The *trans*-Unsaturated Acid Contents of Fats of Ruminants and Non-ruminants

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The discovery by Bertram (1928) of vaccenic (*trans*-octadec-11-enoic) acid in ox-, sheep- and butter-fat has been confirmed by other investigators (Grossfeld & Simmer, 1930; Geyer, Nath, Barki, Elvehjem & Hart, 1947), who also found traces of this acid in lard. In addition, Gupta, Hilditch, Paul & Shrivastava (1950) showed that *trans*-octadec-10-enoic acid was also present in the depot and milk fats of oxen and sheep.

More recently, by use of infrared absorption techniques, Swern, Knight & Eddy (1952) found that beef fat contained not traces, but 5–10% of *trans*-acids consisting of both elaidic (octadec-9-enoic) and vaccenic acids, and Cornwall, Backderf, Wilson & Brown (1953) showed that similar quantities of *trans*-acids were present in butter-fat mainly as monoene, but also as diene and possibly triene acids.

To account for the presence of *trans*-acids in the depot fats of oxen, Swern *et al.* (1952) suggested that they may have been produced endogenously from *cis*-monoethenoid acids, such as oleic acid, by the action of oxidases. However, oxidases occur also in non-ruminants (Bergström & Holman, 1948), but only ruminants deposit substantial amounts of *trans*-acids, as shown by Hartman, Shorland & McDonald (1954). Furthermore, recent work (Khan, Tolberg, Wheeler & Lundberg, 1954) has thrown some doubt on the feasibility of an oxidation mechanism by which a *cis*-unsaturated acid can attain a *trans* configuration without being converted into a hydroperoxide. The conversion of a hydroperoxido-unsaturated fatty acid into an unsaturated fatty acid would require additional changes for which no analogy can be found in the autoxidation processes hitherto studied. On the other hand, Reiser (1951) demonstrated that rumen contents can hydrogenate linseed oil, and this led Hartman *et al.* (1954) to the hypothesis that *trans*-acids might be formed as a result of hydrogenation, which is known to induce *trans*-isomerization of fatty acids (cf. Markley, 1947). In conformity with the hypothesis that *trans*-acids of

ruminant fats arise from hydrogenation of the dietary unsaturated fatty acids in the rumen, it was found that whereas pasture lipids contained only small amounts of *trans*-acids, these acids were present to the extent of 9% in the fatty acids from the rumen contents of pasture-fed sheep.

METHODS

The fats used in this work were generally prepared by steam rendering of the fatty tissues. For small samples and muscle tissues the finely sliced material was dropped into about 10 vol. of boiling 95% ethanol and refluxed for $\frac{1}{2}$ hr. After removal of the solvent and drying *in vacuo* at 100° the fat was extracted in a Soxhlet apparatus with light petroleum (b.p. 40–60°).

The analysis of the *trans*-acids was performed on the fatty acids prepared as described by Hilditch (1947). The procedure outlined by Hilditch to remove unsaponifiable matter was also applied to the lipids from the pasture and rumen contents which contained chlorophyll as well as substantial amounts of unsaponifiable matter.

The method of estimating the *trans*-acid contents (shown in Table 1) was based on the infrared absorption of the *trans* double bond (cf. Shreve, Heether, Knight & Swern, 1950), and the instrument used was a Perkin-Elmer model 21 infrared spectrophotometer (NaCl optics) with 1 mm. cells. The instrumental settings were: gain 8 to 8 $\frac{1}{2}$, response 4/4, slit 180 (manual), with a scanning speed of 60 min./100 cm.⁻¹. The specific extinction coefficient k of the *trans* double bond at 965 cm.⁻¹ was calculated from the extinction coefficients $E[\log_{10}(T_{990}/T_{965})]$, determined from the transmission graphs (spectral region, 990–950 cm.⁻¹) of mixtures of varying proportions of stearic acid and elaidic acid in carbon disulphide (concentration 5%, w/v) compensated with 5% (w/v) stearic acid solution (McDonald, 1954). The mean value obtained for k was 0.473 (range ± 0.02), in good agreement with that calculated from the corresponding figure reported by Ahlers, Brett & McTaggart (1953), namely 0.477. By use of the determined value of k , the *trans*-acid contents of unknown samples (expressed as wt. % of elaidic acid) were calculated from the transmission graphs of 5% (w/v) solutions of the sample, compensated as before with stearic acid.

In certain samples the amounts of diene and triene acids were determined by the procedure of Brice, Swain, Herb, Nichols & Riemenschneider (1952).

