Occurrence of *trans*-Unsaturated Acids in the Faecal Lipids of Ruminants and non-Ruminants

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After the identification of 5–10 % of *trans*-unsaturated acids, mainly elaidic (*trans*-octadec-9-enoic) and vaccenic (*trans*-octadec-11-enoic), in ox fat (Swern, Knight & Eddy, 1952), it has been shown in this Laboratory (Hartman, Shorland & McDonald, 1954, 1955) that substantial amounts of these acids appear in the depot fats of other ruminants (3·5–11·2 %) and in certain marsupials (18·1–21·0 %), but less than 1 % in non-ruminants. Their presence has been attributed (Hartman *et al.* 1954) to the activity of rumen micro-organisms which, as first demonstrated by Reiser (1951), are efficient hydrogenators. Since hydrogenation is known to be accompanied by partial formation of *trans* isomers (Hilditch & Vidyarthi, 1929) bacterial action could account for the presence of *trans* acids in the fats of ruminants and certain marsupials, the stomachs of which contain a microbial population (Moir, Somers, Sharman & Waring, 1954).

In the large intestines of both ruminants and non-ruminants there is a rich bacterial flora and a strongly reducing atmosphere, which in the light of the above-mentioned suggestion of Hartman *et al.* (1954) should be conducive to *trans* isomerization of unsaturated fatty acids. To obtain information on this point faecal lipids from a number of ruminants and non-ruminants were examined for their contents of *trans* acids. The present paper reports the results of this investigation.

MATERIALS AND METHODS

Fresh faeces, collected from animals specified in Table 1, were immersed in boiling 95 % ethanol and refluxed for ½ hr. to effect sterilization. The wild rabbit is known to produce two kinds of faeces: soft mucus-coated pellets, which are reingested (Watson, 1954), and normal hard droppings. Soft pellets were taken from the stomachs of freshly killed animals, whereas hard droppings were collected from caged animals.

After the evaporation of the solvent the residues were dried *in vacuo* on a water bath and extracted in a Soxhlet apparatus with methanol–light petroleum (b.p. 40–60°) (2:1, v/v). The solvents were removed, the residues dissolved in light petroleum and the lipids recovered by evaporating the solvent *in vacuo*. The lipids were saponified with ethanolic potassium hydroxide and the unsaponifiable matter was extracted according to the standard method of the Society of Public Analysts (1933). The fatty acids recovered from the soaps were purified by passing them through silicic acid, according to the procedure of Borgström (1952) suitably modified, and their characteristics were determined. A similar procedure was applied to lipids obtained from dietary materials to determine their contents of *trans*-unsaturated acids, which, however, were found to be insignificant.

The analysis of *trans*-unsaturated acids was performed by the infrared technique as described in a previous paper (Hartman *et al.* 1955), but the instrument settings of the Perkin–Elmer model 21 spectrometer were as follows: gain 8·5, response 4:4, slit fixed at 157 μ; the scanning speed was 32 min./100 cm.$^{-1}$ between 1040 and 960 cm.$^{-1}$ and 86 min./100 cm.$^{-1}$ between 960 and 900 cm.$^{-1}$, the slower scanning being necessary near the maximum of the carboxyl-group absorption at 935 cm.$^{-1}$ where black-out conditions become most apparent. The base-line technique of measuring optical density was used between 1010 and 930 cm.$^{-1}$ The specific-extinction coefficient obtained for pure elaidic acid was 0·500. The accuracy of results for *trans* acids is within $\pm$0·5 % expressed as elaidic acid.

The contents of dienoic and trienoic acids were determined spectroscopically by the method of Brice, Swain, Herb, Nichols & Riemenschneider (1952).

The fatty acid fraction obtained from faecal lipids of the horse was subjected to a more detailed treatment than other samples, with the object of obtaining concentrates of *trans* acids. The acids (32·7 g.) were crystallized from acetone (500 ml.) once at $-20°$ and three times at $-35°$. The combined mother liquors contained the acetone-soluble fractions (15·2 g.) which comprised the bulk of *trans* acids. After the evaporation of the solvent the acids were converted into their methyl esters. The methyl esters were fractionated *in vacuo*; the two main fractions with iodine values of 60–80 were bulked, mixed with an equal weight of methyl arachidate to displace the material otherwise held up in the column during the fractionation, and refractionated. This fractionation led to a concentrate containing 45·6 % of *trans* acids. The difficulty of obtaining high concentrations of *trans* acid mixtures has been described by Cornwell, Backderf, Wilson & Brown (1953).

