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The Hydrogenation of Unsaturated Fatty Acids in the Ovine Digestive Tract

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The natural pasture diet of the sheep contains about 5% of its dry weight as complex lipids. After ingestion these complex lipids are hydrolysed by the rumen micro-organisms, liberating free fatty acids (Garton, 1961). These fatty acids are highly unsaturated and consist predominantly of linolenic acid (60%) with smaller amounts of linoleic acid and oleic acid, while palmitic acid is the major saturated acid (Shorland, Weenink & Johns, 1955; Garton, 1960).

Reiser (1951) showed that when linseed oil was incubated with sheep-rumen contents there was a conversion of linolenic acid into linoleic acid, and he attributed this to hydrogenation by rumen micro-organisms. Analysis of rumen contents indicated that the linolenic acid of pasture grasses was rapidly hydrogenated, forming *trans* unsaturated acids and stearic acid (Shorland *et al.* 1955). This process was believed to be responsible for the peculiar composition of ruminant depot fat with its high content of saturated and *trans* unsaturated fatty acids compared with that of non-ruminants (Ogilvie, McClymont & Shorland, 1961).

Shorland, Weenink, Johns & McDonald (1957) studied the effect of incubating large amounts of oleic acid, linoleic acid and linolenic acid with sheep-rumen contents for 48 hr. at 37°, and showed a limited conversion into stearic acid as well as *trans* and positional isomers of the unsaturated acids. The availability of ^{14}C-labelled unsaturated C_{18} fatty acids has allowed us to investigate the hydrogenation processes under more physiological conditions, by using shorter (20–180 min.) incubations and fatty acid loadings more equivalent to the dietary intake. In addition, the observations have been extended to other regions of the digestive tract, and evidence has been obtained that hydrogenation also occurs in the caecum and colon.

EXPERIMENTAL

Samples. Rumen contents for experiments *in vitro* were withdrawn from Clun Forest sheep with rumen fistulae (Dawson, Ward & Scott, 1964) fed on a hay–oats diet (5:1, w/w; 1200 g./day). The micro-organisms from the rumen contents were obtained by high-speed centrifugation (15000*g* for 1 hr.) of the strained rumen liquor. Samples for comparison of fatty acid composition were collected from the rumen, duodenum, ileum, caecum and colon immediately after slaughtering sheep fed *ad lib.* on a mixed pasture diet. Each section of the tract was tied off and removed from the animal, and the contents were expressed. Faeces were collected into a calico faecal bag tied on to the animal.

Incubation of samples. Rumen contents were strained through muslin and incubated in an artificial rumen as described by Dawson *et al.* (1964). In certain large-scale preparative experiments they were incubated in a flask at 39° by using the same anaerobic gassing as with the artificial rumen. Ileum, caecum and colon contents were also incubated in this way after diluting to a more fluid consistency with 0·05–0·1 vol. of 0·9% sodium chloride.

Extraction and titration of long-chain fatty acids. Lipids were extracted from the samples and the total fatty acid fraction (free and combined) was prepared by methods described by Dawson *et al.* (1964). The large amounts of very short-chain fatty acids that are present particularly in the rumen, e.g. acetic acid and propionic acid, were eliminated during the process. The ethereal solution of fatty acids was diluted threefold with acetone and titrated with 0·02 N-NaOH in methanol from a Conway burette by using Cresol Red as an indicator and stirring with a stream of CO_2-free air.

Gas–liquid chromatography. The methyl esters of the long-chain fatty acids were prepared and examined by gas–liquid chromatography according to methods reported by Scott, Ward & Dawson (1964) with both a Pye Argon chromatograph, with analytical and preparative columns, and a Pye Panchromatograph. Polyethylene glycol adipate on Gas Chrom 'Z' (100–120 mesh; Applied Science Laboratories, State College, Pa., U.S.A.)–Celite (10% w/w) columns were prepared after initially purifying the polymer by precipitating it from a chloroform solution with methanol. This resulted in a column of high resolving power for the C_{18} acids. Short-chain monocarboxylic fatty acids resulting from the oxidation of long-chain unsaturated fatty acid esters (see below) were chromatographed as the free acids on 10% polyethylene glycol adipate on Celite at 100°. After hexanoic acid had emerged the temperature was increased to 150° to collect acids up to and including pelargonic acids. Longer-chains monocarboxylic acids (C_{10}–C_{17}) were separated from short-chain dicarboxylic acids by trituration of the anhydrous acids with light petroleum (b.p. 40–60°) (Rudloff, 1956), which took the monocarboxylic acids into solution. Dicarboxylic acids from the same oxidation were converted into the methyl

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esters with diazomethane. The short-chain dicarboxylic acids (C_2–C_6) were chromatographed at 125° on 10% polyethylene glycol adipate on Celite, and the longer-chain acids (C_9–C_{18}) on the same column at 180°.

