

# Developing rat brain: changes in cholesterol, galactolipids, and the individual fatty acids of gangliosides and glycerophosphatides

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**SUMMARY** Groups of brains from rats of various ages—7 to 275 days—were analyzed for their contents of galactolipids (cerebroside + cerebroside sulfate), cholesterol, and the individual fatty acids of the gangliosides and glycerophosphatides. Ganglioside stearate was found to accumulate at a steady pace during the first 20 days of life, then more slowly for at least 32 days more, then to decrease. Ganglioside arachidate, on the other hand, accumulated steadily throughout the period covered. The glycerophosphatide acids showed several inversions in the ratios between selected pairs of acids, the trend being toward increasing unsaturation and chain length. Higher contents of 22:6 and 20:4 acids than previously reported were found, presumably because of improved methods. The galactolipids and cholesterol were deposited at very similar rates after about 15 days, the molar ratio of the deposits (cholesterol/galactolipids) being about 2.2, the value found for purified myelin.

**KEY WORDS** gangliosides · glycerophosphatides · cerebroside · sulfatides · cholesterol · fatty acids · rat · brain · age · docosahexaenoic acid · gas-liquid chromatography response · myelin

**M**OST OF THE data in this paper were obtained as part of a study with labeled acetate (1) of the in vivo metabolism of a number of lipids in rat brain. Some of the findings extend previous findings over a wider age span, using improved methods. Others are new, particularly the changes in fatty acid composition of brain glycerophosphatides and gangliosides.

Abbreviations: GLC, gas-liquid chromatography; NANA, *N*-acetyl neuraminic acid. Fatty acids are identified by the number of carbon atoms and the number of double bonds.

## METHODS

Rats (Sprague-Dawley strain) of five different ages were purchased and kept for different lengths of time. The rats referred to in the accompanying paper (1) as having been injected with labeled acetate at 7 days of age yielded the values shown in the figures of this paper for 7, 9, 17, and 37 days. The rats injected at 13 days yielded data for 13, 15, 23, and 43 days; those injected at 22 days yielded data for 22, 24, 32, and 52 days. Each of these groups comprised 5 rats, while 10 rats were used for the 143-day group and 9 for the 275-day group. The brains were pooled during extraction of the lipids and aliquots of the extract were analyzed using the methods described in the accompanying paper (1).

For the GLC analysis of the ester-linked fatty acids, obtained from a Florisil column after alkaline methanolysis, a known amount of pure 23:0 methyl ester (Applied Science Labs., State College, Pa.) was added as internal standard. For the analysis of the ganglioside fatty acids, obtained by acidic methanolysis from partially purified gangliosides, a known amount of 22:0 ester was added. In the case of the radioactive ganglioside acids, quantification was combined with collection, using a GLC apparatus with thermal conductivity detector. The other acids were analyzed by means of a GLC apparatus with flame ionization detector (2).

The amounts of cholesterol and galactolipids (cerebroside plus sulfatide) were determined gravimetrically from fractions eluted from the Florisil column (1).

## RESULTS AND DISCUSSION

### *Body and Brain Weights*

Figure 1 shows the average body and brain weights. Although the rats were of different ages when purchased,

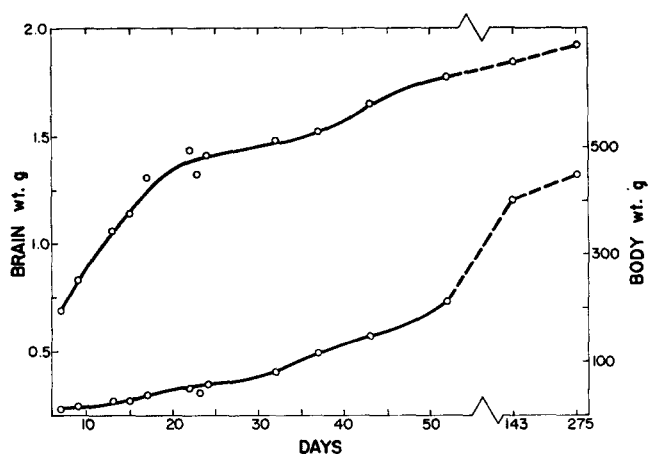


FIG. 1. Rat body and brain weights as a function of age. The upper curve is for the brain weights.

and therefore spent different amounts of time in two environments, the points fall on a fairly smooth line. Only the points around 23 days show some scatter, possibly due to sensitivity to the stress of weaning. It should be noted that the brain weights appear to be rising even up to 275 days.

