Branched-chain fatty acids in lipids of the newly born lamb

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SUMMARY The fatty acid compositions of the lipids of wool, carcass, liver, brain, and perirenal fat of two newly born lambs have been determined. The fatty acids from the lipids of carcass and internal organs of the lambs contained 0.2% of branched-chain acids, compared with 3% of branched acids in the depot fat of an adult sheep. This observation is presumed to reflect the low permeability of the placenta to maternal lipids. On the other hand, the fatty acid and α-hydroxy acid fractions from the wool wax of the lambs contained 62 and 44% of branched-chain acids respectively, compared with 80 and 54% of branched acids in the corresponding fractions from the wool wax of adult sheep. Since the rate of production of wool wax in the fetal lamb is as great as in the adult, relative to wool production, the results suggest that the branched acids are the product of the sebaceous glands.

Shorland and co-workers (1) found in the fatty acids from the depot fats of ruminant animals about 2% of branched-chain acids of the *iso* and *anteiso* series. These were shown to result from chain extension by rumen microorganisms of the low molecular weight branched acids formed by deamination of amino acids (2). The esterified fatty acids in wax from sheep's wool contain about 80% of branched acids which also belong to the *iso* and *anteiso* series (3, 4) and for which an origin from amino acids has likewise been postulated (5). It has not, however, been conclusively established whether the branched-chain acids of wool wax originate in the rumen like those in the depot fat, or whether they may be synthesized to some extent by the sebaceous glands.

Popják (6) concluded that the fetal rat and rabbit are almost entirely isolated from the lipids of the mother. If this were true for the sheep, the body fats of the fetal or neonatal lamb would be almost entirely devoid of branched-chain acids, and comparison of the fatty acid composition of lamb and sheep wool wax might indicate whether the latter has been influenced by lipids originating elsewhere than in the sebaceous gland.

EXPERIMENTAL METHODS

Origin of the Lambs

Lamb 1 was born in the field and was kept in good health without feeding until killed a day later. Lamb 2, born in an observation pen to a mother harnessed to prevent suckling, was killed several hours later without feeding. Both lambs were Merinos, as was the adult sheep which was maintained on pasture.

Extraction of the Lipids

Most of the wool was removed with clippers and the wax was extracted with light petroleum during 6 hr in a Soxhlet apparatus. The lambs were skinned and dissected and the various parts separately homogenized with 10 ml/g of chloroform–methanol 1:2 (v/v). The suspension was filtered, and the solids were successively extracted with similar volumes of chloroform–methanol 1:1 and hot chloroform. In each case the extracts were combined and washed with 30% aqueous ethanol, and the solvent was evaporated under reduced pressure.

Examination of the Lipids

(a) *Body Fats*

Glycerides were separated from sterols, sterol esters, and polar lipids by chromatography on Florisil, essentially as described by Carroll (7), except that pretreatment of the adsorbent was necessary in order to obtain reproducible results. It was suspended in water and acetic acid was added until the mixture was acid to bromocresol green. The solid was collected on a filter and washed twice with methanol by resuspension and filtration. After drying at

room temperature the solid was activated by heating at 240° for 4 hr and allowed to cool in a desiccator. It was brought to the desired activity by shaking with water, usually 10% by weight for the present purpose, and stored in a sealed container.

Elution of the lipid fractions was effected by increasing proportions of ether in hexane, as described by Carroll (7), except that final elutions were made with methanol–ether 1:4 and 1:1 and with pure methanol. The polar lipids eluted in this way were presumably principally phospholipids.

