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# Composition of cerebroside acids as a function of age*

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## SUMMARY

The brains of from 11 to 30 rats of various age groups—23 to 418 days old—were pooled and analyzed for total lipids, total cerebrosides, and the individual cerebroside acids. Cerebroside deposition is evident over the range of ages studied, and its contribution to the total deposition becomes increasingly important with increasing age. Cerebronic acid is by far the major cerebroside acid, but appreciable amounts of the $\alpha$-hydroxy $C_{23}$ and $C_{22}$ acids are also present. The unsaturated acids constitute a minor element, and the contribution of the hydroxy unsaturated acids is least. The odd-numbered acids show the greatest increases with age, compared to the other acids, while the hydroxy unsaturated acids show little accumulation except during the earliest period studied. Degradation experiments with the saturated hydroxy acids show that the hydroxyl groups are in the alpha position.

The brains of rats of five different age groups have been analyzed for their contents of total lipids, total cerebrosides, and the individual major cerebroside acids. In the period of life covered—approximately the first half of the life span—marked differences in the contents and deposition rates of the individual cerebroside acids were found.

## EXPERIMENTAL

The analyses were carried out as described in the accompanying paper (1). Male rats of the Sprague-Dawley strain [1] were obtained in groups of from 11 to 30 rats. Since the exact ages of each group were unknown, we report only the average body weights. The approximate ages of the groups were 22, 31, 65, 170, and 418 days. The animals were killed with ether, and the entire brains were removed and kept frozen until they could be pooled, weighed, and extracted.

## RESULTS AND DISCUSSION

Table 1 shows the values obtained with the lipids and cerebrosides. Each time-point reveals that an increase in brain and cerebroside weight has occurred in the interval. Cerebroside becomes an increasingly important fraction of the brain, even in the middle age period, when the brain weight has almost stabilized and the lipid content has actually started to decrease. The findings of Folch and co-workers (2) with mouse cerebrosides are similar to our results, but there the brain weight appears to fall off at a rather early age (after 90 days). Bürger's data on human brain indicate that cerebroside deposition stops at about 16 years and brain weight starts to fall at about 30 years (3). It would appear that the rodent brain matures more slowly than the human brain with respect to reaching the cerebroside plateau. The methods used by Folch and Bürger for determining cerebrosides were not as specific as ours, and the increasingly large contribution of cerebroside sulfate to the analytical values would probably exaggerate the increases they observed.

Table 2 shows the results of the fractionation of the cerebroside acids into four groups. The percentage distribution data show that the saturated hydroxy acids constitute by far the major group. Somewhat lower are the saturated normal (nonhydroxy) acids, then the unsaturated normal acids, and the unsaturated hydroxy acids constitute the smallest group. Our data confirm the estimates of Klenk (4).

The effect of age on the saturated acids is to decrease the importance of the normal acids during the

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[1] Holtzman Rat Co., Madison, Wis.



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second month of life. The saturated hydroxy acids, after showing a slight dip in the second month, rise to even higher values. The normal unsaturated acids are quite constant in percentage, but there is a small drop in later life. The unsaturated hydroxy acids show a rise during the fourth week of life but decline in percentage during the remaining period covered. Thus it is apparent that each of the four groups of acids is affected differently by age.

Looking at the actual weights of the four groups, we see that there is a considerable increase in weight in all the categories. This increase continues throughout the period covered except for the unsaturated acids, which start to decrease in the last stage. The rate of accumulation of the unsaturated hydroxy acids seems to slow down first, and relatively little is deposited after the surge which comes during the fourth week of life.

*Contents of the Individual Cerebroside Acids.* Tables 3 and 4 list the amount of each of the longer saturated acids. Next to each weight is a number, obtained by dividing the weight at that point by the weight in group A. Thus the number indicates the relative increase in each particular acid with age.

All the acids, except lignoceric, show a continuous rise with age. Lignoceric deposition seems to cease during the fourth week and the third through fifth months, but speeds up considerably in later life. The shorter normal acids, mainly stearic and palmitic, vary erratically, and part of these probably arise from the impurity known to be present in the isolated cerebrosides. The shorter hydroxy acids, on the other hand, increase regularly with age and are probably all authentic members of the cerebroside acids. They comprise only 3 to 4 per cent of the hydroxy acids. The percentage increase is least for lignoceric and behenic acids, and greatest for the odd-numbered acids within each group. Within the hydroxy group, the $C_{21}$ acid shows the greatest percentage increase and the $C_{23}$ acid reaches a level higher than that of lignoceric (at point D). At this age the three most plentiful acids are the $C_{22, 23, 24}$ hydroxy saturated acids.

Table 5 shows the data for the unsaturated acids of two groups of rats. Here, by far, the major components are the $C_{24}$ acids. The normal acids include an appreciable quantity of oleic acid, part of which may be from an impurity. The acid showing the greatest increase with age is the $C_{21}$ hydroxy acid, while hydroxy nervonic acid accumulates only slightly during the second to sixth month. The marked percentage increase of the odd-numbered saturated acids in later life is not too noticeable here.