#### Brain Lipids

Figure 2 shows the amount of total lipids (minus water-extractable materials) per brain. Like total brain weight, the lipids were still accumulating after 200 days. As shown before with mice (3), the rate of accumulation is maximal during the period of most rapid myelination. However, almost as much lipid is deposited in later life: 79 mg between 30 and 275 days, vs. 91 mg between 7 and 30 days. As can be seen from data shown below, much of the increase in later life is due to galactolipid and cholesterol accumulation.

Myelination appears to be complete in the rat brain at roughly 50 days, as judged from histological observations (4), so it would appear from Fig. 2 that considerable deposition of nonmyelin lipids takes place after this point.

#### Ganglioside Fatty Acids

Figure 3 shows the amount of stearate in the gangliosides of each brain. The rate of accumulation is strikingly steady during the first 21 days of life. For the next 31 days (approximately) about 120  $\mu$ g are laid down, an increase of 29%. Stearate was found to be the major acid of the gangliosides, as reported by others; it ranged between 83 and 91% of the total acids. The low percentages were found in the 7-day, 143-day, and 275-day groups. The amount of 16:0 per brain was little affected by age (except for a single high point of 44  $\mu$ g at 143 days), ranging around 20  $\mu$ g after about 12 days. The longer acid, 20:0, increased steadily over the period covered,

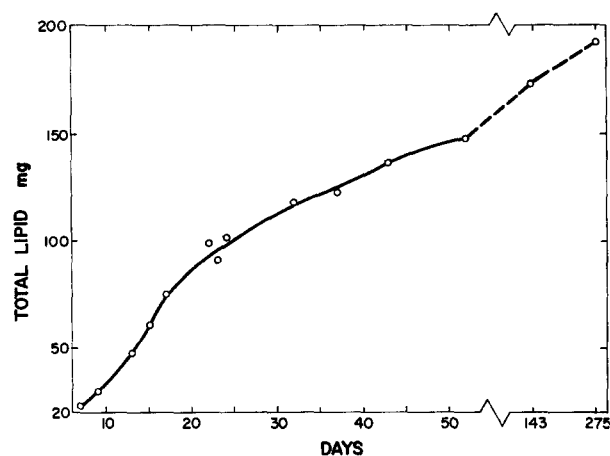


FIG. 2. Weight of rat brain lipids as a function of age.

going from 10  $\mu$ g at 7 days to 51  $\mu$ g at 275 days. The amount of ganglioside oleate, which is very small, was not determined.

Since gangliosides appear to be present primarily or entirely in neurones (5), and since the formation of new neurones apparently slows down greatly at an early age (6, 7), it may be tentatively concluded that the neurones (once formed) accumulate additional ganglioside for an appreciable time.

Previous age studies of ganglioside accumulation in rat brain, reported as amount of NANA and hexose *per gram*, have given curves qualitatively equivalent to ours (8-10). The values given by James and Fotherby (9) for "adult" rats indicate that some individuals have an appreciably lower concentration of gangliosides, presumably as the result of further aging. This supports the validity of our two last time points, which indicate that some ganglioside is actually lost from the neurones in later life, or that appreciable numbers of neurones are gradually lost from rat brain after 52 days. Folch et al. (3) found a curve like ours for mice, the peak amount of ganglioside appearing at about 90 days, followed by a 12% drop in the next 90 days. Svennerholm (11) found no distinct changes with age in older human brains with

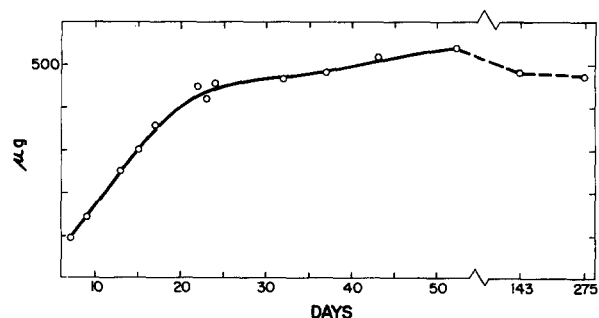


FIG. 3. Ganglioside stearate content of whole rat brain as a function of age.