The fatty acids liberated from the triglyceride and polar lipid fractions on saponification were converted into methyl esters by refluxing with methanol–1% H_2SO_4 for 3 hr. Saturated and unsaturated methyl esters were separated by treatment with mercuric acetate (8), followed by chromatography on Florisil (10% water), from which the saturated esters were eluted with hexane and the mercury compounds with ether and ether–methanol. The unsaturated methyl esters recovered by treatment of the mercuric acetate adducts with 3% HCl in methanol were rechromatographed to remove polar contaminants. Portions of the samples of methyl esters were converted into hydrocarbons, first by reduction with $LiAlH_4$ in ether to mixtures of alcohols, which were then heated with iodine (2-fold excess) and red phosphorus (20% of the weight of iodine) for 1 hr at 100°. The resulting alkyl iodides were purified by percolation through alumina in hexane, then recovered, dissolved in ether, and reduced to hydrocarbons by addition of excess $LiAlH_4$ in ether. The products were recovered by addition of water, then dilute H_2SO_4, and evaporation of the ether layer after drying over Na_2SO_4.

In order that the hydrocarbon mixtures should be as free as possible of unreacted intermediates and by-products they were refluxed with ethanolic KOH for 30 min, then recovered in hexane, and chromatographed on alumina (activity I). The hydrocarbon mixtures recovered from the hexane eluates were then shaken with hydrogen and Adam's catalyst to reduce any olefinic compounds. The products thus obtained, usually in 80–90% yield, showed only saturated hydrocarbon absorption in the infrared spectrum.

Unsaturated methyl esters were hydrogenated by shaking with hydrogen and Adam's catalyst prior to their conversion to hydrocarbons.

(b) *Wool Waxes*

The samples of wool wax were examined by the method of Downing, Kranz, and Murray (4). This consisted of saponification of the wax by refluxing in 0.5 N ethanolic KOH for 1 hr, followed by dilution with 2 volumes of water and extraction of the unsaponifiable material into hexane. The aqueous layer was treated with excess calcium chloride solution and the precipitated calcium salts of the fatty acids were collected, extracted twice with hot acetone to remove any remaining unsaponifiable material, and then converted into the methyl esters of the fatty acids by refluxing for 3 hr with methanol–benzene–H_2SO_4 (50:50:1, v/v/v, 25 ml/g acids). The methyl esters were recovered by filtration to remove calcium sulfate, washing with 25% aqueous ethanol, and evaporation of the benzene layer. Equally good results were obtained by the use of dry HCl in place of H_2SO_4 in the methylation step, and the filtration step was then rendered unnecessary.

Samples of methyl esters of the wool wax acids were chromatographed on Florisil and the unhydroxylated esters which were eluted with hexane–benzene 9:1 were examined by gas–liquid chromatography (GLC) for the presence of unsaturated compounds.

The main portions of the methyl esters derived from wool wax acids were reduced with $LiAlH_4$ in ether to a mixture of α,β-diols, α,ω-diols, and monohydric alcohols (4). The mixture was allowed to stand in acetone containing one drop of H_2SO_4 for 2 hr in order to convert the α,β-diols into their isopropylidene derivatives, the solution was then made alkaline with ethanolic KOH and the solvent evaporated. The residue was taken up in hexane and chromatographed on alumina (Woelm, neutral, activity III) from which the isopropylidene derivatives of the α,β-diols were eluted with hexane, the monohydric alcohols with chloroform–benzene 1:2 and the α,ω-diols with chloroform–ethanol 2:1. A sample of the α,β-diol ketals was examined by GLC for the presence of unsaturated compounds.

The α,β-diols were recovered by treatment of the ketals with ethanol containing one drop of HCl, followed by evaporation of the solvent on the steam bath. The α,β-diols and the monohydric alcohols were converted into the corresponding mixtures of hydrocarbons by the method described in (*a*) above. The diols derived from the ω-hydroxy acids were not examined.

(c) *Brain Hydroxy Acids*

The α-hydroxy acids from the brain lipids were examined as described previously (9).

