J. Lipid Research, April, 1961
Volume 2, Number 2

The lipid residues in cytolipin H*

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[Received for publication September 29, 1960]

SUMMARY

Analyses were done of the individual fatty acids in two preparations of cytolipin H and the lipid bases in one preparation. A number of fatty acids were present, lignoceric acid predominating. No 2-hydroxy fatty acids were present. Of the lipid bases, 93% was sphingosine and 7% was a more unsaturated residue (dehydrosphingosine?). No dihydrosphingosine was present. Analyses of a number of cytolipin preparations showed them to be similar to cerebrosides derived from nonnervous animal tissues in having a nonuniform distribution of normal saturated fatty acids: C_{24}, C_{22}, and C_{16} acids were present in high concentration, and C_{20}, C_{18}, and C_{14} acids were present in very low concentration.

On the basis of elementary and group analyses, cytolipin H, a lipid hapten isolated from human epidermoid carcinoma, was reported to contain fatty acid, a lipid base, glucose, and galactose in equimolar proportions (1). The carbohydrate residues were recently found to have the configuration of the disaccharide 4-O-β-D-galactopyranosyl-D-glucopyranose (lactose) (2). However, although evidence was presented indicating the presence of both fatty acid and a long-chain base, the lipid residues were not identified. A close correspondence of the carbon content of cytolipin H with that calculated for a molecule containing cerebronic acid and sphingosine led to the suggestion that these residues did indeed constitute the lipid portion of the cytolipin H molecule. It was subsequently found that 2-hydroxy fatty acids were absent. Scarcity of material prevented a conventional solution to this problem at the time, and micromethods had to be developed that would permit analysis of the fatty acid content of the original sample. A second specimen of cytolipin H was analyzed with respect to both its fatty acids and the long-chain base. These studies are presented in this report.

* This work was supported by grants from the National Science Foundation, the American Cancer Society, the United States Public Health Service (Research Grant C-2316 from the National Cancer Institute), and Interdisciplinary Grant 2M-6418 from the National Institute of Mental Health.

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METHODS AND MATERIALS

Cytolipin H was isolated from a human tumor (1). Analyses for this preparation and a second one isolated independently have been presented (1, 3). Fatty acid analysis was carried out, after hydrolysis and isolation, by paper chromatographic methods (4) and gas-liquid partition chromatography (5, 6). The analytical method for sphingosine and its derivatives was based on the formation of long-chain aldehydes followed by their separation using gas-column chromatography (6).

RESULTS

These studies were begun assuming that 2-hydroxy fatty acids were present in the cytolipin H molecule.

Fatty Acids in the Original Specimen. The material (13.7 mg) was hydrolyzed with 8 ml of 1.2 N HCl in 90% aqueous ethanol by heating under reflux for $2^1/_2$ hours. After neutralizing to pH 2.0, the ethanol was removed under a stream of nitrogen. Water (4 ml) was added and the acids and esters were extracted with five 10-ml portions of ether. After evaporating the ether, the residue was refluxed with aqueous 1.3 N NaOH. The hydrolyzate was brought to pH 2.0, extracted immediately with ethyl ether, and the ether was washed with water to avoid polymerization of hydroxy fatty acids. Evaporation of the solvent under nitrogen yielded 7.0 mg of fatty acids (fraction 1). A sample was taken for chromatographic analysis, and the re-

mainder (6.15 mg) was purified over the Mg^{++} salt (7), giving 2.3 mg of material (fraction 2) in which cerebronic acid, if present, would have been concentrated. Ninhydrin analysis (8) showed fraction 1 to contain about 10% sphingosine, whereas fraction 2 was free of sphingosine. The neutralization equivalent of fraction 2 was 373. Analysis of the fatty acids in both fractions by the paper chromatographic method is shown in Table 1 (preparation 1). Neither the total

TABLE 1. LIPID RESIDUES IN TWO PREPARATIONS OF CYTOLIPIN H

	Preparation 1		Preparation 2*	
	Paper Chromatography		Paper Chromatography	Gas-Liquid Chromatography
	Fract 1	Fract 2		
	per cent	*per cent*	*per cent*	*per cent*
Fatty acids				
Lignoceric (C_{24})	65.	96.	42.	46.
Behenic (C_{22})	5.	4.	16.	10.5
Arachidic (C_{20})	0.5			
Stearic (C_{18})	3.		tr.	1.
Oleic	15.			
Palmitic (C_{16})	11.		23.	27.
Myristic (C_{14}) (+ palmitoleic)	0.5		7.	0.5
Other			12.?†	15.5‡
Lipid bases				
Sphingosine				93.
Dihydrosphingosine				
Unknown (dehydrosphingosine?)				7.