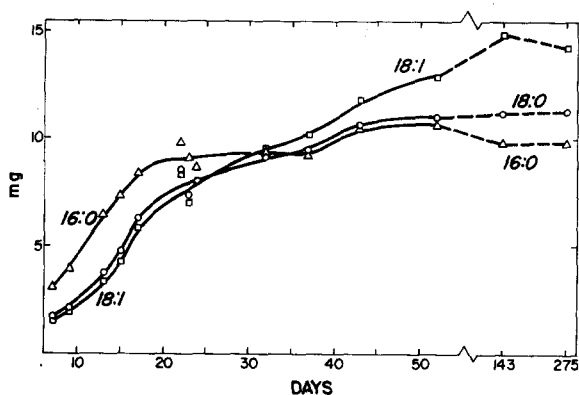


Fig. 4. Glycerophosphatide stearate, oleate, and palmitate content of whole rat brain as a function of age.

respect to the concentration (in dry gray matter) of ganglioside NANA; however, the total dry weight of human brain declines appreciably with age in later life (12), so that the total amount of ganglioside must also decline in later life.

#### Glycerophosphatide Fatty Acids

The amounts per brain of the three most common ester-linked acids are shown in Fig. 4. It can be seen that 16:0 is the most prominent acid in early life but is surpassed at around 32 days by 18:0 and 18:1. The ratio of 16:0/18:0 drops fairly regularly with increasing age; at 7 days it is 1.81 and at 275 days it is 0.87. There is a similar crossover between 18:0 and 18:1 at 24 days. The ratio of 18:1/18:0 is about 0.89 during the second week of life, then rises to 1.27 by 275 days. Thus it would be possible to estimate the maturity of a rat brain by determining the ratio of these three acids. The changing ratios reveal that there is an increase in average chain length and degree of unsaturation with increasing age. The deposition of 16:0 and 18:0 in the rat glycerophosphatides seems to stop by 52 days.

Biran and Bartley (13) found similar changes in rat brain ester-linked acids, using only three age points. As they suggested, the changing ratios may reflect changing ratios in the different types of glycerophosphatides. Alternatively, the changes may reflect changing fatty acid composition in individual complex lipids. At least one brain lipid, sphingomyelin, shows increasing unsaturation and average chain length with age (14).

Figure 5 shows the changes in the less common acids, 20:4 (arachidonic), 22:5 $\omega$ 6, and 22:6. As with the shorter acids, there is a crossover point (at 23 days) after which the longer, more highly unsaturated acid becomes more prominent. Thus a comparison of the 22:6/20:4 ratio also gives an indication of the maturity level of a brain.

The high contents of 20:4 and 22:6 that we find, compared with some other workers (13, 15), are striking. The

relative concentration of 22:6 ranges between 11.2 and 14.3% of the total glycerophosphatide acids. Our higher values probably are the result of using both a rapid, mild method of isolation, which keeps air oxidation to a minimum, and temperature programming during GLC, which probably makes quantification of the very slow-eluting acids more accurate. Once the methyl esters are isolated (from the Florisil column) their stability seems to be somewhat enhanced, as reported by others (16). Similar high values for 20:4 and 22:6 have been obtained with human brain (17, 18).

The values reported here for 20:4 and 22:6 are probably lower than they should be, for analysis of a mixture of 22:6 (distilled at Hormel Institute and sent sealed in an inert atmosphere) and 22:0 yielded an area response for 22:6 that was 21% lower than expected from the gravimetric values. By comparing the actual weight of the brain methyl esters isolated with the weight calculated from the GLC data, we found the recovery from the GLC method to be 85–90%. Part of this loss is due to failure to measure the areas of the trace peaks, but part is undoubtedly due to the lower response for the highly unsaturated esters. These observations suggest that the polyenoic acids are present in considerably higher proportions than is generally appreciated.