Since the dry matter of faeces is known to contain a large proportion of bacteria, it appeared desirable to determine the distribution of *trans* acids between bacterial and non-bacterial lipids. These separations were carried out

Table 1. *Characteristics of non-volatile fatty acids from various faecal lipids*

Species	Date collected	No. and sex of specimens	Diet	Fatty acids (%, w/w of dry matter)	Characteristics of fatty acids: Saponification equiv.	Iodine value (Wijs)	*trans* Acids (as %, w/w, of elaidic acid)	Remarks
(*a*) Non-ruminants								
Horse	6 June 1956	1 (M.)	Pasture	6·66	296·6	37·8	14·1	Adult; Wallaceville Animal Research Station, Dep. of Agriculture
Domestic rabbit (hard droppings)	27 August 1956	4 (F.)	Cabbage and food pellets	1·46	293·1	73·5	10·8	Toxicology Dep., Medical School, University of Otago, Dunedin
Wild rabbit (*Oryctolagus cuniculus* L.)								
Hard droppings	11 August 1956	2 (M.) 2 (F.)	Pasture	3·67	327·1	67·6	17·9	Animal Ecology Section, D.S.I.R., Taita
Soft pellets	15 May 1957	6 (M.) 3 (F.)	Pasture	3·84	310·5	50·0	17·2	Adults; Kourarau. Shot at the peak of the reingestion period
Rat	February 1956	5 (M.)	Fat-free (cf. Longenecker, 1939)	2·46	322·9	46·5	10·0	Nutrition Research Dep., Medical School, Dunedin
(*b*) Ruminants								
Cow	14 September 1956	1	Pasture	1·88	328·8	38·9	9·3	Plant Chemistry Laboratory, Palmerston North
Sheep	14 September 1956	1 (M.) 4 (F.)	Pasture	5·75	316·5	27·4	12·5	Adults, Plant Chemistry Laboratory, Palmerston North

Table 4. *Characteristics of bacterial and non-bacterial fatty acids from horse and sheep faeces*

			Bacterial fatty acids			Non-bacterial fatty acids			
Species	Date collected	No. and sex of specimens	Saponification equiv.	Iodine value	*trans* Acids (%, w/w)	Saponification equiv.	Iodine value	*trans* Acids (%, w/w)	Wt. of bacterial fatty acids / Wt. of non-bacterial fatty acids
Horse	22 July 1957	1 (M.)	300·8	39·6	10·0	298·3	34·8	8·6	0·389
Sheep	20 August 1957	3 (M.) 2 (F.)	327·8	25·9	12·9	321·1	36·8	15·8	0·495

Table 2. *Contents of dienoic and trienoic acids in fatty acids from animal faeces*

Values are expressed as % (w/w) of total fatty acids.

Species	Conjugated acids: Dienoic	Conjugated acids: Trienoic	Non-conjugated acids: Dienoic	Non-conjugated acids: Trienoic
Horse	1·2	Nil	0·7	0·9
Domestic rabbit (hard pellets)	2·9	0·5	18·6	2·3
Wild rabbit				
Hard pellets	3·7	0·6	4·3	10·9
Soft pellets	1·8	Nil	1·7	4·5
Rat	2·6	0·5	2·5	1·0
Cow	1·7	0·5	3·8	2·1
Sheep	1·0	Nil	0·9	1·3

Table 3. *Fractionation data of methyl esters of 'acetone-soluble' fatty acids from horse faeces, and the* trans *acid contents of fractions*

Weight put in column: 4·5 g. of esters + 4·5 g. of methyl arachidate.

Fraction no.	Wt. (g.)	M.p.	Iodine value	Saponification equiv.: Esters	Saponification equiv.: Acids (unsaponifiable matter removed)	*trans* Acids (%, w/w)
1	0·27	3–4°	42·6	—	—	12·9
2	0·27	− 3 to − 2	44·4	—	—	38·5
3	1·01	− 8·5 to − 7·5	44·7	316·6	293·4	32·6
4	1·91	3–5	66·8	309·1	295·1	45·6
5	0·51	1·9–2·0	54·1	316·1	303·6	19·6
Residue	4·85	45·0–46·0	8·5	339·2	314·2	5·5

