions involved, due to variability in the active bacteria in the faecal microflora.

These results indicate that essential polyunsaturated fatty

Table 3 Fatty acids (FAME) in control (unincubated) and incubated (4 h at 37 °C) faecal homogenates in the presence of emulsions of linolenic acid

Fatty acid (methyl esters)	Linolenic acid added Control† ($g\,100\,g^{-1}$)	Incubated† ($g\,100\,g^{-1}$)
C16:0	8·64 ± 0·83	9·27 ± 3·02
C18:0	9·62 ± 2·26	11·91 ± 5·69
Unidentified	tr	tr
C18:1trans	tr	0·59 ± 1·17
C18:1cis	4·46 ± 3·53	6·30 ± 5·82
C18:2trans,trans	0·76 ± 1·51	0·56 ± 1·11
C18:2cis,trans	0·91 ± 1·81	5·05 ± 6·69
C18:2cis,cis	4·82 ± 3·98	13·41 ± 4·93*
C18:3all trans	tr	2·41 ± 2·78
C18:3cis/trans‡	tr	6·50 ± 1·25**
C18:3all cis	70·80 ± 8·67	22·94 ± 14·62**
C20:3cis/trans‡	tr	16·47 ± 4·55**
C20:4all cis	tr	3·98 ± 2·18*
C22:0	tr	0·62 ± 0·71

† Mean ($n = 4$) ± S.D.
tr, Below minimum level for quantification.
* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.
‡ Includes cis,cis,trans and cis,trans,trans.

Table 2 Fatty acids (FAME) in control (unincubated) and incubated (4 h at 37 °C) faecal homogenates in the presence of emulsions of linoleic acid

Fatty acid (methyl esters)	Linoleic acid added Control† ($g\,100\,g^{-1}$)	Incubated† ($g\,100\,g^{-1}$)
C16:0	9·63 ± 1·61	10·69 ± 2·45
C18:0	10·09 ± 0·62	15·03 ± 2·89*
Unidentified	tr	1·14 ± 2·28
C18:1trans	tr	4·22 ± 5·61
C18:1cis	5·03 ± 3·72	11·36 ± 8·09
C18:2trans,trans	tr	4·11 ± 3·26
C18:2cis,trans	2·70 ± 5·40	10·59 ± 1·59
C18:2cis,cis	72·56 ± 3·99	36·35 ± 3·06***
C18:3all trans	tr	4·69 ± 4·11
C18:3cis/trans‡	tr	1·01 ± 2·02
C18:3all cis	tr	0·44 ± 0·89
C20:3cis/trans‡	tr	tr
C20:4all cis	tr	tr
C22:0	tr	0·37 ± 0·73

† Mean ($n = 4$) ± S.D.
tr, Below minimum level for quantification.
* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.
‡ Includes cis,cis,trans and cis,trans,trans.

acids can be altered by hydrogenation by the colonic microflora. The extent to which linoleic and linoleic acids were hydrogenated in the present study suggests that this effect would seriously undermine conclusions drawn from *in vivo* balance studies. If hydrogenation is ignored in such studies, the calculated uptake of unsaturated acids will be exaggerated and the calculated uptake of saturated fatty acids correspondingly underestimated. The 4 h incubation period used in these *in vitro* studies was short compared to the *in vivo* colonic transit time, which may vary from 12 to over 60 h. It might thus be expected that *in vivo* hydrogenation of unabsorbed unsaturated fatty acids in the colon would be more extensive than reported here.

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