Collection of effluent fatty acids and their esters. The methyl esters of long-chain monocarboxylic fatty acids (C_{10}–C_{18}) and the dimethyl esters of dicarboxylic acids (C_9–C_{14}) were collected from the preparative-column effluent by electrostatic precipitation (Scott *et al.* 1964). The short-chain dicarboxylic acids (C_2–C_6) were collected by bubbling through 12 cm. of toluene scintillation-counting fluid (Scott *et al.* 1964). This was shown to give a satisfactory recovery of dimethyl malonate (80%). Short-chain fatty acids (C_1–C_9) were collected by bubbling through 12 cm. of a mixture of 1 M-hyamine in methanol (Nuclear Enterprises Ltd.) and toluene scintillation-counting fluid (1:9, v/v). The recovery of acetic acid was found to be 95%.

Infrared spectroscopy. The C_{18} monoenoic acid and dienoic acid fractions purified by successive gas–liquid chromatography on Apiezon L and polyethylene glycol adipate columns were examined in CS_2 solution in a Perkin–Elmer Infracord apparatus with a 0·1 mm. microcell. The presence of a peak at 965 $cm.^{-1}$ was taken to be indicative of the presence of a *trans* isomer.

Thin-layer chromatography. The methyl esters of the geometrical isomers of C_{18} mono-, di- and tri-enoic acids were separated by thin-layer chromatography by a method kindly demonstrated to us by Dr B. de Vries of Unilever Research Laboratories, Vlaardingen, Holland (de Vries, 1962, 1963). The thin-layer chromatograph consisted of Silica Gel G (Merck) impregnated with 30% (w/w) silver nitrate. Troublesome blackening of the plates was considerably diminished by storage in darkness at 4° instead of at room temperature. Trienoates were separated with benzene–diethyl ether (9:1, v/v), and dienoates and monoenoates by benzene–light petroleum (b.p. 40–60°) (9:1, v/v). The fatty acid methyl esters were located by spraying with 0·2% (w/v) 2,7-dichlorofluorescein in ethanol and examination under ultraviolet light. After radioactive scanning (see below) the methyl esters were recovered by sucking up the appropriate area of the gel into a polythene-tipped pointed glass tube containing a no. 4 sintered-glass filter. The gel was shaken out of the tube with the aid of a mechanical vibrator and spread on a planchet for radioactive assay. The methyl esters were extracted by introducing the gel into a tube containing 5 ml. of diethyl ether–methanol–benzene (3:1:1, by vol.). The tube was shaken at 39° for 90 min. and centrifuged, and the residue extracted in like manner. The combined extracts were evaporated to dryness *in vacuo*, and to the residue containing some silver nitrate was added 1 ml. of water. The methyl ester of the fatty acid isomer was extracted by washing three times with 2 ml. of light petroleum (b.p. 40–60°). The recoveries were approx. 50–70%; the lost radioactivity adhered strongly to the silica gel.

Oxidation of unsaturated fatty acids. Oxidation at the double bond was carried out by the method of Rudloff (1956), and the mono- and di-carboxylic acids were examined by gas–liquid chromatography. The fatty acid methyl esters (0·1–1 mg.) were oxidized by using the reagent volumes described by Scheuerbrandt & Bloch (1962) scaled up tenfold. A blank oxidation was observed to give a number of small peaks on gas–liquid chromatography, but most of these were eliminated by washing the permanganate and periodate reagents with chloroform. Satisfactory oxidation was obtained of oleic acid and elaidic acid, giving azelaic acid and pelargonic acid, and of *cis*-octadec-11-enoic acid, giving heptanoic acid and a C_{11} dicarboxylic acid.

For the examination of the radioactive short-chain monocarboxylic acids a carrier mixture of C_2–C_9 acids (1 mg. of each) was added and the pH adjusted to about 9. The mixture was evaporated to dryness at 100° under N_2 and the residue was treated with 0·4 ml. of anhydrous diethyl ether containing 8·4% (w/w) of HCl. The supernatant liquor containing the free acids was examined by gas–liquid chromatography.

For the examination of long-chain monocarboxylic acids and all dicarboxylic acids, the mixture resulting from oxidation was evaporated to dryness *in vacuo*. The residue was treated with 0·5 ml. of N-HCl, and the acids were extracted five times with 1·5 ml. of diethyl ether. Carrier carboxylic acids were added when available before methylation and gas–liquid chromatography.

Radioactive counting techniques. Gas-flow counting, liquid-scintillation counting and effluent scanning of the gas–liquid chromatograms were carried out as described by Scott *et al.* (1964). Thin-layer chromatographic plates were scanned on a trolley which was moved at a constant rate under a collimated (0·5 mm. slit width) mica-windowed Geiger–Müller tube. This was connected to a rate-meter, and the output current, integrated over 5–25 sec., was continuously traced with a pen recorder moving at the same rate as the trolley. (We are indebted to Mr J. L. Bounden for constructing this apparatus.) The efficiency was only 1–2% with ^{14}C, but good resolution was obtained with the radioactivities available.

