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Occurrence of 2-hydroxy fatty acids in animal tissues*

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SUMMARY

The contents of 2-hydroxy fatty acids in rat spleen, kidney, lung, sciatic nerve, and skin, and in bovine plasma, were determined by a copper precipitation method, and the proportions of the various hydroxy acids were determined by gas-liquid chromatography. It was observed that there are two groups of hydroxy acids, short chain (16h:0 as major component) and long chain (24h:0 as major component). The relative proportions of the two groups varied with the tissue. No 2-hydroxy acids could be found in liver and epididymal fat. The 2-hydroxy acids of brain are not liberated by mild alkali and therefore occur only in amide linkage. The amide-bound fatty acids of brain gangliosides and sphingomyelin account for none, or only a very small fraction, of the total brain hydroxy acids.

Only in a few lipids are 2-hydroxy fatty acids known to occur, those found in animal central nervous system, wool fat, and certain microbial organisms (1, 2, 3). Cerebroside and cerebroside sulfate are the best characterized of the lipids containing the 2-hydroxy acids, and both lipids have been claimed to occur in many tissues. Moreover, various types of glycolipids apparently have been found in every tissue thus far examined. It therefore seemed possible that 2-hydroxy acids also are widely distributed.

We have recently developed a highly sensitive and specific method for isolating, determining, and characterizing the 2-hydroxy acids, even in the presence of large amounts of the ordinary acids (4). The method is based on the ability of 2-hydroxy fatty acids to form very insoluble copper chelates, which can then be analyzed by gas-liquid chromatography (GLC) for the individual hydroxy acids, and by colorimetry for the copper content. This paper describes the application of the method for analysis of various rat tissues and bovine plasma. It also presents evidence that the brain hydroxy acids are bound primarily to cerebroside and cerebroside sulfate.

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EXPERIMENTAL METHODS

Tissue Hydroxy Acids. Liver, kidney, lung, spleen, and epididymal fat were obtained from seven mature rats, and the tissues were pooled and extracted with chloroform–MeOH 2:1 (5). Cleavage with MeOH–HCl, saponification, removal of nonsaponifiable materials, esterification, and isolation of the polar esters by Florisil chromatography were carried out as described before (6). The polar esters were resaponified and the free acids were treated with cupric oleate (0.1 ml) and absolute EtOH (0.3 ml) as described in the accompanying paper (4). The copper content of the precipitate was determined by titration and the hydroxy acids were analyzed, as acetoxy methyl esters, by GLC on silicone and polyester columns. Identification of the peaks was made by comparison with standards and by comparison of the peak positions on both columns.

The skin sample was obtained, with the accompanying hair, from the abdomen of a 285-g male rat. The extraction was carried out by refluxing with 20 volumes of chloroform–MeOH under magnetic stirring for 2 hr, then leaving at room temperature over night. In this case, the lipids were treated by the short procedure (4), involving high temperature saponification with 15 ml of KOH–propylene glycol, and purification with a 3.5-g silica gel column. The column was eluted

with more benzene than usual (30 ml/g silica gel) to remove as much cholesterol and normal acids as possible before elution of the polar acids.

Sciatic nerves were obtained from two male rats, weighing about 470 g. The nerves were dissected out from their origin to the knee, including the branches to the thigh. Twenty grams of lyophilized bovine plasma[1] was homogenized with 10 ml water and 200 ml chloroform–MeOH. Both these lipid samples were processed like the skin lipids, but only 1 g silica gel was used.

The Ester-Linked Fatty Acids. The lipids from 30 g of pig brain were saponified by stirring with 1 liter of 1 N NaOH for 16 hr at 37°. Such mildly alkaline conditions have been shown to cleave ordinary esters while leaving sphingomyelin (7, 8) and cerebroside/cerebroside sulfate (9) unaffected. After acidification, the lipids were partitioned by adding chloroform–MeOH. The lipids in the chloroform layer were made alkaline and were extracted to remove the nonsaponifiable materials. The resultant soaps were esterified and the esters treated as with the rat liver.