The observed high, and increasing, levels of brain 20:4 and 22:6 underline the importance of the suggestion (19) that it might be wise to increase our intake of antioxidants. Granules resembling brain lipofuscin, the granules accumulating in neurones with age, can be induced to form in rats by use of a vitamin E-deficient diet (20). Human heart lipofuscin granules are rich in polyunsaturated fatty acids (21).

A number of observations about the minor acids of the glycerophosphatides are of interest. While the amount of 22:5 $\omega$ 6 rises 5.7-fold between 7 and 52 days, the isomeric acid, 22:5 $\omega$ 3, rises only 1.7-fold. Since the proportions of the highly unsaturated acids in brain can be in-

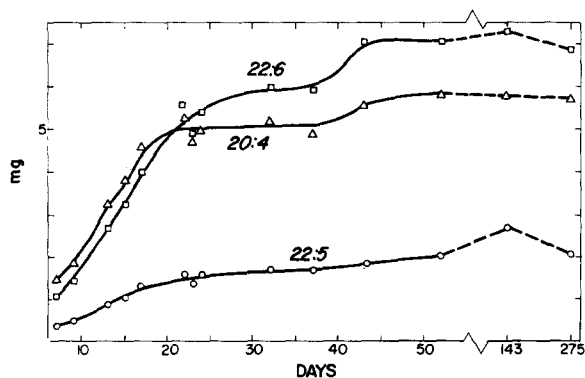


Fig. 5. Changes with age in amounts per brain of ester-linked polyunsaturated acids: arachidonic, docosapentaenoic, and docosahexaenoic. The 22:5 acid is apparently the  $\omega$ 6 isomer (31).

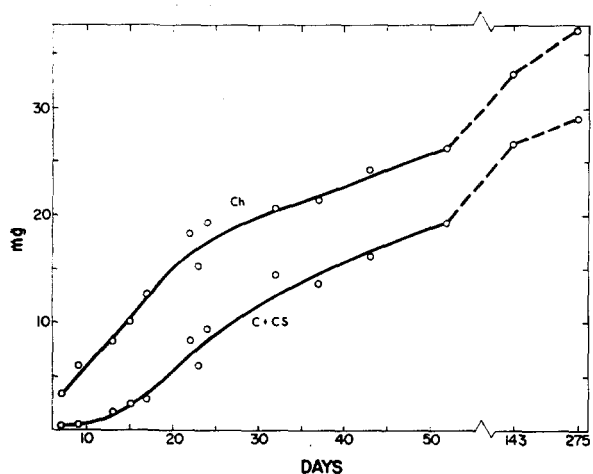


FIG. 6. Age changes in cholesterol (upper curve) and cerebroside + sulfatide of whole rat brain.

fluenced by diet (22–24), this difference may merely reflect a characteristic of changes in blood levels rather than a change in brain metabolism.

With our gas chromatograph the peaks for 18:3 and 20:1 coincide. The combined peak is barely visible in the 7-day rat but rises greatly, in a relative sense, to a level of 1.08 mg per brain at 52 days. This value is 9% of the stearate value. We found the 20:1 acid in pig brain to be a mixture of three isomers, primarily 20:1 $\omega$ 7 and 20:1 $\omega$ 9 (25). Linoleic acid, a minor acid in brain, rises at a rate which parallels 18:0 fairly closely (at 1/15th the level).

#### Cholesterol and Cerebroside

Figure 6 shows the values for brain cholesterol and cerebroside (plus sulfatide). Since the brains of very young animals contain desmosterol (26), which is not separated from cholesterol in our procedure, the early values for cholesterol are too high. Cholesterol and cerebroside accumulate over a somewhat longer period than glycerophosphatides. A similar time course has been found for cholesterol and galactolipids in mouse brain (3), and for cerebroside alone in rat brain (27). Since sulfatide accumulation continues during the period studied here (28), the increasing values for the mixed galactolipids in Fig. 6 are due to both components.