MATERIALS

Geometrical isomers of unsaturated C_{18} acids and many long-chain dicarboxylic acids were kindly supplied by Dr B. de Vries, Vlaardingen. Linolenic acid, linoleic acid and oleic acid (CFP grades) were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; elaidic acid was obtained from K and K Laboratories, Long Island City 1, N.Y.; and methyl *cis*-octadec-11-enoate was a gift from the National Institutes of Health, Bethesda, Md., U.S.A. [U-^{14}C]Linolenic acid, [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid were purchased from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Table 1 shows the fatty acid composition of the lipids in the diet that the sheep received during most of the experiments reported in this section. As is to be expected from previous results on pasture grass (Shorland *et al.* 1955; Garton, 1960), the diet is extremely rich in unsaturated C_{18} acids (Tables 1 and 2), and of these linolenic acid predominates. The drying of the pasture grass to produce hay had not caused any substantial loss of linolenic acid by autoxidation. The particulate matter in the rumen contents consisting of bacteria, protozoa and undigested food particles shows a picture in high

contrast with that of the diet soon after feeding (30 min.): the predominant acids are palmitic acid, stearic acid and linolenic acid (Table 1). At 6 and 24 hr. after feeding there is a substantial decrease in the amount of palmitic acid present, owing presumably to the passage of rumen contents down the digestive tract and possibly absorption through the rumen wall (McCarthy, 1962). At the same time a tenfold decrease in the concentration of unsaturated C_{18} acids occurs, again owing presumably in small part to similar losses, but mainly to the hydrogenation of such acids to stearic acid. The stearic acid content of the rumen fluid did not decrease with time, and in fact had increased slightly at 6 hr. after feeding, which indicated that any losses were compensated for by its metabolic formation from unsaturated C_{18} acids. The analysis of rumen contents obtained through a fistula was, however, complicated by the fact that the sample obtained by suction and coarse filtration was undoubtedly more fluid than the mass of the material present in the rumen, and as such may not have been representative. In fact, in subsequent experiments it was found that the less fluid portion, consisting apparently of undigested foodstuff, was exceptionally rich in saturated fatty acids, particularly stearic acid and palmitic acid, whereas the percentage of unsaturated acids was small. Since these saturated acids were virtually all in the free form, it seemed likely that once formed during the digestive processes they became attached to large food particles in the rumen.

The concentration of anteiso branched-chain C_{15} acid at various times after feeding suggested that it was metabolically formed from the foodstuffs, possibly from the isovaleric acid formed by amino acid deamination (Allison, Bryant, Keeney & Katz, 1961). This acid has been isolated from mutton fat by Hansen, Shorland & Cooke (1953).

The fatty acids present in the faeces were predominantly stearic acid and palmitic acid, and the unsaturated acids occurred only in low concentration (Table 1). Calculation showed that the amount of C_{18} fatty acid excreted daily was about 11% of the dietary intake of C_{18} acids. Straight-chain C_{15} and C_{17} acids and a branched-chain C_{15} acid occurred as minor constituents and were probably of microbial origin. The faeces also contained a very polar fatty acid which was probably hydroxystearic acid. The presence of all these acids in human faeces has been noted by James, Webb & Kellock (1961) and Gompertz & Sammons (1963).

The time-course of hydrogenation of unsaturated fatty acids brought about by rumen micro-organisms was studied by following the fatty acid composition of the rumen contents in the intact sheep and in an artificial rumen (Dawson *et al.* 1964). The results presented in Fig. 1 show that, on feeding, the percentage of unsaturated C_{18} fatty acids increased markedly, whereas that of stearic acid decreased. The palmitic acid content of the rumen showed no change. This is to be expected, since the dietary fatty acids had a high percentage of unsaturated C_{18} acids and a very low content of stearic acid, whereas the percentage of palmitic acid was similar to that in the rumen contents. The percentage of unsaturated C_{18} acids began to fall after 1 hr., and by 5 hr. had declined nearly to the prefeeding level: a concomitant increase in the percentage of stearic acid occurred (Fig. 1). Similar changes

Table 1. *Fatty acid composition of diet, particulate fraction from rumen contents and faeces*

Experimental details are given in the text. The notation used for the acids in this and subsequent Tables is that used by Ahrens *et al.* (1959).

		Rumen 'microbial' fraction (μmoles/100 ml.)			
Acid	Diet (μmoles/100 g.)	After 0·5 hr.*	After 6 hr.*	After 24 hr.*	Faeces (μmoles/100 g. wet wt.)
14:0	16·4	0·6	1·0	0·3	6·2
15:0 (anteiso branched)	0	1·3	3·1	0·6	15·9
15:0	8·0	1·4	2·4	0·9	35·8
16:0	810·2	25·5	14·6	12·1	181·8
16:1, 16:2	Trace	1·0	0·6	0·3	6·4
17:0	Trace	0·3	0·5	0·4	30·3
17:1	0	0·6	0·8	0·3	24·6
18:0	48·6	22·5	27·5	21·3	431·1
18:1, 18:2 (non-conjugated), 18:3 (non-conjugated)	2731·1	20·3	10·9	2·4	51·5

* Time interval after giving the diet consisting of 1000 g. of dried pasture grass + 200 g. of oats.

occurred in the artificial rumen, when supplied with hay and oats (Fig. 1) or pure linolenic acid (1 μmole/ml.).