Analysis of Lipids Free of Phosphatide-Ganglioside. One interesting question is whether 2-hydroxy acids occur in brain in lipids other than the known forms, cerebroside and cerebroside sulfate. This was answered in part by analyzing the total lipids of rat brain and lipids that were passed through a column of Florisil, which removes gangliosides and sphingomyelin. A 1.93-g rat brain was extracted and duplicate aliquots of the extract were analyzed directly by the short method (4). Additional duplicate aliquots were evaporated to dryness and, in chloroform–MeOH 2:1, were passed through columns containing 50 mg Florisil/mg crude lipids. Elution was carried out with the same solvent, 25 ml/g Florisil. The lipids were then analyzed as above for hydroxy acids.

Other details of methodology were as described in the accompanying paper (4).

RESULTS

Distribution of 2-Hydroxy Acids. Table 1 shows the results obtained by the copper analysis method. It is evident that hydroxy fatty acids do exist in several tissues, but not in all. Liver and epididymal tissue did not yield precipitates with the copper reagent. In the conditions employed here, the lower limit of sensitivity for precipitation was probably around 0.05 μmole (on the basis of experiments with 18h:0), so it may be estimated that less than 0.003% of hydroxy acid exists in liver fatty acids and even less than that in epididymal fat. The negative finding for liver was also obtained by examining the polar acids from a Florisil separation.[2] Lead tetraacetate oxidation of these acids did not give rise to any aldehydes; this, too, is a very sensitive test but somewhat more inconvenient.

Small but definite amounts of 2-hydroxy acids are found in the other non-nervous tissues. The highest concentration per gram of tissue is observed in skin, while the highest concentration per gram of fatty acids is found in spleen. The low concentration in skin lipids reflects the large amount of attached adipose tissue, which is presumably, like epididymal fat, free of 2-hydroxy acids. It is likely that the sebaceous fat in the skin, and the lipids adhering to the hair, are rich in 2-hydroxy acids. Wool fat is very high in such acids (10), although investigators of human skin (11, 12) and mouse skin (13) lipids did not indicate that these occur. Using a rough guess of 27 g for the weight of skin on a 285-g rat, and our observed figure of 0.405 μmoles hydroxy acid/g of skin, we estimate the total skin content per rat to be about 11 μmoles, a figure very close to that for brain.

TABLE 1.
CONTENT OF 2-HYDROXY ACIDS IN VARIOUS TISSUES

Tissue	Weight of Fatty Acids Analyzed	Amount of 2-Hydroxy Acids Found: Total Amount Isolated		Amount per g of Tissue	Amount per Rat	Percent of Total Acids‡
	mg	*mg*	*μmoles*†	*μmoles*	*μmoles*	
Brain	407	31.8	87.9	7.14	12.6	5.9
Liver	593	0.0	0.0	0.0	0.0	0.0
Epididymal fat	8621	0.0	0.0	0.0	0.0	0.0
Spleen	65	0.4	0.96	0.235	0.137	0.41
Lung	293	0.6	2.01	0.192	0.288	0.19
Kidney	406	1.5	4.35	0.280	0.622	0.29
Sciatic nerve	54*	2.2	2.61	13.6	1.3	1.3*
Skin	1504*	1.4	4.02	0.405		0.07*
Plasma	1384*	0.4	0.80	0.003		0.016*

* In these cases, the fatty acids were not isolated. Weight refers to total lipids. The percentages were calculated from the total lipids and are therefore lower, comparatively, than the other figures in the last column.

† Based on copper content in chelate.

‡ Calculated on a molar basis, assuming average molecular weight of total fatty acids is 275.

The data for sciatic nerve show that it contains 2-hydroxy acids and that the concentration per gram of tissue is even higher than in brain. If all peripheral nerves are similar in these respects, the peripheral system must rank close to skin and brain in total content of 2-hydroxy acids per rat. The possibility must be considered that the hydroxy acids of spleen, kidney, lung, and skin are present only in the nerves present

[1] Pentex, Inc., Kankakee, Ill. This amount corresponds to about 235 ml plasma.

[2] Hajra, A. K., and N. S. Radin, unpublished experiments.

in those organs. However, it is unlikely that this site is the only one since (1) liver contains no detectable amount, yet contains nerves; (2) plasma contains no nerves, yet contains hydroxy acids; and (3) the distribution pattern of the individual acids varies somewhat with the tissue (see next section).