on samples of horse and sheep faeces. Horse faeces were treated according to the method outlined by Sperry (1928), but the separation was only moderately successful, as judged by microscopic examination, owing to the injurious effect on the micro-organisms of the mercuric chloride added as preservative. The bacterial fraction of sheep faeces was isolated by following the procedure applied recently by Garton & Oxford (1955) to rumen bacteria, with suitable modifications. Faeces (400 g.) were thoroughly mixed with 2 l. of 0·9% sodium chloride solution and filtered through cheese cloth to remove fibrous and coarse material. The filtrate was centrifuged for 10 min. at 2000 rev./min. and the deposited sludge was mixed with fresh sodium chloride solution and recentrifuged. Microscopic examination of stained smears, followed by plate counts, revealed that the distribution of bacteria between the aqueous suspension and the residue was about 12:1. The suspension was evaporated *in vacuo* and dried. This material (essentially dry bacteria) and the combined dried residues from filtration and centrifugal separation (practically free from bacteria) were extracted three times with boiling ethanol–ether (3:1, v/v) and filtered. The filtrates were evaporated *in vacuo*, the residues taken up in light petroleum and after the removal of the solvent the lipids were treated as already described.

RESULTS

The animal species, their diets and the characteristics of the non-volatile fatty acids obtained from their faecal lipids are listed in Table 1. The contents of dienoic and trienoic acids in the fatty acids from faecal lipids appear in Table 2. The methyl esters of the 'acetone-soluble' fatty acid fraction obtained from horse faeces were, as already mentioned, fractionated twice, the results of the second fractionation being shown in Table 3. As the saponification equivalents of the esters and of the fatty acids obtained from the esters by saponification, extraction of unsaponifiable matter and acidification pointed towards the presence of acids with more than 18 carbon atoms, a sample (0·2 g.) of fraction no. 4 was hydrogenated and crystallized twice from acetone. The crystals (0·12 g.) melted at 38–39° and gave no m.p. depression with pure methyl stearate. This indicated that the unsaturated acids of this particular fraction were of the C_{18} type. The filtrate contained some viscous material, the identification of which was beyond the scope of the present investigation. Further evidence that fraction no. 4 contained *trans*-unsaturated acids was provided by the spectroscopic examination of the hydroxylated products resulting from alkaline permanganate oxidation of a sample (0·25 g.) in the cold according to the method of Lapworth & Mottram (1925). The hydroxylated products, in contrast with the original acids, showed no *trans* acid content.

The characteristics of the fatty acids from bacterial and non-bacterial lipids of horse and sheep faeces appear in Table 4.

DISCUSSION

The iodine values of fatty acids from faeces (Table 1) are considerably lower than those of the corresponding depot fats (see Hartman *et al.* 1955) and the same applies to the dienoic and trienoic acid contents (Table 2) in non-ruminants. This points towards extensive hydrogenation, which combined with the presence of 9·3–17·9% of *trans* acids in faecal lipids seems to support our hypothesis (Hartman *et al.* 1954) for the origin of these acids in the fats of ruminants. In view of the presence of fermentative bacterial flora in the rumen and in the post-absorptive region of the gut a similar mechanism of *trans* acid formation in both organs is not unexpected. However, in animal depot fats a correlation was found between the iodine value and the content of *trans*-unsaturated acids (Hartman *et al.* 1955), whereas no such correlation could be found in faecal lipids.

Garton & Oxford (1955) raised the question whether *trans* acids in animal organisms are in fact bacterial lipids. The figures in Table 4 indicate that the percentage of *trans* acids in bacterial lipids approximates that in non-bacterial lipids, and therefore bacterial lipids account proportionately for the presence of these acids. Their share may amount to some 30% of the total quantity of *trans* acids, although wide variations are, of course, possible. These considerations apply only to *trans* acids from the living bacteria, as it cannot be decided at the present stage to what extent 'non-bacterial' lipids in faeces are of bacterial origin. Similar deductions should be applicable to *trans* acids of the rumen lipids, but in this instance the share of living bacteria would be much smaller.

The above results do not support Hofmann & Tausig's (1955) explanation of the origin of vaccenic acid in animal fats. Hofmann & Tausig stated the difficulty of explaining the formation of vaccenic acid from oleic acid by a *cis–trans* oxidation mechanism (Swern *et al.* 1952), because it involves a selective shift of the 9:10 double bond to the 11:12 position, and suggested the following alternative: 'Our demonstration that certain bacteria are rich sources of *cis*-vaccenic acid points to the intestinal bacteria as a likely source of *trans*-vaccenic acid in the lipids of higher animals. The *cis*-isomer elaborated by bacteria may undergo *cis–trans* isomerisation during its absorption or transport to the tissues.'