In further experiments two sheep were killed, and the contents from various regions of the digestive tract were removed and analysed for C_{18} fatty acids (Table 2). These sheep had been allowed to eat a hay diet *ad lib.* and, although the individual results for digesta from a given organ vary widely between the two animals, some consistent trends between the various parts of the digestive tract are apparent. Thus there was a large decrease in the percentage of total unsaturated C_{18} fatty acids between the diet and the rumen contents and an accumulation of *trans* C_{18} monoenoic acid, and the stearic acid content increased markedly in both cases. In the ileum the percentage of unsaturated C_{18} acids appeared to increase appreciably although the *trans* C_{18} monoenoic acid was almost entirely absorbed. In the caecum the combined linolenic acid and linoleic acid content was decreased, and there was an accumulation of *trans* C_{18} monoenoic acid (Table 2). One explanation of these changes occurring in the caecum could be that it is a further site where unsaturated fatty acids can be hydrogenated. It is possible that the appreciable variation in the results of the two animals can be explained by the food intake before death. Whereas sheep I had very fluid contents and minimal digesta in the lower digestive tract, sheep II had much undigested foodstuff in the rumen with appreciable digesta in the remainder of the tract.

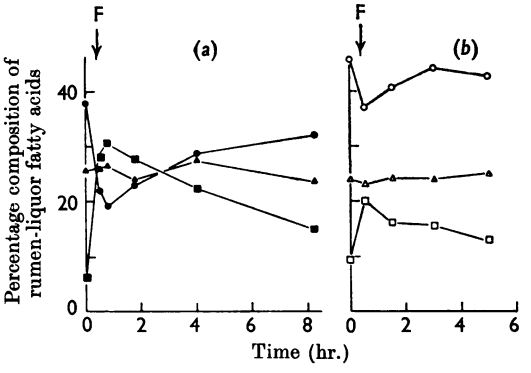

Fig. 1. Hydrogenation of unsaturated fatty acids by rumen micro-organisms (*a*) *in vitro* and (*b*) *in vivo*. F indicates time of giving 1 g. of diet *in vitro* and 1200 g. of hay–oats diet *in vivo*. Experimental details are given in the text. ■ and □, Unsaturated C_{18} acids; ● and ○, stearic acid; ▲ and △, palmitic acid.

Hydrogenation of ^{14}C*-labelled unsaturated fatty acids by rumen micro-organisms.* The hydrogenation process was studied by feeding an artificial rumen with [U-^{14}C]linolenic acid, [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid. The [U-^{14}C]linolenic ester used gave, on gas–liquid chromatography, only one radioactive peak which coincided with the emergence of methyl linolenate. On thin-layer chromatography of the methyl [U-^{14}C]linolenate, 87 % of the radioactivity was in the all-*cis* form, 8 % contained one double bond with a *trans* configuration, 4 % contained two *trans* double bonds and 1 % was all-*trans*.

On incubation of this [U-^{14}C]linolenic acid (1 μmole/ml.) in the artificial rumen, it was rapidly hydrogenated and after 20 min. 64 % of the radioactivity was present in a non-conjugated dienoic acid, which was shown by infrared spectroscopy and thin-layer chromatography to be largely the *cis–cis* isomer (Expts. 1 and 2 in Table 3). At the same time there was an appreciable formation of a ^{14}C-labelled fatty acid of unknown composition having retention volumes (relative to $C_{16:0}$ acid) of 3·88 on 10 % polyethylene glycol adipate at 180° and 2·28 on 10 % Apiezon L at 200°. This could indicate that it was a conjugated C_{18} dienoic acid of unknown configuration. After 3–4 hr. incubations both dienoic acids had largely disappeared, and a C_{18} monoenoic acid and stearic acid had accumulated (Expts. 5 and 6 in Table 3). The monoenoic acid fraction was examined by infrared spectro-

Table 2. *Comparison of* C_{18} *fatty acids in diet, faeces and various parts of the digestive tract of the sheep*

Experimental details are given in the text. Values are expressed as molar percentages of the total C_{18} fatty acids.