* Hydrolytic conditions were different for the two methods; see text.
† Acids migrating more rapidly than myristic.
‡ C_{23}, 5.5%; unknowns between C_{22} and C_{24}, 10%.

fatty acid fraction nor the purified fraction contained any 2-hydroxy fatty acids; over 90% of the total fatty acid was composed of lignoceric (65%), oleic (15%), and palmitic (11%) acids.

Cupric Acetate Test. Prior knowledge of the absence of hydroxy fatty acids had been obtained by developing a quantitative modification of the cupric acetate test. It has been shown by Radin *et al.* (9)[1] that 2-hydroxy fatty acids form insoluble colorless chelates in ethanol with cupric acetate, whereas unsubstituted fatty acids give a blue color. We observed that all saturated acids (C_{14} to C_{24}) formed deep blue precipitates in methanol at 20°; unsaturated acids (oleic, linoleic) formed similar precipitates at −5°, as did 10-hydroxy and 12-hydroxy stearic acids. In contrast, 2-hydroxystearic and 2-hydroxylignoceric acids formed colorless or very pale blue precipitates at −5°. Solution of the precipitate and measurement of the blue color provided a quantitative assay. To a solution of 5 μmoles of fatty acid in 1 ml of methanol, 1.6 ml of 0.005 M cupric acetate in absolute methanol was added. After standing at 5° for 30 minutes, the precipitate was centrifuged at 5° and the supernatant solution removed. The precipitate was dissolved in 7.5 ml of *m*-xylene and the solution read against this solvent in a Coleman model 14 spectrophotometer at 680 mμ using a red filter. Results obtained with different mixtures of stearic and 2-hydroxystearic acids are shown in Figure 1*A*. A similar curve represents mixtures of lignoceric and cerebronic acids (Fig. 1*B*). Based on the neutralization equivalent, the purified fatty acid fraction of cytolipin H (preparation 1) gave a trace of color in excess of that obtained with a sample of lignoceric acid containing no cerebronic acid. This result indicated that no 2-hydroxy fatty acid was present.

Recalculation of Empirical Formula. The empirical formula for cerebronyl sphingosyl glucosidogalactoside is $C_{54}H_{103}O_{14}N$, for which the calculated percentages of C, H, and N are 65.5, 10.5, and 1.41, respectively. The percentages found for cytolipin H were 65.5, 10.3, and 1.38. An empirical formula calculated as a weight average of the fatty acids shown in Table 1 for preparation 1 is $C_{51.9}H_{98.4}O_{13}N$, for which the calculated percentages are C, 65.8; H, 10.5; and N, 1.44. The agreement between these values and those found for cytolipin H is still close, and thus the results based on elemental composition present a picture consistent with those based on analysis of larger structural units. The per cent hexose and iodine number for the revised empirical formula, 38.4 and 31.9, respectively, compare favorably with the values found, namely, 36.7 and 32.

Lipid Residues in a Second Preparation. A sample of 0.4 mg of a second preparation of cytolipin H was

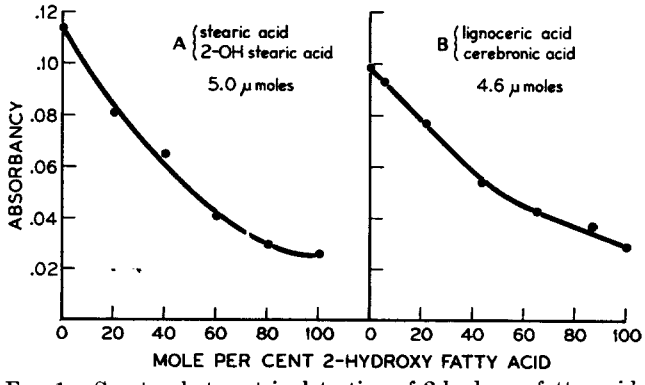

FIG. 1. Spectrophotometric detection of 2-hydroxy fatty acids in the presence of unsubstituted acids by the formation of colorless copper chelates.