Gas–Liquid Chromatography (GLC)

Gas–liquid chromatographic examinations were carried out with the apparatus and methods previously described (4) and also with an F and M Scientific Corporation Model 609 instrument with flame ionization detector. Columns were 0.17 in. (i.d.) by 12 ft, packed with 10% SE-30 silicone elastomer (Griffin and George Ltd. London) on Celite 545 (60–80 mesh). The straight-chain components in the mixtures of methyl esters derived from body fats were determined by chromatog-

TABLE 1 YIELDS OF LIPID FROM TISSUES OF NEONATAL LAMBS

Section	Field-Born Lamb (Lamb 1) Wt of tissue*	Field-Born Lamb (Lamb 1) Wt of lipid	Pen-Born Lamb (Lamb 2) Wt of tissue*	Pen-Born Lamb (Lamb 2) Wt of lipid
	g	*g*	*g*	*g*
Wool	15.1	1.75	13.6	1.4
Heart and lungs	182	5.0	182	4.75
Liver	72.5	1.9	89	2.1
Intestines	200	5.5	—	—
Brain	78.4	4.6	62	3.55
Perirenal tissue	23.0	5.8	27	9.1
Carcass	1610	18.8	1920	38.7

* Wet weights of tissue are given except for the wool, which was allowed to dry in air at room temperature. The field-born lamb weighed 3.5 kg, the pen-born 4.3 kg.

raphy at 240° of the saturated and unsaturated fractions separated by the mercuric acetate treatment and the unsaturated methyl esters were also chromatographed on a column of ethylene glycol adipate at 175° for examination of the polyunsaturated esters. The methyl esters of the unhydroxylated acids of wool wax, and the isopropylidene derivatives of the α,β-diols derived from the wool wax α-hydroxy acids, were chromatographed on the SE-30 column at 280° and with a linear temperature program from 150–285°.

For examination of the branched-chain compounds the hydrocarbon mixtures derived from the methyl esters derived from body fats were chromatographed at 220° with a 6 × 0.17 in. column of Linde Molecular Sieve 5A inserted before the main column. This was preconditioned by passage of nitrogen at 260° for several hours, followed by repeated 5μl charges of hydrocarbon derived from wool wax acids (4) until a constant composition for the hydrocarbon mixture was registered by the detector. The sieve column then absorbed every trace of normal hydrocarbons and allowed the branched-chain hydrocarbons to be estimated, both as regards relative amounts of each chain length in an individual sample and for comparison of the total amount of branched compounds in each sample. This method was not relied upon for determination of the absolute amount of branched compounds in the samples. This was determined for the methyl esters of the saturated fatty acids from perirenal fat of an adult sheep by means of the apparatus incorporating a density meter. The hydrocarbons derived from these esters were then used as a standard for comparison with the hydrocarbon mixtures derived from the lamb fats, using the F and M instrument and the molecular sieve. Such comparisons were made at a constant combination of instrument settings, the most suitable for 1 μl charges being range 10, attenuation 64.

The identities of components in the mixtures chromatographed were inferred from comparison of retention times with those of authentic compounds (4) chromatographed either simultaneously or as soon as possible before or after, and assignments of identity were checked by graphs of the logarithm of retention time against the number of carbon atoms per molecule.

TABLE 2 GENERAL COMPOSITION OF LIPIDS FROM TISSUES OF NEONATAL LAMBS

Component	Weight Per Cent of Total Lipid: Lamb 1 Carcass	Lamb 1 Liver	Lamb 1 Perirenal	Lamb 2 Carcass	Lamb 2 Liver
N	1.00	2.06	1.29	—	—
P	1.56	2.16	0.78	—	—
Sterol esters	2	4	—	1.5	3
Triglycerides	44	2	72	52	13
Sterols	8	3	1	6	8
Polar lipids	46	91	27	40	75

RESULTS

The yields of lipid obtained from various parts of the lambs are shown in Table 1. Considerable differences existed between the weights of certain tissues in the two animals and in the yields of lipid from some tissues. The lower yields of lipid from the carcass and perirenal tissue of lamb 1 probably resulted from the longer time for which it had remained alive without feeding. However, the smaller brain weight of the second animal reflects individual variation, as the skull was noticeably smaller. In neither animal were significant reserves of fat deposited and the only depot which could be dis-