	Acid (methyl ester)	Diet	Rumen contents	Duodenum	Ileum	Caecum	Colon	Faeces*
Sheep I	18:2 + 18:3 (non-conjugated)	66	7·8	4·4	28·5	11·2	12·2	3·3
	18:1 *cis*	32	6·4	17·5	29·0	7·9	38·4	1·9
	18:1 *trans*	—	4·0	38·8	Trace	56·6	Trace	5·4
	18:0	2	81·8	39·3	42·5	24·3	49·4	89·4
Sheep II	18:2 + 18:3 (non-conjugated)	66	23·0	22·5	43·3	10·7	19·6	—
	18:1 *cis*	32	6·6	6·2	24·7	41·3	27·2	—
	18:1 *trans*	—	11·2	9·2	Trace	9·9	9·2	—
	18:0	2	59·2	62·1	32·0	38·1	44·0	—

* Faeces were collected from another sheep on a similar diet.

scopy and thin-layer chromatography, and was found to be predominantly *trans*. When [U-^{14}C]-linolenic acid at a much lower concentration (namely 0·004 μmole/ml.) was added to the artificial rumen it was hydrogenated extremely rapidly, so that at 20 min. 68 % of the radioactivity was present as stearic acid and by 2 hr. 95 % (Expts. 3 and 4 in Table 3). No radioactivity could be detected in fatty acids of chain length less than C_{18} after incubating rumen contents with [U-^{14}C]linolenic acid. Thus no evidence was obtained for a conversion of stearic acid into palmitic acid as suggested by Garton (1961).

On incubation of [1-^{14}C]linoleic acid (0·02 μ-mole/ml.) in the artificial rumen 93 % was converted into stearic acid in 90 min.; the small amount of monoenoic acid that accumulated was predominantly the *trans* isomer (Expt. 7 in Table 3). The original [1-^{14}C]linoleic contained no ^{14}C-labelled stearic acid or C_{18} monoenoic acid. There was a rapid conversion of [1-^{14}C]oleic acid into stearic acid when it was incubated for 60 min. in the artificial rumen. On isolation of the residual monoenoic acid fraction a small percentage of the radioactivity (3 %) was found by thin-layer chromatography to be in acids having the *trans* configuration (Expt. 8 in Table 3). The [1-^{14}C]oleic acid as received contained about 10 % of *trans* double bonds but it was purified by thin-layer chromatography as the methyl ester.

The position of the double bonds in both the dienoic acid and monoenoic acid fractions formed by the hydrogenation of linolenic acid was investigated by oxidation with permanganate–periodate at the double bond. The fractions were isolated by gas–liquid chromatography on 10 % Apiezon L to separate the unsaturated C_{18} acid esters from methyl stearate, followed by gas–liquid chromatography on 10 % polyethylene glycol adipate to partition the monoenoic acid and dienoic acid components. When the collected fractions were rechromatographed on 10 % polyethylene glycol adipate each ran as a single peak. There was a wide distribution of radioactivity in the monocarboxylic acids formed by oxidation of the monoenoic acid fraction, the maximum radioactivity being in valeric acid. However, no radioactivity was present in monocarboxylic acids of chain length greater than C_9. Table 4 shows the distribution of the double bond in the monoenoic acid fraction calculated from the radioactivity pattern. The predominant positional isomers were octadec-13-enoic acid and octadec-14-enoic acid with a gradual decrease in the amount of acids with double-bond position on either side of these two isomers.

Oxidation of the non-conjugated dienoic acids formed from linolenic acid showed a complex pattern of radioactivity in the mono- and di-carboxylic acids formed (Table 5). The short-chain monocarboxylic acids were labelled almost exclusively in the acetic acid and propionic acid fractions, suggesting that the double bond most remote from the carboxyl group was predominantly at C-15 or C-16. The labelled short-chain dicarboxylic acids were mainly succinic acid with a small amount of glutaric acid; of the longer-chain acids undecanedioic acid and dodecanedioic acid were predominant. Although a completely definitive interpretation of the results is not possible, the pattern of acids formed by oxidation suggests that the main dienoic acids have double bonds at C-11 or C-12 and C-15 or C-16.

Table 3. *Hydrogenation of* [U-^{14}C]*linolenic acid,* [1-^{14}C]*linoleic acid and* [1-^{14}C]*oleic acid by rumen micro-organisms*

Experimental details are given in the text. The substrate was emulsified with clear rumen liquor and added to the artificial rumen.

	Percentage distribution of radioactivity in fatty acid methyl ester							
Expt. no.	1	2	3	4	5	6	7	8
Incubation time (min.)	20	20	20	120	180	240	90	60
Substrate added	[U-^{14}C]-Linolenic acid (10 μc; 60 μmoles)	[U-^{14}C]-Linolenic acid (10 μc; 60 μmoles)	[U-^{14}C]-Linolenic acid (30 μc; 0·25 μmole)	[U-^{14}C]-Linolenic acid (30 μc; 0·25 μmole)	[U-^{14}C]-Linolenic acid (10 μc; 60 μmoles)	[U-^{14}C]-Linolenic acid (10 μc; 60 μmoles)	[1-^{14}C]-Linoleic acid (25 μc; 1 μmole)	[1-^{14}C]-Oleic acid (5 μc; 1 μmole)
Acid								
18:0	1	Trace	68	95	39	60	93	83
18:1 *cis*	3	2	3	3	5	4	1	14
18:1 *trans*			18		28	36	4	3
18:2 *cis–cis*	64	41	11	1	11	0	2	0
18:2 *cis–trans* or *trans–cis*		7				0		0
18:2 *trans–trans*		1				0		0
18:2 (conjugated?)	21	34	0	0	3	0	0	0
18:3 (non-conjugated)	11	15	Trace	1	14	0	0	0