It appears that in view of the low iodine value and considerable *trans* acid contents of intestinal bacteria (cf. Table 4), the latter do not represent a rich source of *cis*-octadec-11-enoic acid as does *Agrobacterium tumefaciens* and other bacteria investigated by Hofmann and his co-workers. Further, partial hydrogenation of dietary linoleic acid (and polyunsaturated acids) would account not only for *trans*-isomerization but also for positional isomers, including those with the double bond in the 11:12 position (Allen & Kiess, 1956).

Note added in proof. After this paper had gone to Press, it was found that the infrared technique, as used in the present investigation for the determination of *trans*-unsaturated acids in lipids, was subject to a possible source of error owing to interference from background absorption. While the more recent results obtained with horse faecal lipids were not materially different from those reported here, the values found for ruminant faecal lipids varied somewhat according to the base-line applied. These observations might lead to some revision of the results now presented, although no changes in the basic conclusions are envisaged. Re-investigation of the infrared method is now in progress and it is hoped to report on it at a later date.

SUMMARY

1. The faecal lipids of several ruminants and non-ruminants were found to contain considerable amounts of *trans* acids (9–18% of the fatty acid fraction).

2. The percentage of *trans* acids in bacterial and non-bacterial faecal lipids was similar, the bacteria accounting for approximately 30% of the total amount of these acids.

3. By analogy with the formation of *trans* acids in the rumen, the origin of these acids in the faecal lipids is attributed to the hydrogenating action of intestinal bacteria.

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Studies on the Protein of Fish Skeletal Muscle

4. ULTRACENTRIFUGAL ANALYSIS OF CODLING EXTRACTS*

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There is very little information about the ultracentrifugal behaviour of fish-muscle protein and virtually none for marine species. Hamoir (1955*a*, *b*) has shown the ultracentrifugal diagram of an extract of carp muscle made at low ionic strength and also presented data on certain purified carp myosin and actomyosin fractions and on the crystallized proteins myoglobin (from carp and tuna), tropomyosin and nucleotropomyosin (both from carp). In addition, the sedimentation coefficients of a number of crystalline proteins derived from the myogens of carp (Henrotte, 1952, 1954, 1955; Hamoir, 1957) and of flounder (Henrotte & Dresse, 1955) have been published.

It was therefore of interest to supplement the electrophoretic data previously obtained on codling extracts (Connell, 1953) with ultracentrifuge data. It was also hoped to elucidate some of the problems of the existence in fish extracts of a protein corresponding in its sedimentation behaviour to that of rabbit myosin. Roth (1947) was unable to detect the presence of L-myosin (Schramm & Weber, 1942) in extracts made from carp muscle which normally extract this protein from rabbit muscle, and only components corresponding to S-myosin or actomyosin could be observed. Nevertheless, electrophoretic components corresponding to β-myosin are present in rapidly made extracts of both carp (Hamoir, 1949, 1951) and codling (Connell, 1954). However, with the usual myosin-isolation procedures only heavy or polydisperse preparations were obtained by these authors from such extracts. Some of these results appeared to be due to inattention to a number of factors, e.g. the absolute freshness of the tissue from which the extract was obtained, the rapidity of working up and examining the extract, the temperature and the pH. Special attention has therefore been paid to such details of procedure. In particular, it is now known that rabbit myosin suffers an apparently irreversible aggregation and may be kept in a monodisperse condition for only a few hours at room temperature or a few days at 0° (Laki & Carrol, 1955; Holtzer, 1956; Holtzer & Lowey, 1956; Rupp & Mommaerts, 1957); cod myosin is even more unstable (J. J. Connell, unpublished work) and its handling poses special problems. Since myosin at or below its isoelectric point is denatured in the presence of salts (Edsall, 1930), acid-extracting solutions as used by Hamoir (1955*a*, *b*) have been avoided.

The electrophoretic and ultracentrifugal diagrams of extracts of increasing ionic strength were compared; also the amount of protein extracted, the ATP-sensitivity [Portzehl, Schramm & Weber, 1950; see also Table 3 (footnote) of the present paper] and sometimes the actin-combining power of the extracts were measured to indicate when myosin or actomyosin, or both, were being extracted.

* Part 3: Connell, J. J. (1954).

EXPERIMENTAL

Materials

Live cod (*Gadus callarias* L.) have been used for all these experiments. The fish were never more than about 40 cm. in length.

Buffers. Buffers of pH 7·5 and 6·5 were made up respectively as follows: 0·00338M-KH_2PO_4 + 0·0155M-Na_2HPO_4 (*I* 0·05) and 0·0217M-KH_2PO_4 + 0·0095M-Na_2HPO_4 (*I* 0·05). Potassium chloride was added to these buffers to bring the total ionic strength up to 0·1, 0·2, 0·3 and 0·5.