Table 1. *Characteristics of some animal fats with special reference to their trans-acid contents*

Species	Sample	Date collected	No. and sex of specimens	Diet	Characteristics of fat			Remarks
					Saponification equiv. (Wijs)	Iodine value (as % w/w, of elaidic acid)	<i>Trans</i> -acids (as % w/w, of elaidic acid)	
<i>(a) Miscellaneous</i>								
Rat	Body fat	May-July 1952	5 (M.) 5 (F.)	Non-ruminants Fat-free (cf. Longenecker, 1939)	299.5	58.4	Not detected*	Rats placed on diet at 45 g. and killed approx. 2 months later at 160 g.
Pig	Perinephric	25. viii. 53	—	Skim-milk	288.2	49.9	0.9	Commercial sample, Longburn Freezing Works, Longburn
Horse	Perinephric	4. xi. 47	—	Pasture	283.6	91.6	Not detected	Two fatty horses (cf. Shortland, Bruce & Jessop, 1952)
Rabbit	Abdominal cavity fat	7. vi. 51	18 (F.)	Pasture	285.1	144.7	Not detected	Guavas Forest, near Wai-pawa, Hawkes Bay
Pheasant	Abdominal and external tissue fat	7. vii. 54	1 (M.) 1 (F.)	Clover and inkweed seeds	286.6	90.0	Not detected	Galatea Estate, near Whakatane; wild adult birds
<i>(b) Marsupials</i>								
Wallaby (<i>Thylogale eugene</i>)	Perinephric	15. xii. 54	1 (M.)	Pasture	292.5	76.6	18.1	Adult, Lake Okareka, near Rotorua
Wallaby	Perinephric	15. xii. 54	1 (F.)	Pasture	292.1	73.6	19.2	Adult, Lake Okareka, near Rotorua
Quokka (<i>Setonix brachyurus</i>)	Perinephric	26. ix. 54	6 (F.)	Sheep nuts	277.5	73.2	21.0	Adult experimental animals, Zoology Dept., University, Western Australia
Opossum (<i>Trichosurus vulpecula</i>)	Perinephric	17. vii. 54	2 (F.)	Tree-foliage and fruit	287.1	86.1	Not detected	Adult animals, good condition, Orongorongo Valley, near Wellington
Ox	Perinephric	29. x. 53	—	Ruminants Pasture	286.6	40.7	4.5	Commercial sample, Gear Meat Co. Ltd., Wellington
Ox	Oleo-oil and tallow	—	—	—	—	—	5-10	Swern <i>et al.</i> (1952)
Cow	Butterfat	—	—	Pasture with grain and hay supplement	—	—	5.0-9.7	Cornwell <i>et al.</i> (1953)
Fallow deer (<i>Dama dama</i>)	Perinephric	12. v. 52	1 (F.)	Pasture	295.3	30.8	3.5	Shortland (1953)
Sambur (<i>Cervus unicolor</i>)	Mesenteric	16. x. 54	1 (F.)	Pasture	277.6	34.3	4.4	Adult with 12 lb. embryo, taken near Bulls
Goat	Perinephric	17. vii. 54	1 (M.)	Pasture (in bush)	292.6	36.4	8.9	Adult, lean, Orongorongo Valley, near Wellington
Goat	Perinephric	17. vii. 54	1 (F.)	Pasture (in bush)	286.7	34.8	9.6	Adult, better condition, from Orongorongo Valley, near Wellington
Goat	Perinephric	26. vii. 54	1 (M.)	Five-finger and <i>Rhipogonum scandens</i>	285.9	47.4	11.1	Kid, wild, Mt. Bruce, near Masterton
Goat	Perinephric	26. vii. 54	1 (F.)	As above	294.8	47.1	6.8	Adult, wild, Mt. Bruce, near Masterton
Sheep	Perinephric	13. xi. 53	—	Pasture	289.1	44.0	11.2	Commercial sample, Gear Meat Co. Ltd., Wellington

* Less than 0.2% of *trans*-acids.

RESULTS AND DISCUSSION

From Table 1 it can be seen that there are wide differences in the iodine values of the fats examined, even where the dietary fat (pasture fat) is similar, and that there is some connexion between these values and the contents of *trans*-acids. From pasture-fed animals fats with highest iodine values (91.6–144.7) show no detectable amounts of *trans*-acids, those intermediate in iodine value (73.2–76.6) have the highest *trans*-acid contents (18.1–19.2%), and those with lowest iodine values (30.8–40.7) contain 3.5–9.6% of *trans*-acids. It has been pointed out elsewhere (Shorland, 1953) that pasture fatty acids consist mainly (60–70%) of linolenic acid (iodine value 274.1), and that whereas the fats of pasture-fed ruminants are of relatively low iodine value (30–50) and contain only traces of this constituent, pasture-fed non-ruminants, such as horses and rabbits, deposit fats of relatively high iodine value (90–150) containing considerable amounts (16–42%) of linolenic acid.

fat has been found (Shorland, 1953) to correspond closely to that of rye-grass (Table 2) and there is some evidence that leaf fats in general have a somewhat similar fatty acid composition (cf. Hilditch, 1947). Whereas in pasture-fed horses and rabbits the C_{18} triene and diene acids are deposited in approximately the same ratios in the diet (triene/diene, 5.4), in the depot fats of the ruminants studied, and in the wallaby, this ratio is shown to be considerably modified (triene/diene, 0.43–1.18). The dietary fat of the quokka differed from that of the other animals studied in containing greater proportions of diene than of triene acids (triene/diene, 0.19) and this ratio was not greatly modified (triene/diene, 0.29) in the depot fat.