Table 4. *Oxidative degradation of monoenoic acids formed from* [U-^{14}C]*linolenic acid in the artificial rumen*

Experimental details are given in the text. The percentage composition of the monoenoic acid fraction was calculated from the radioactivity of the monocarboxylic acids formed by oxidation, with allowance made for the number of carbon atoms. There were no radioactive monocarboxylic acids beyond pelargonic acid.

Monocarboxylic acid from oxidation	Radioactivity (counts/100 sec., corrected for background)	Composition of monoenoic acid fraction (acid)	(%)
Formic acid	106	Octadec-17-enoic acid	2
Acetic acid	395	Octadec-16-enoic acid	6
Propionic acid	1032	Octadec-15-enoic acid	10
Butyric acid	2811	Octadec-14-enoic acid	21
Valeric acid	3789	Octadec-13-enoic acid	22
Hexanoic acid	2776	Octadec-12-enoic acid	14
Heptanoic acid	2777	Octadec-11-enoic acid	12
Octanoic acid	1551	Octadec-10-enoic acid	6
Pelargonic acid	2108	Octadec-9-enoic acid	7

Table 5. *Acids formed on oxidation of non-conjugated dienoic acids produced during hydrogenation of* [U-^{14}C]*linolenic acid*

Experimental details are given in the text. The percentage composition of each fraction was calculated from the radioactivity, with allowance made for the number of carbon atoms.

Acid	Radioactivity (counts/100 sec.)	Composition (%)
Monocarboxylic acid		
Acetic acid	5046	55
Propionic acid	5494	40
Butyric acid	597	3
Valeric acid	342	1
Hexanoic acid	220	1
Short-chain dicarboxylic acids		
Malonic acid	327	6
Succinic acid	4437	57
Glutaric acid	2416	25
Adipic acid	1449	12
Long-chain dicarboxylic acids		
Azelaic acid	5848	15
Sebacic acid	3284	8
Undecanedioic acid	20716	44
Dodecanedioic acid	13337	26
Tridecanedioic acid	3211	5
Tetradecanedioic acid	1071	2

Hydrogenation of linoleic acid and oleic acid in the caecum and colon. Ileum, caecum and colon contents were also incubated with labelled unsaturated C_{18} acids. Table 6 shows that with the caecum and colon contents there was a conversion of [1-^{14}C]-linoleic acid into C_{18} monoenoic acid which mainly had the *trans* configuration. With the digesta from the caecum there was no detectable hydrogenation of [1-^{14}C]oleic acid. With the colon contents oleic acid was slowly converted into stearic acid and possibly a *trans* monoenoic acid. On the other hand, when ileum contents were incubated for 2 hr. with [U-^{14}C]linolenic acid there was no evidence of hydrogenation.

Gas–liquid chromatographic data. Although the retention volumes of some of the C_{18} acids have been published (Beerthuis, Dijkstra, Keppler & Recourt, 1959; Litchfield, Reiser & Isbell, 1963; James, 1963), a more complete comparison of the behaviour of the geometrical isomers on 10% Apiezon L and 10% polyethylene glycol adipate columns is given in Table 7. Since we were unable to discover any data in the literature on the gas–liquid chromatography of dicarboxylic acid dimethyl esters and short-chain monocarboxylic acids on 10% polyethylene glycol adipate, their retention volumes are reported in Table 8.

DISCUSSION

The present results confirm the findings of previous workers (Shorland *et al.* 1957) that the rumen is a site of hydrogenation of unsaturated fatty acids. The principal acid formed after a 20 min. incubation was shown to be a non-conjugated *cis–cis* dienoic acid. From the oxidation data of this fraction it cannot be completely excluded that double-bond migration had taken place in the direction of the carboxyl group of the molecule, since the short-chain dicarboxylic acids (mainly succinic acid and glutaric acid) formed on oxidation may have been derived from this end of the fatty acid molecule. However, since no double bond was found in the monoenoic acid fraction lower than C-9 it seems reasonable to conclude that the short-chain dicarboxylic acids were formed by end oxidation of the carbon chain between the two double bonds. If this was so, the double bond nearest the carboxyl group was predominantly at C-11 or C-12 and the other double bond at C-15 or C-16, since only acetic acid and propionic acid were formed on oxidation.