Distribution Patterns of the 2-Hydroxy Acids. The proportions of the individual acids in each tissue (except brain) are shown in Table 2.

TABLE. 2 PERCENTAGE COMPOSITION OF 2-HYDROXY ACIDS OF VARIOUS TISSUES

Fatty Acid	Sciatic Nerve	Spleen	Kidney	Lung	Plasma	Skin
10h:0	tr*			tr		0.4
11h:0	tr					0.6
11h:1						0.03
12h:0	0.3	0.1		0.2	tr	0.08
12h:1	tr				0.6	0.1
13h:0	tr	0.4	0.9	0.5		1.0
14h:0	0.7	0.3	0.1	0.08	0.5	0.6
14h:1						0.2
15h:0	tr	0.08	0.08	0.1	0.7	0.8
15h:1	tr				tr	1.7
16h:0	0.3	0.5	1.9	7.5	17.3	36.5
16h:1					tr	12.2
17h:0	0.3	0.1	0.3	0.5	4.6	5.2
17h:1				0.2		1.5
18h:0	0.5	1.2	1.6	3.5	10.8	3.7
18h:1			0.3	0.6	0.7	1.9
19h:0	0.2	0.1	0.1	0.5	1.5	0.3
19h:1						0.4
20h:0	0.7	1.9	5.9	4.2	1.6	0.7
20h:1	0.3					0.4
21h:0	0.2	0.5	1.6	1.6	1.0	tr
21h:1	0.1	0.1				tr
22h:0	27.2	19.1	26.4	20.7	9.7	2.8
22h:1	0.7	0.5				0.2
23h:0	4.6	11.0	20.4	17.7	17.4	1.4
23h:1					0.4	0.5
24h:0	56.8	47.5	35.0	35.4	26.3	15.4
24h:1	7.2	10.8	3.2	3.6	6.2	2.3
25h:0	tr	2.5	1.4	1.7	0.7	2.0
25h:1	tr	0.4	0.2	0.6		0.4
26h:0	tr	1.2	0.3	0.5	0.2	2.7
26h:1	tr	2.1	0.4	0.5		0.4

* tr = trace, a barely visible peak.

A general observation can be made that there seem to be two groups of hydroxy acids—those clustering around 24 carbon atoms in length, and those clustering around 16 carbon atoms. Sciatic nerve, spleen, lung, and kidney are primarily in the cerebronic acid group and resemble brain in that respect (4, 6, 14). Like brain of mature animals, all these tissues contain considerable proportions of 23h:0. It would be interesting to determine whether the content of 23h:0 rises with age, as in brain (14). Curiously enough, the tissue most like brain anatomically (sciatic nerve) contains the least 23h:0. Sciatic nerve also differs from the other tissues in having only traces of the 25- and 26-carbon homologs. This finding is similar to that of Baker (15), who found that the ester-linked fatty acids of sciatic nerve had a lower average molecular weight than those of brain.

The data for plasma and skin show the presence of high concentrations of 16h:0 and adjacent acids, although cerebronic acid and its neighbors are still major acids. Indeed, plasma has a very high content of 23h:0. Lung seems to fall between the two extremes of skin and sciatic nerve, showing 7.5% 16h:0 and 3.5% 18h:0. Skin has the greatest variety, particularly in unsaturated acids (compare 16h:1) and odd-numbered acids (15h:0, 15h:1, 17h:0, 17h:1, 25h:0). Downing et al. (10) also reported a high proportion of shorter-chain 2-hydroxy acids in wool fat, together with a very high content of branched chain acids. We found no branched chain acids in skin but there is an unidentified peak (3.8%), which is near 13h:0 on DEGS and 15h:0 on SE-30. It is likely that the high branching in wool fat is characteristic of ruminant animals, which absorb large amounts of odd-numbered and branched-chain acids from the bacteria in their digestive tracts (16). Another factor to consider is that wool fat is a secretion, whereas we analyzed whole skin. The *normal* acids of skin may contain small amounts of branched acids (11–13).