TABLE 3 FATTY ACID COMPOSITION OF LIPIDS OF NEWLY BORN AND ADULT SHEEP (WEIGHT PER CENT)

Fatty Acid	Lamb: Carcass Glycerides	Lamb: Carcass Polar Lipids	Lamb: Liver Glycerides	Lamb: Liver Polar Lipids	Lamb: Perirenal Glycerides	Adult Perirenal Fat Glycerides
Saturated C_{14}	0.5	0.5	1.6	1.2	0.5	2.4
C_{15}	0.2	0.2	0.2	1.4	0.2	1.0
C_{16}	25	18	24	27	18	17
C_{17}	0.3	0.2	0.2	0.8	0.2	2.3
C_{18}	15	16	12	21	19	30
C_{19}	0.1	0.4	—	0.4	0.1	0.5
Branched (total)	0.2	0.2	0.3	0.4	0.2	3.0
Total saturated	41	35	38	52	38	56
Unsaturated C_{14}	0.4	0.2	0.4	0.3	0.2	0.05
C_{15}	—	—	0.1	—	—	0.1
C_{16}	2.0	2.8	6.6	7.2	1.4	2.2
C_{17}	0.3	0.2	0.4	1.0	0.2	0.6
C_{18}	57	53	54	31	61	40
C_{19}	—	8	—	8	—	—
Branched (total)	—	—	—	—	0.015	0.05
Total unsaturated	60	64	61.5	47.5	63	43

cerned was in the perirenal tissue, in which the proportion of lipid was very much lower than in similar tissues from adult animals. Data indicating the amounts of various classes of constituents in the lipids obtained from carcass, liver, and perirenal tissue for the two lambs are shown in Table 2, where the amount of the "phospholipid" fraction includes polar lipid unrecovered from Florisil chromatography. The fatty acid compositions determined for triglycerides and polar lipids from these tissues are given in Table 3. The figure of 3% for branched acids in adult sheep fat is in agreement with previous findings (1, 2). Where the proportions of branched-chain acids were extremely low, as in the case of the unsaturated acids from lamb and sheep body fats (Table 3) it was difficult to determine the absolute amounts of branched acids, and these two figures are subject to considerable uncertainty, possibly as much as ±50%. The results for the two lambs were very similar and the mean values are reported. It was noticed that in the lamb body fats the branched-chain acids were of greater average chain length than those in the body fat of the adult sheep (Table 4). The acids from the fat of the adult sheep contained 0.5% of a component which from its retention time appeared to be the 3,7,11,15-tetramethylhexadecanoic acid found in ruminant lipids (14, 15). This acid was not detected in the lamb fat, and no other multibranched acids were detected in any of the mixtures examined. Polyunsaturated acids were present only in trace amounts in the glycerides and are not shown. However, the C_{20}, C_{22}, and C_{24} compounds amounting to about 8% in the polar lipids consisted principally of this class of acids, the C_{20} group being composed almost entirely of the trienoic and pentaenoic acids.

The samples of wool wax obtained from the lambs were similar to wax from adult sheep in that upon saponification they gave approximately equal quantities of long-chain acids and unsaponifiable material. The acid fractions contained the same three classes of long-chain acid as have been found in the adult wax, and in similar proportions, namely α-hydroxy acids (40%), ω-hydroxyacids (5%), and unhydroxylated acids (55%).

TABLE 4 CHAIN-LENGTH DISTRIBUTION IN THE BRANCHED-CHAIN FATTY ACID FRACTIONS OF PERIRENAL FAT GLYCERIDES OF NEWLY BORN AND ADULT SHEEP (WEIGHT PER CENT)

Acid	Lamb	Adult
C_{14}	0.6	3.4
C_{15}	8.4	30
C_{16}	3.1	12
C_{17}	54	47
C_{18}	20	6.7
C_{19}	13	0.7

TABLE 5 COMPOSITION OF UNHYDROXYLATED FATTY ACIDS FROM WOOL WAX OF NEWLY BORN LAMBS AND ADULT SHEEP (WEIGHT PER CENT)