The finding of appreciable acetic acid on oxidation of the dienoic acid can be contrasted with the

Table 6. *Hydrogenation of* [U-^{14}C]*linolenic acid,* [1-^{14}C]*linoleic acid and* [1-^{14}C]*oleic acid in the lower digestive tract of the sheep*

Experimental details are given in the text. The substrate was emulsified with 5% albumin solution and added to a flask containing ileum, caecal or colon contents. The mixture was incubated at 39° under anaerobic conditions.

	Percentage distribution of radioactivity in fatty acid methyl ester						
Section	Ileum	Caecum				Colon	
Expt. no.	1	2	3	4	5	6	7
Incubation time (min.)	120	90	180	90	180	180	180
Substrate added ...	[U-^{14}C]-Linolenic acid (5 μc; 0·05 μmole)	[1-^{14}C]-Linoleic acid (5 μc; 0·2 μmole)	[1-^{14}C]-Linoleic acid (5 μc; 0·2 μmole)	[1-^{14}C]-Oleic acid (5 μc; 0·2 μmole)	[1-^{14}C]-Oleic acid (5 μc; 0·2 μmole)	[1-^{14}C]-Linoleic acid (5 μc; 0·2 μmole)	[1-^{14}C]-Oleic acid (5 μc; 0·2 μmole)
Acid							
18:0	0	2	Trace	0	0	Trace	12
18:1 *cis*	0	3	6	99	100	13	85
18:1 *trans*	0	62	44	1	0	45	3
18:2 non-nonjugated	0	33	50	0	0	42	0
18:3 non-conjugated	100	0	0	0	0	0	0

Table 7. *Gas–liquid chromatography of* C_{18} *fatty acid methyl esters*

Experimental details are given in the text.

Acid (methyl ester)	Retention volume relative to methyl palmitate: On 10% Apiezon L (200°)	On 10% polyethylene glycol adipate (180°)
Octadecanoic acid (stearic acid)	2·27	1·93
cis-Octadec-6-enoic acid	1·93	2·08
cis-Octadec-9-enoic acid (oleic acid)	1·96	2·11
cis-Octadec-12-enoic acid	1·98	2·14
trans-Octadec-9-enoic acid (elaidic acid)	2·05	2·11
cis–cis-Octadeca-9,12-dienoic acid (linoleic acid)	1·85	2·56
cis–trans-Octadecadienoic acid *trans–cis*-Octadecadienoic acid *trans–trans*-Octadecadienoic acid } non-conjugated	1·95	2·60
cis–trans-Octadeca-9,11-dienoic acid or *trans–cis*-octadeca-10,12-dienoic acid	2·42	3·59
trans–trans-Octadeca-9,11-dienoic acid or *trans–trans*-octadeca-10,12-dienoic acid	2·92	4·33
cis–cis–cis-Octadeca-9,12,15-trienoic acid (linolenic acid)	1·85	3·27
Octadecatrienoic acid non-conjugated geometrical isomers	1·95	3·27

results of Shorland *et al.* (1957), who did not detect this acid as an oxidation product of the dienoic acid formed by incubation of rumen contents with linolenic acid. It implies that during the hydrogenation there can be a migration of the double bond from C-15 to C-16. Such a migration has been demonstrated by chemical hydrogenation with a nickel catalyst (Sreenivasan *et al.* 1963). The demonstration that this also occurs in the rumen might explain the presence of *trans*-octadec-16-enoic acid in the depot fat of the sheep (Hansen, 1963), since such an acid was found to be present in the monoenoic acid fraction formed on hydrogenation.

The structure of the unknown fatty acid that accumulated during short-term incubation of linolenic acid in the artificial rumen cannot be assessed from the present results. However, its retention volumes on gas–liquid chromatography suggested that it was neither a monoenoic acid nor a trienoic acid. Of the possible dienoic acids the non-conjugated, as well as the conjugated *trans–trans*, *trans–cis* and *cis–trans* forms could be eliminated because of their behaviour on gas–liquid chromatography (Table 7). This suggested the possibility that it may have the conjugated *cis–cis* configuration, but no markers were available to test this hypothesis and too little was formed to measure its absorption

Table 8. *Gas–liquid chromatography of mono- and di-carboxylic acids*

Free monocarboxylic acid	Retention volume relative to hexanoic acid on 10% polyethylene glycol adipate (100°)
Acetic acid	0·14
Propionic acid	0·21
Butyric acid	0·32
Valeric acid	0·57
Hexanoic acid	1·00

Dicarboxylic acid (dimethyl ester)	Retention volume relative to methyl palmitate on 10% polyethylene glycol adipate (150°)
Succinate	0·12
Glutarate	0·18
Adipate	0·27
Pimelate	0·40
Suberate	0·60
Azelate	0·88
Sebacate	1·32
Undecanedioate	1·96
Dodecanedioate	2·95
Tridecanedioate	4·44

characteristics. It disappeared almost completely as the hydrogenation continued, and no evidence was obtained for the accumulation of a dienoic acid resistant to further hydrogenation, as described by Shorland *et al.* (1957).