[1] N. S. Radin. Personal communication.

hydrolyzed by refluxing for $2^1/_2$ hours with 4 ml of 1.2 N HCl in 90% ethanol. After adjusting the hydrolyzate to pH 2, evaporating the ethanol, and extracting with ether, the ether-extractable material was refluxed with dilute NaOH. The mixture was acidified to pH 2, extracted with ether, and the fatty acids were analyzed by paper chromatography. The results are shown in Table 1 (preparation 2).

For gas-liquid chromatographic analysis, 2.0 mg of cytolipin H was hydrolyzed for 6 hours at 100° with methanol-2 N HCl in a sealed tube. The lipid bases and fatty acid esters were separated and analyzed (6). The results of these analyses are shown in Table 1. Although the fatty acid analyses by the two methods were not identical, the results are in reasonably good agreement, especially in view of the difference in conditions of hydrolysis. Lignoceric acid was again the predominant fatty acid residue, although it was present in lesser amount than in the first preparation. Behenic and palmitic acids were present to a much greater extent in the second preparation. These three acids constituted 80% to 85% of the fatty acids in both preparations. About 10% of the fatty acids in the second preparation could not be identified with either of the analytical methods.

The lipid base is seen (Table 1) to be mainly sphingosine with a small quantity of an unknown base (presumably dehydrosphingosine). No dihydrosphingosine was present.

DISCUSSION

The results of this study show that the lipid residues in cytolipin H are neither a single fatty acid nor a single lipid base. Mixtures are obtained that may vary somewhat in composition, as is the case with many lipids isolated from natural sources. Whereas some of this variation may occur in the original tissue, much is attributable to selection that occurs during the process of purification. It is possible, however, to discern an interesting feature, namely, that the distribution of saturated fatty acids in cytolipin H is the same as it is in other glycolipids (from tissues apart from the nervous system). Thus in the two preparations of cytolipin H reported here, 80% to 85% of the fatty acid residues were composed of C_{24}, C_{22}, and C_{16} saturated acids. Only very small quantities of C_{20}, C_{18}, and C_{14} saturated acids were present. In a third preparation of cytolipin H, the combined percentage of C_{24}, C_{22}, and C_{16} acids was 95%. This may be compared with 93% for these three acids found, by Marinetti *et al.* (10), in a preparation of glucocerebroside from the spleen of a patient with Gaucher's disease, and 84% in a preparation of glucocerebroside from bovine spleen, by Skipski *et al.* (4). In two preparations of bovine spleen cytolipin, 91% and 86% of the fatty acids were found in this group. A summary of the results with these and another preparation is shown in Table 2. The reason

TABLE 2. FATTY ACID CONTENT OF CEREBROSIDES AND CYTOSIDES ISOLATED FROM NON-NERVOUS ANIMAL TISSUES

Preparation	Lignoceric	Behenic	Palmitic	Arachidic	Stearic	Myristic
Cytolipin H (prep. 1)*	65	5	11	0.5	3	0.5
Cytolipin H (prep. 2)†	44	13	25		1	0.5
Cytolipin H (prep. 3)*	62	10	23		5	
Bovine spleen cytolipin						
(prep. 1)†	28	19	48		2	1
(prep. 2)†	40	26	25	2	4	1
(prep. 3)*	65	10	25			
Bovine spleen glucocerebroside (4)†	46	27	11		4	
Gaucher spleen glucocerebroside (10)	46	27	20	3	3	

* Analysis by paper chromatography.
† Values are average of analyses by both paper and gas-liquid partition chromatography.

for the relative absence of C_{14}, C_{18}, and C_{20} acids from these two groups of sphingolipids is obscure, but may be related to the specificity of biosynthetic enzymes. This implicates a common pathway for the two types of substances (cerebroside and cytoside), and suggests that cytolipin may be synthesized by acylation of sphingosyllactoside (11).