The highest percentages of *trans*-acids were found in the fats of the quokka (21.0%) and the wallaby (18.1–19.2%). In contrast with these two marsupials, the remaining marsupial studied, the opossum, did not contain any detectable amount of *trans*-acids in its depot fat; nor did any of the other non-ruminants examined, except the pig.

Table 2. *The contents of di- and tri-unsaturated acids in animal fats as compared with those present in the diet*

Values expressed as % (w/w) of total fatty acids.						
Species	Fat	Conjugated		Non-conjugated		Reference
		Diene	Triene	Diene	Triene	
Pasture-fed animals and diet						
Rye-grass (<i>Lolium perenne</i>)	Glycerides	0.95	0.08	10.65	62.72	Shorland (1953)
(a) Miscellaneous						
Non-ruminants						
Wild rabbit	Abdominal	0.7	0.1	7.2	42.3	Shorland (1953)
Horse	Total fatty tissues	1.0	Trace	3.3	16.1	Shorland <i>et al.</i> (1952)
(b) Marsupials						
Wallaby	Perinephric	3.76	0.23	2.85	4.18	—
Wallaby	Perinephric	3.26	0.13	3.00	3.90	—
Ruminants						
Sambur	Stomach mesentery	0.57	0.04	1.07	1.89	—
Fallow deer	Perinephric	Trace	Trace	1.1	1.0	Shorland (1953)
Ox	Intermuscular	0.5	Trace	0.2	0.3	Shorland (unpublished work)
Sheep	External tissue	0.54	0.18	0.48	0.79	
Animals fed on special diets						
Quokka	Perinephric	2.9	0.37	6.37	2.08	—
Diet of quokka	Extracted fat	2.23	0.23	29.47	5.92	—
Opossum*	Perinephric	0.20	0.03	9.94	9.32	—

* Diet not known with certainty, but includes leaf material whose fat probably resembles that of pasture.

The relatively high iodine values (73.2–86.1) of the fats of the marsupials examined suggested that these animals also deposit appreciable amounts of the highly unsaturated acids of the diet, and this was confirmed by the results shown in Table 2.

The diet of the wallabies used in this work consisted mainly of pasture supplemented with tree foliage. The fatty acid composition of the pasture

The *trans*-acids found in small proportion (0.9%) in this animal could be attributed to the *trans*-acids in the residual butter-fat of the skim-milk diet. Without exception the fats of all ruminants so far examined contained substantial amounts (3.5–11.1%) of *trans*-acids.

In determining the origin of *trans*-acids in animal fats it is obviously important to know the

amounts of these acids in the diet. Examination of pasture and forage plants, including rye-grass, clover and rape, showed that the fatty acids, which form 2–3 % of the moisture-free tissues, contained from 0.6 to 2.2 % of *trans*-acids, and in the small amount of fat (4.6 % of the dry weight) present in the diet of the quokka no *trans*-acids were detected. It appears, therefore, that the *trans*-acids in the fats of ruminants and of the two marsupials examined (Table 1) arise mainly from some transformation within the animal subsequent to or during digestion. An examination of the lipids extracted from fresh rumen contents of pasture-fed sheep (Table 3) showed that the *trans*-acids formed a considerable part (8.3–10.5 %) of the total fatty acids.

They established that the lower fatty acids produced in the upper stomach were almost completely absorbed by the time the ingesta reached the fundus. In view of the similarity between the digestive processes of sheep and of the quokka, the presence of *trans*-acids in the depot fat of the quokka (Table 1) is therefore not unexpected. The high content of *trans*-acids in the perinephric fat of the wallaby might be similarly explained. On the other hand, *trans*-acids were not found in the depot fat of the opossum. The reason for this is uncertain, but whereas the quokka and the wallaby can deal with fibrous material, the opossum lives more on foliage and fruit and may not therefore possess a ruminant-like digestion.