The hydrogenation of the dienoic acid to a monoenoic acid was apparently again accompanied by considerable double-bond migration, and the resultant monoenoic acid fraction had the *trans* configuration. This is likely to account for most of the *trans* monoenoic acid formed during the hydrogenation of linolenic acid and linoleic acid, since the conversion of oleic acid into *trans* isomers appeared to be minimal under the present conditions (cf. Shorland *et al.* 1957). In the mixture of isomers present in the monoenoic acid fraction the frequency of occurrence of the double bond was grouped around a maximum at C-13 and C-14 (Table 4), which could suggest that these positions are the most stable.

These results, which show a substantial migration of the double bond, can be compared with the chemical hydrogenation of linolenic acid; hydrogenation with a metallic catalyst results in a similar considerable migration of the double bond and *trans* acid formation, whereas hydrogenation with hydrazine does not produce any such isomerism (Sreenivasan *et al.* 1963; Scholfield, Jones, Nowakowska, Selke & Dutton, 1961). Clearly in the experiments where 60 μmoles of linolenic acid were incubated with rumen contents the capacity of the hydrogenation system must have been overloaded, since decreasing the amount of added substrate to 0·25 μmole resulted in a greatly increased percentage hydrogenation. Under our conditions the hydrogenation of linoleic acid and oleic acid appeared to be no slower than that of linolenic acid, in contrast to Shorland *et al.* (1957).

The contents of the ileum showed a greater percentage of unsaturated C_{18} acids compared with either the rumen or duodenal contents (Table 2). This could have resulted either from the selective absorption of stearic acid or the influx of unsaturated fatty acids via the bile or from the intestinal mucosa (Burr, McPherson & Tidwell, 1960). It is also apparent that the *trans* monoenoic acids formed in the rumen were almost completely absorbed in the ileum and presumably became incorporated into the depot fats (Hartman, Shorland & McDonald, 1954).

The unsaturated fatty acids passing to the caecum can then undergo a further hydrogenation, which continues in the colon. The absence of hydrogenation of [U-^{14}C]linolenic acid when incubated with ileum contents suggests that the micro-organisms responsible may not be merely those which pass out from the rumen and which are likely to be killed by the acidic secretion of the abomasum. In our experiments the caecum contents were able to hydrogenate linoleic acid to a *trans* C_{18} monoenoic acid, but there appeared to be little further hydrogenation to stearic acid (Expts. 2 and 3 in Table 6). This would explain the renewed accumulation of *trans* C_{18} monoenoic acids seen in the caecum (Table 2). On the other hand, the colon contents seemed to be capable of slowly causing the hydrogenation of oleic acid, so that by the time digesta were excreted the fatty acid present was predominantly stearic acid.

The ability of the sheep to carry out hydrogenation of unsaturated fatty acids in its lower digestive tract can be contrasted with that of man. With six individuals, Gompertz & Sammons (1963) noted hydrogenation of erucic acid to behenic acid in one only who possessed a megacolon and where the long retention of the faeces is likely to lead to an increased chance of microbial hydrogenation. Further, the hydrogenation of [^{14}C]oleic acid to stearic acid was negligible in a patient with steatorrhoea (Sammons, Frazer, Gompertz & Morgan, 1962).

SUMMARY

1. When [U-^{14}C]linolenic acid was incubated for short periods in an artificial rumen, it was rapidly hydrogenated to two types of dienoic acids, subsequently to a C_{18} monoenoic acid and finally into stearic acid.

2. The isomers in the major dienoic acid fraction had *cis–cis* non-conjugated configurations, and the double bonds were largely at C-11 or C-12 and C-15 or C-16.

3. The monoenoic acids formed were largely *trans*, with the double bond predominantly at C-13 or C-14.

4. Rapid hydrogenation of [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid occurred in the artificial rumen. The former gave a *trans* monoenoic acid as an intermediary.

5. The *trans* C_{18} monoenoic acids passing from the rumen were almost quantitatively absorbed in the ileum.

6. The unsaturated C_{18} acids present in the ileum digesta were hydrogenated in the caecum and colon, so that nearly all the acids in the excreta were saturated.

7. The hydrogenation of [1-^{14}C]linoleic acid by caecal and colon contents resulted again in the formation of substantial amounts of *trans* C_{18} monoenoic acids.

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Preparation of Potassium 2-Deoxy-2-[^{35}S]sulphoamino-D-glucose

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Although it is well known that the biological activity of exogenous samples of the sulphated aminopolysaccharide heparin disappears rapidly *in vivo* (cf. Loomis, 1959, 1961), the sequence of events involved in the disposal of the injected polymer is still not fully understood. Up to the present, studies on the metabolic fate of heparin have been based on observations made after the injection of unlabelled biosynthetic preparations of the polymer (Wilander & Holmgren, 1938; Wilander, 1939; Jacques, 1939; Reinert & Winterstein, 1939; Copley & Schendorf, 1941; Piper, 1947;