Saturated and unsaturated acids differ markedly in that the latter are almost entirely $C_{24}$ acids, while the former contain more shorter acids. Comparing within the two groups the distribution of saturated acids, we see that the third most plentiful acid is the $C_{23}$ hydroxy acid and the $C_{20}$ normal acid. In general, it appears that the content and position in the dis-

TABLE 1. CEREBROSIDE CONTENT OF BRAINS AS A FUNCTION OF RAT WEIGHT

| | Rat Group | | | | |
|---|---|---|---|---|---|
| | A (26 rats) | B (30 rats) | C (11 rats) | D (12 rats) | E (15 rats) |
| Rat weight, g. | 51.2 | 80.6 | 243 | 449 | 476 |
| Brain weight (wet), g. | 1.48 | 1.61 | 1.92 | 2.09 | 2.18 |
| Total lipids, mg. per brain | 106 | 134 | 177 | 210 | 194 |
| Purified cerebrosides, mg. per brain | 5.05 | 8.9 | 14.6 | 21.8 | 24.0 |
| Cerebrosides as percentage of total lipids | 4.8 | 6.7 | 8.3 | 10.4 | 12.4 |
| Galactose content of cerebrosides, percentage | 20.8 | 21.0 | 21.0 | 21.4 | 20.6 |

TABLE 2. DISTRIBUTION OF THE CEREBROSIDE ACIDS *

| | Rat Group | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| *Percentage Composition of the Total Cerebroside Acids* | | | | | |
| Saturated normal acids | 30 | 28 | 20 | 20 | 24 |
| Unsaturated normal acids | 11 | 13 | 12 | 12 | 10 |
| Saturated hydroxy acids | 52 | 47 | 59 | 61 | 62 |
| Unsaturated hydroxy acids | 6.8 | 12.1 | 9.1 | 6.7 | 4.8 |
| *Amounts per Brain, in mg.* | | | | | |
| Saturated normal acids | 0.68 | 1.09 | 1.30 | 1.97 | 2.60 |
| Unsaturated normal acids | .25 | .51 | .75 | 1.17 | 1.05 |
| Saturated hydroxy acids | 1.17 | 1.82 | 3.74 | 5.90 | 6.88 |
| Unsaturated hydroxy acids | .15 | .48 | .58 | .65 | .53 |
| Total | 2.25 | 3.89 | 6.36 | 9.68 | 11.05 |

* For further description of the five rat groups see Table 1 and text.

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TABLE 3. DISTRIBUTION OF THE SATURATED NORMAL ACIDS *

| Number of C Atoms | Rat Group | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| 24 | 285 *1.0* | 287 *1.0* | 580 *2.0* | 585 *2.1* | 1099 *3.9* |
| 23 | 19 *1.0* | 26 *1.4* | 60 *3.2* | 107 *5.6* | 127 *6.7* |
| 22 | 109 *1.0* | 131 *1.2* | 174 *1.6* | 217 *2.0* | 264 *2.4* |
| 21 | 5 *1* | 4 *1* | 11 *2* | 14 *3* | 26 *5* |
| 20 | 37 *1.0* | 61 *1.6* | 113 *3.0* | 209 *5.6* | 246 *6.6* |
| under 20 | 229 | 576 | 357 | 835 | 836 |

* Values for the acids refer to the micrograms of acid per rat brain. Numbers in italics are ratios indicating the increase in content of each acid.

TABLE 4. DISTRIBUTION OF THE SATURATED HYDROXY ACIDS *

| Number of C Atoms | Rat Group | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| 24 | 715 *1.0* | 1080 *1.5* | 2208 *3.1* | 3420 *4.8* | 4080 *5.7* |
| 23 | 66 *1.0* | 119 *1.8* | 390 *6.0* | 875 *13.5* | 972 *14.8* |
| 22 | 342 *1.0* | 536 *1.6* | 925 *2.7* | 1309 *3.8* | 1352 *4.0* |
| 21 | 4 *1* | 4 *1* | 23 *6* | 36 *9* | 94 *24* |
| 20 | 18 *1.0* | 32 *1.7* | 93 *5.1* | 157 *8.7* | 179 *9.9* |
| under 20 | 29 | 50 | 100 | 105 | 201 |

* Values for the acids refer to the micrograms of acid per rat brain. Numbers in italics are ratios indicating the increase in content of each acid.

TABLE 5. DISTRIBUTION OF THE UNSATURATED CEREBROSIDE ACIDS *

| Number of C Atoms | Rat Group | | | |
|---|---|---|---|---|
| | Normal Acids | | Hydroxy Acids | |
| | B | D | B | D |
| 24 | 346 | 866 | 374 | 422 |
| 23 | 8 | 22 | 15 | 36 |
| 22 | 22 | 43 | 37 | 48 |
| 21 | 3 | 5 | 10 | 64 |
| 20 | 9 | 33 | 4 | 13 |
| under 20 | 121 | 196 | 37 | 64 |

* Values for the acids refer to the micrograms of acid per rat brain.

tribution pattern of each cerebroside acid undergo highly characteristic changes with age. One possible explanation for this individuality is that the distribution varies greatly with location in the brain and the developmental changes in each section of the brain are highly characteristic. Alternatively, the difference may be on a cellular level rather than brain area. It is planned to analyze the cerebrosides of different areas when a less laborious method can be devised.