The GLC curves for lung and sciatic nerve were distinctive in that a rapid elution of material was observed just after injection of the samples. The eluted material came out over a period of several minutes showing little by way of peaks, and the base line was approached only gradually. This material did not, however, interfere with the determination of the esters. Another observation was that the weight of the 2-hydroxy esters of sciatic nerve was higher than that expected from the copper determination. It would appear from these findings that these tissues contain a polar lipid, which is precipitated during chelate formation but which does not form a copper derivative. The acetoxy methyl esters of sciatic nerve, skin, lung, kidney, and pig brain were examined by thin-layer chromatography using Skellysolve B–absolute ether 85:15 (17), as described before (4). All five samples gave strong spots with R_f 0.36 for the acetoxy esters, as well as very faint spots at the origin and with R_f 0.53. However, a strong spot was also found with R_f 0.79 in the case of sciatic nerve, a weaker spot from lung, less in kidney, and none in the others.

The two major spots from sciatic nerve were scraped off the glass plate and extracted with ether (17). The ether was washed once with water and the lipid portion examined by GLC. The material with R_f 0.36 gave the same 2-hydroxy acid pattern observed with the original sample, while the material with R_f 0.79 was shown to be the source of the indistinctly eluted lipid. Presumably the indistinct elution is the result of decomposition during chromatography. The material with R_f 0.79 is not a mixture of normal esters, as these have a higher R_f in this solvent system. It is not likely that the unknown material is an artifact of high-temperature saponification, which was used for the sciatic nerve sample, as the lung sample was obtained by HCl–MeOH cleavage and low-temperature saponification, yet also showed some of the material. The other tissues, which were treated like lung, contained negligible amounts, so it would appear that sciatic nerve and lung are characterized by the unknown, slightly polar substance.

Linkage of the 2-Hydroxy Acids of Brain. The ester-linked acids of brain, obtained as described under Experimental Methods, did not yield a precipitate with cupric oleate. Thus it would appear that the 2-hydroxy acids in brain occur bound only to amino groups, presumably those of sphingosine. The known sphingolipids of brain are cerebroside, cerebroside sulfate, ceramide (18), sphingomyelin, and various gangliosides and other ceramido-polysaccharides. The first three of these are eluted from Florisil (containing 7% added water) by chloroform–MeOH 2:1, so we have a simple method for a preliminary examination of the distribution of hydroxy acids in the sphingolipids. The total 2-hydroxy acid content of a single rat brain (1.93 g) was found to be 19.7 μmoles, while the acid content in the Florisil effluent from the same rat was 19.4 μmoles, 1.5% less. This difference is in the range of variation in the method, so it appears that the sphingomyelin and ceramido-polysaccharides contain very little or no 2-hydroxy acids. It should be added that thin-layer chromatography of the Florisil effluent with chloroform–MeOH–water 24:7:1 (19) showed that sphingomyelin and ganglioside were absent, but there was a trace of lipid in a position corresponding to cephalin. O'Brien and Rouser have recently reported finding hydroxy acids in gangliosides and sphingomyelin (20), but the amounts present must constitute a very small fraction of the amount in total brain.

The 2-hydroxy acids of wool fat appear to be ester-linked. The mode of linkage in other tissues is yet to be elucidated.

DISCUSSION

The data in this paper show that 2-hydroxy acids are more widely distributed in animal tissues than was indicated by previous work, yet their absence from liver and epididymal fat is correspondingly thought-provoking. Polar acids do exist in liver, as shown by Florisil chromatography, but their hydroxyl groups are located past the 2-position. A branched-chain hydroxy acid was isolated from liver incubated *in vitro* with labeled mevalonic acid (21), but apparently this has not yet been found to occur naturally. At least one of the naturally occurring polar acids of liver contains a carbonyl group.[2] Prostaglandin E is a dihydroxy monoketo fatty acid found in seminal plasma and possibly lung (22).

Acids that seem to be polar have been reported in two sphingolipids, red cell globoside (23) and tumor cytolipin H (ceramido-lactose) (24), but these have not been identified as 2-hydroxy acids. We have also found a polar acid in preparations of Gaucher cerebrosides from spleen, but these, too, do not seem to be 2-hydroxy acids.

The occurrence of two clusters of 2-hydroxy acids in skin, lung, and plasma suggests that there are two enzyme systems involved in their biosynthesis—one specializing in 24h:0 and its neighbors, and one specializing in 16h:0 and its neighbors.

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