	Normal			Branched		
Acid	Lamb 1	Lamb 2	Adult	Lamb 1	Lamb 2	Adult
C_{13}	–	0.06	0.05	–	0.5	0.4
C_{14}	0.8	3.5	1.4	0.5	1.8	2.6
C_{15}	1.5	0.6	1.1	1.6	3.9	7.3
C_{16}	6.4	6.5	3.0	4.6	4.1	6.7
C_{17}	0.6	0.3	0.3	2.1	1.8	3.9
C_{18}	4.0	2.9	1.7	3.7	2.9	4.5
C_{19}	0.3	0.2	0.2	2.8	4.7	5.6
C_{20}	2.0	1.8	0.9	5.8	4.7	5.6
C_{21}	0.3	0.1	0.2	2.8	3.0	5.6
C_{22}	2.1	1.8	1.1	2.4	1.9	2.6
C_{23}	0.6	0.3	0.3	1.6	1.8	2.8
C_{24}	9.8	8.6	3.5	6.7	3.8	3.5
C_{25}	0.3	0.4	0.3	7.0	7.4	6.4
C_{26}	7.0	7.1	2.4	10	7.1	5.6
C_{27}	0.3	0.1	0.2	7.0	5.9	6.4
C_{28}	1.4	2.9	1.1	2.6	2.4	2.5
C_{29}	–	–	0.8	1.0	2.4	3.5
C_{30}	–	0.7	0.3	0.1	0.6	1.4
C_{31}	–	–	–	–	1.2	3.1
Total	37	38	19	62	62	80

The α-hydroxy and unhydroxylated acids each contained both straight-chain and branched-chain compounds, but no detectable amounts of unsaturated acids, again as in the wax of adult sheep. The results obtained for the compositions of the unhydroxylated acids and α-hydroxyacids are shown in Tables 5 and 6, where they are compared with similar fractions from adult sheep. The ω-hydroxy acids were not examined in detail in the present work. The unhydroxylated acids from the wool waxes of the two lambs contained almost identical total amounts of branched-chain acids, although there were considerable differences in the proportions of individual constituents. This appears to

TABLE 6 COMPOSITION OF α-HYDROXY ACIDS FROM WOOL WAX OF NEWLY BORN LAMBS AND ADULT SHEEP (WEIGHT PER CENT)

	Normal			Branched		
Acid	Lamb 1	Lamb 2	Adult	Lamb 1	Lamb 2	Adult
C_{14}	1.8	1.0	2.3	0.06	0.5	0.5
C_{15}	1.0	1.0	0.9	0.16	0.2	1.1
C_{16}	46	42	40	1.6	1.5	1.4
C_{17}	1.8	2.0	0.7	0.2	0.2	0.4
C_{18}	6.1	7.2	2.1	25	28	24
C_{19}	0.1	0.1	0.1	2.2	2.4	3.1
C_{20}	0.2	0.2	0.3	1.8	1.8	3.2
C_{21}	–	0.05	0.1	0.5	0.6	3.6
C_{22}	0.2	0.2	0.3	2.4	2.5	3.6
C_{23}	0.1	0.1	–	1.6	1.5	5.2
C_{24}	0.7	0.7	0.2	4.8	5.0	5.8
C_{25}	–	0.1	–	1.6	1.5	2.7
Total	58	55	47	42	46	53

be characteristic of wool wax acids, as it was quite a marked feature of the waxes from a number of lambs of the same strain maintained for several months after birth on the same diet of reconstituted cows' milk (D. T. Downing, unpublished results).

The α,β-diols derived from the α-hydroxy acids from the brains of lambs 1 and 2 amounted to 99 and 65 mg respectively, compared with 112 mg per brain from a pooled sample of brains from adult sheep (9). The chain-length compositions determined for the hydrocarbons derived from the brain α-hydroxy acids are shown in Table 7. No branched constituents were detected in these hydrocarbons.