*Identification of the Saturated Hydroxy Acids.* These acids were characterized further to demonstrate that they are indeed $\alpha$-hydroxy acids. A sample of the methyl esters (37.4 mg.) was saponified with KOH-alcohol-benzene (5) for 4 hours at 83°C in a screw-cap test tube (6), and the free acids were obtained by acidification and ether extraction. The dried acids were heated for 2 hours at 42°-43°C with 3 ml. of oxidation solution in a flattened test tube protected with a $CaCl_2$ tube. The oxidation solution consisted of 1 g. of dried potassium acetate, 1 g. of lead tetraacetate (recrystallized from acetic acid), and 20 ml. of acetic acid (redistilled after refluxing about 5 hours with $KMnO_4$). The potassium salt speeds the oxidation (7). The mixture was stirred magnetically until the hydroxy acids dissolved. Test runs with synthetic cerebronic acid gave the theoretical yield of $CO_2$ under these conditions. The reaction was stopped by adding 7 ml. of a solution made from 1 g. of KI, 5 g. of sodium acetate, and 10 ml. of water, which reduced the tetraacetate and formed iodine (8). About 50 ml. of water was added to dissolve the lead iodide. The iodine was reduced with 10 per cent aqueous thiosulfate, the aldehydes were extracted with ether, and the ether was washed with water and evaporated off. The aldehydes were promptly dissolved in 4 ml. of acetic acid, and small portions of $KMnO_4$ were added while the mixture was stirring at room temperature. After 30 minutes, when the purple color seemed permanent, 30 ml. of water was added and $K_2S_2O_5$ and 5N $H_2SO_4$ were added to dissolve the $MnO_2$ and reduce the excess permanganate. The acids were extracted with ether and esterified by refluxing 3 hours with half-saturated methanolic HCl. The yield of esters, 34.3 mg., was 99.5 per cent (calculated for cerebronate).

The chromatographic analysis of these esters is shown in Figure 1 and can be compared with the curve of Figure 2 in the preceding paper (1). The distribution of the various acids is similar in the longer region, but additional peaks have appeared in the shorter region. These arise from overoxidation, presumably by the permanganate; a trial run with synthetic cerebronic acid disclosed that about 7 per cent of the acid was degraded by two carbon atoms instead of one.

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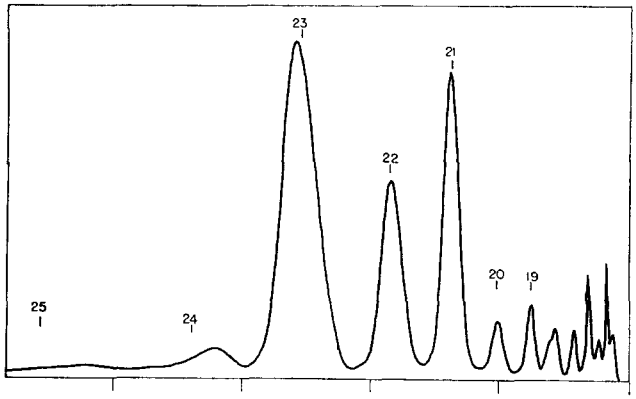


FIG. 1. Gas chromatographic analysis of degradation products of the cerebroside saturated hydroxy acids. The vertical lines under the abscissa represent 10-minute intervals.

This correction was applied to the cerebroside acid degradation products and the mass ratios of the three major esters were found to agree well with the values obtained from the methoxy esters. The ratios for the ethers of the $C_{22, 23, 24}$ esters were 23:16:61; for the degradation products, the ratios were 24:16:61. It may be noted that the retention times for the degradation products matched the expected times particularly well.

The curve for the degradation products quite clearly reveals a longer acid; the corresponding ether peak is more difficult to see. Judging by the retention times in both curves, this trace component is not a straight chain $\alpha$-hydroxy acid.

## REFERENCES

1. Kishimoto, Y., and N. S. Radin. *J. Lipid Research* **1:** 72, 1959.
2. Folch-Pi, J. In *Biochemistry of the Developing Nervous System,* edited by H. Waelsch, New York, Academic Press, Inc., 1955, p. 121.
3. Bürger, M. In *Experimental Research on Ageing,* edited by F. Verzar, Basel, Berkhäuser Verlag, 1956, p. 101.
4. Klenk, E. *Z. physiol. Chem., Hoppe-Seyler's* **166:** 268, 1927.
5. Chibnall, A. C., S. H. Piper and E. F. Williams. *Biochem. J.* **55:** 707, 1953.
6. Entenman, C. In *Methods in Enzymology,* edited by S. P. Colowick and N. O. Kaplan, New York, Academic Press, Inc., 1957, vol. 3, p. 299.
7. Criegee, R., and E. Büchner. *Chem. Ber.* **73:** 563, 1940.
8. Cordner, J. P., and K. H. Pausacker. *J. Chem. Soc.* 1953: 102.

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