The small amounts of diene and triene acids in

Table 3. *trans*-Acids present in the fatty acids of sheep-rumen contents

Date collected	No. of sheep	Approx. vol. of fluid (ml.)	Lipids		
			Unsaponifiable (g.)	Fatty acids (g.)	<i>trans</i> -Acids in fatty acids (% w/w)
29. i. 54	3	2000	4.09	8.12	10.5
14. iv. 54	7	9000	11.88	21.92	8.3

The presence of such high proportions of *trans*-acids in the rumen contents as compared with those of the dietary fat is in agreement with the earlier suggestion of Hartman *et al.* (1954) that the rumen is the site of formation of the *trans*-acids in ruminants. Referring to this work, Garton & Oxford (1955) have suggested that rumen bacteria might contain *trans*-acids. This is quite possible, but in view of the small quantities of bacterial lipids isolated by Garton & Oxford [0.04–0.09 g. of lipids (iodine value 23.8)/l. of rumen contents] as compared with the amounts of *trans*-acids reported in the present paper (0.2–0.4 g. of *trans*-acids/l. of rumen contents) most of these acids seem to be extraneous to bacteria, although strict comparison between the two experiments is not advisable because of the different diets. This is confirmed by our further experiments (Shorland, Weenink & Johns, unpublished work), in which linolenic acid incubated with filtered rumen fluid (low in fat content and free of *trans*-acids) was effectively hydrogenated with the formation of 15 % of *trans*-acids.

The occurrence of *trans*-acids in the depot fats of certain marsupials (Table 1) might appear not to conform to the hypothesis just mentioned. However, recent investigations by Moir, Somers, Sharman & Waring (1954) have established that the macropod marsupial, the quokka (*Sentoria brachyurus*) (and possibly other marsupials) possesses a ruminant-like digestion, the stomach contents containing a rich microbial population similar to but rather simpler than those of sheep.

the fats of ruminants, as compared with those of the quokka and the wallaby (Table 2), suggest that the hydrogenation processes are more extensive in ruminants than in these animals. It is conceivable that the conversion of diene and triene acids in the ruminants may proceed to a considerable extent as far as the saturated acid stage, so that the amounts of *trans*-acids formed would be correspondingly less than in the wallaby and the quokka, where the polyene acids have not been so completely hydrogenated. In ruminant depot fats the proportions of polyene acids are small (1–3 %), so that the *trans*-acids must be mainly monoene acids, as suggested by the work of Swern *et al.* (1952). However, a faint peak at 985 cm.⁻¹ was observed in the perinephric fats of the ox and the female goat, indicating the presence of traces of *trans-trans*-conjugated acids (cf. Jackson, Paschke, Tolberg, Boyd & Wheeler, 1952). The occurrence of small amounts of polyene *trans*-acids in butter-fat was also reported by Cornwell *et al.* (1953). No evidence was found in the present investigation of peaks at 982 and 948 cm.⁻¹, which shows the absence of *cis-trans*-conjugated systems. As mentioned earlier, there are considerable proportions of diene and triene acids in the depot fats of the quokka and the wallaby, so that the possible occurrence of *trans-trans*-unsaturated acids in these fats is not excluded.

In considering the influence of the dietary fat on the composition of the depot fats, Shorland (1952) has noted that the depot fats of ruminants are not

appreciably influenced by the nature of the dietary fat. The present work suggests that certain marsupials are not unlike ruminants in this respect, inasmuch as these animals also modify the dietary fat in the alimentary tract by hydrogenation of highly unsaturated acids.

SUMMARY

1. An examination of the depot fats of ruminants showed the presence of substantial amounts (3.5–11.2 %) of *trans*-acids.

2. The fats of non-ruminants were found to contain not more than 0.9 % of *trans*-acids, apart from those of certain marsupials, such as the wallaby and the quokka, in which the *trans*-acid contents varied from 18.1 to 21.0 %.

3. From the available evidence it appears that the *trans*-acids arise mainly from hydrogenation of dietary unsaturated acids by bacteria in the rumen, or in the rumen-like stomach in marsupials.

Investigations on the effects of rumen contents on unsaturated fatty acids is being continued, and it is hoped that the results of the investigations now in progress will be published shortly.

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Synthesis of Citrovorium Factor in Rats Given Chloretone

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Numerous clinical and experimental observations have indicated that ascorbic acid may function in certain anaemias (Vilter, 1947). Pernicious anaemia has responded to ascorbic acid therapy, and a deficiency of ascorbic acid favours the development of megaloblastic anaemia in monkeys and in human patients (May, Nelson, Lowe & Salmon, 1950; May, Sundberg & Schaar, 1950). Although pteroylglutamic acid does not minimize the need for ascorbic acid (Silverman & Mackler, 1951), megaloblastic

anaemia of scorbutic monkeys is alleviated by pteroylglutamic acid or, in smaller doses, by citrovorum factor (*N*⁵-formyltetrahydropteroylglutamic acid) (May, Sundberg & Schaar, 1950). Correction of scurvy with ascorbic acid abolishes megaloblastic anaemia without any administration of pteroylglutamic acid or citrovorum factor (May, Sundberg & Schaar, 1950). Dietary ascorbic acid is reported to promote growth in chicks and storage of pteroylglutamic acid in their livers