When the carbon content of cerebronyl sphingosyl glucosidogalactoside ($C_{54}H_{103}O_{14}N$) was calculated, it was not appreciated that a similar result could be obtained for the behenic acid derivative ($C_{52}H_{99}O_{13}N$). The high result obtained in the determination of neutralization equivalent was particularly misleading in this regard, and the absence of 2-hydroxy acids from non-nervous animal tissues[2] stimulated us to develop methods for investigating this aspect of structure on the limited quantities of material at hand. These studies reinforce the statement (10) that older methods of single-value characterization of fatty acid content cannot be accepted without reservation, since a range of fatty acids is usually present in these compounds. The recent report (12) that the fatty acid in the glucocerebroside isolated from the spleen of a patient with Gaucher's disease was behenic acid very probably can be explained as the resultant of the fortuitous average of the right amount of C_{24} and C_{16} acids with a relatively smaller quantity of the C_{22} acid. Behenic acid has

[2] We are indebted to Professor E. Klenk for calling this fact to our attention.

not been found to predominate in any of the glycolipid preparations we have studied.

The lipid base in cytolipin H is almost entirely sphingosine. No dihydrosphingosine was present in the preparation studied, and a small quantity (7%) of an undetermined base (6), presumably an unsaturated derivative of sphingosine, was found. In comparison, two preparations of bovine spleen cytolipin were found to have 5% and 8% of dihydrosphingosine, the latter preparation also containing a small amount (3%) of the unknown base. The reported differences in degree of unsaturation between preparations of cytolipin H and bovine spleen cytolipin are thus found to be due to inconstant variations (from preparation to preparation) in the unsaturation of both fatty acid and lipid base residues. In view of the degree of variation in fatty acid composition indicated by the presence of oleic acid to the extent of 15% of the fatty acid in the first preparation of cytolipin H and its absence from the second preparation, the degree of unsaturation cannot be considered a reliable criterion of difference between human tumor and bovine spleen cytolipins (3).

Since the structure and configuration of the carbohydrate residues of cytolipin H have been shown to be that of the disaccharide lactose (2), the identification of the fatty acid and lipid base residues almost completes the structural identification. What must still be confirmed is that the glycosidic bond forms with the primary hydroxyl group of sphingosine (10), and what must still be determined is the configuration of this bond. This latter point of structure has still not been rigorously established for cerebrosides (13). The availability of antibody that reacts with cytolipin may make it possible to use the sensitive immunochemical method of hapten inhibition to provide answers to both questions when proper models are available.

We wish to thank Dr. Lewis Gidez for carrying out some of the early gas-liquid chromatographic analyses of fatty acids, and Mr. Stuart M. Arfin, Mr. Harvey Friedman, and Miss Kay Anthony[3] for capable technical assistance.

REFERENCES

1. Rapport, M. M., L. Graf, V. P. Skipski, and N. F. Alonzo. *Cancer* **12:** 438, 1959.
2. Rapport, M. M., L. Graf and J. Yariv. *Arch. Biochem. Biophys.*, in press.
3. Rapport, M. M., L. Graf and N. F. Alonzo. *J. Lipid Research* **1:** 301, 1960.
4. Skipski, V. P., S. M. Arfin and M. M. Rapport. *Arch. Biochem. Biophys.* **87:** 259, 1960.
5. Horning, E. C., E. A. Moscatelli and C. C. Sweeley. *Chem. & Ind. (London)*, 751, 1959.
6. Sweeley, C. C., and E. A. Moscatelli. *J. Lipid Research* **1:** 40, 1959.
7. Klenk, E. *Z. physiol. Chem., Hoppe-Seyler's* **166:** 268, 1927.
8. Lea, C. H., and D. N. Rhodes. *Biochem. J.* **56:** 613, 1954.
9. Radin, N. S., J. R. Brown and F. B. Lavin. *J. Biol. Chem.* **219:** 977, 1956.
10. Marinetti, G. V., T. Ford and E. Stotz. *J. Lipid Research* **1:** 203, 1960.
11. Cleland, W. W., and E. P. Kennedy. *J. Biol. Chem.* **235:** 45, 1960.
12. Rosenberg, A., and E. Chargaff. *J. Biol. Chem.* **233:** 1323, 1958.
13. Carter, H. E., D. S. Galanos and Y. Fujino. *Can. J. Biochem. and Physiol.* **34:** 320, 1956.

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