TABLE 7 COMPOSITION OF α-HYDROXY ACIDS FROM BRAIN (WEIGHT PER CENT)

Acid	Lamb 1	Lamb 2	Adult
C_{16}	2.6	1.5	0.8
C_{17}	0.5	0.4	0.8
C_{18}	15	14	6.5
C_{19}	0.4	0.2	0.6
C_{20}	1.3	1.2	2.0
C_{21}	0.1	0.1	2.5
C_{22}	23	22	19
C_{23}	4.4	5.0	12
C_{24}	47	49	44
C_{25}	1.3	1.9	5.5
C_{26}	5.2	5.1	6.0

DISCUSSION

It is apparent from Table 3 that the concentration of branched acids in the body fat glycerides of neonatal lambs is only about 10% of that in the fats from adult sheep. This is in accordance with the hypothesis of the exclusion of maternal lipids by the placenta as discussed by Popják (6), with the result that a very high proportion of the lipid in the fetus is material which has been synthesized there de novo. In spite of this effect the composition of the fatty acids from the wool wax of the lambs was similar to that in the adult sheep, and the total concentration of branched acids in both α-hydroxy acid and unhydroxylated acid fractions of lambs' wool wax was 80% of the corresponding figure for wool wax of adult sheep. It would therefore seem that exogenous lipids do not have a great effect on the fatty acid composition of wool wax.

Although the proportion of branched acids in the body fats of the lambs was much smaller than in mature animals it was not as small as anticipated from the work discussed by Popják. This might be ascribed to a number of causes, including poor screening efficiency of the placenta for branched acids, species difference in placental screening efficiency, or increased permeability of the placenta at the end of the gestation period. That branched- and straight-chain acids can be differentiated in metabolic processes is indicated by the work of Gerson et al. (10). Apparently the microbial lipids absorbed from the gut are devoid of branched-chain unsaturated acids (2), and the very low proportion of these in the body fats suggests that the saturated branched-chain acids, unlike the normal acids such as stearic, are resistant to desaturation in animal tissues.

As shown in Table 4 the relative proportions in the various chain lengths of the branched acids from body fats were somewhat different between the newly born and adult animals. It is presumed that this is due to differences in metabolism of the branched acids according to chain length, either as it affects the screening efficiency of the placenta or the fate of the branched acids that reach the fetus.

In an earlier study of the α-hydroxy acids from sheep brain (9) it was noted that these acids contained little or no branched compounds, although the general pool of fatty acids available in the animal contains 2–3% of branched acids. The lack of branched acids in brain cerebroside hydroxy acids could have been due to near completion of myelination at the time of birth, with the consequence that the cerebrosides would have been formed when branched acids were presumed to be virtually absent. However, branched acids were readily detected and estimated in other tissues from the newly born lambs. Furthermore, an appreciable part of the brain cerebrosides is apparently laid down after birth, when an even greater proportion of branched acids will be present in the system. This non-incorporation of branched acids into the brain cerebrosides would seem to support the conclusion of Fulco and Mead (11) that the fatty acids of brain cerebrosides are synthesized largely or entirely de novo from acetate and do not incorporate preformed long-chain fatty acids from the general metabolic pool.

Hajra and Radin (12) have shown that a higher proportion of acids of odd carbon number are present in rat brain cerebrosides in mature animals than in the young. This is now seen to be the case also with the hydroxy acids from sheep brain, and is presumed to be the result of progressive decarboxylation of the hydroxy acids with age, accompanied by rehydroxylation (13).

The author is indebted to Dr. A. T. Dick, C.S.I.R.O. Division of Animal Health, and to Messrs. A. R. Culvenor and J. S. Ebery of Sandon, Victoria for supplying the lambs. N and P analyses were performed by Dr. K. W. Zimmermann, C.S.I.R.O. and University of Melbourne Microanalytical Laboratory.

Manuscript received July 26, 1963; accepted December 9, 1963.

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