The two curves of Fig. 6 rise at similar rates (in terms of mg/day deposited) after approximately 15 days. Converting the weight deposition rates to a molar basis yields a ratio of deposition rates of about  $840/387 = 2.2$  for cholesterol/galactolipid. This ratio is close to those reported for isolated myelin (29, 30) so that it is possible to conclude tentatively that the cholesterol and galactolipids deposited after about 15 days are utilized largely for the synthesis of myelin or membranes of similar composition. Both lipids have been found in extramyelin fractions following subcellular fractionation, but it is not

certain how much of this is due to degradation of myelin during homogenization or to deposition during the second week of life. This question might be answered by performing an age study with subcellular fractions.

Our finding that cholesterol and galactolipid accumulate in "myelin proportion" after the period when histologists report no further myelin deposition could mean that axons (and their accompanying myelin) continue to increase in length even in later life. Histological observation would not detect this type of increased myelin deposition.

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#### REFERENCES

1. Kishimoto, Y., W. E. Davies, and N. S. Radin. *J. Lipid Res.* **6**: 525, 1965.
2. Radin, N. S. *J. Chromatog.*, in press.
3. Folch, J., J. Casals, A. Pope, J. A. Meath, F. N. LeBaron, and M. Lees. In *The Biology of Myelin*, edited by S. R. Korey. Harper & Bros., New York, 1959, p. 131.
4. Jacobson, S. *J. Comp. Neurol.* **121**: 5, 1963.
5. Lowden, J. A., and L. S. Wolfe. *Can. J. Biochem.* **42**: 1587, 1964.
6. Miale, I. L., and R. L. Sidman. *Exptl. Neurol.* **4**: 277, 1961.
7. Altman, J. *Anat. Record* **145**: 573, 1963.
8. Pritchard, E. T., and P. L. Cantin. *Nature* **193**: 580, 1962.
9. James, F., and K. Fotherby. *J. Neurochem.* **10**: 587, 1963.
10. Burton, R. M., L. Garcia-Bunuel, M. Golden, and Y. M. Balfour. *Biochemistry* **2**: 580, 1963.
11. Svennerholm, L. *Acta Soc. Med. Upsalien.* **62**: 1, 1957.
12. Bürger, M. In *Experimental Research on Aging*, edited by F. Verzar. Birkhaeuser Verlag, Basel, 1956, pp. 101–111.
13. Biran, L. A., and W. Bartley. *Biochem. J.* **79**: 159, 1961.
14. Stållberg-Stenhagen, S., and L. Svennerholm. *J. Lipid Res.* **6**: 146, 1965.
15. Johnston, P. V., K. C. Kopaczyk, and F. A. Kummerow. *J. Nutr.* **74**: 96, 1961.
16. Wren, J. J., and A. D. Szczepanowska. *J. Chromatog.* **14**: 405, 1964.
17. Foote, J. L., R. J. Allen, and B. W. Agranoff. *J. Lipid Res.* **6**: 518, 1965.
18. O'Brien, J. S., D. L. Fillerup, and J. F. Mead. *J. Lipid Res.* **5**: 329, 1964.
19. Tappel, A. L. In *Symposium on Foods: Lipids and Their Oxidation*, edited by H. W. Schultz. Avi Publishing Co., Westport, 1962, pp. 367–386.
20. Sulkin, N. M., and P. Srivani. *J. Gerontol.* **15**: 2, 1960.
21. Hendley, D. D., A. S. Mildvan, M. C. Reporter, and B. L. Strehler. *J. Gerontol.* **18**: 250, 1963.
22. Marco, G. J., L. J. Machlin, E. Emery, and R. S. Gordon. *Arch. Biochem. Biophys.* **94**: 115, 1961.
23. Biran, L. A., W. Bartley, C. W. Carter, and A. Renshaw. *Biochem. J.* **93**: 492, 1964.
24. Mohrhauer, H., and R. T. Holman. *J. Neurochem.* **10**: 523, 1963.
25. Kishimoto, Y., and N. S. Radin. *J. Lipid Res.* **5**: 98, 1964.

26. Kritchevsky, D., and W. L. Holmes. *Biochem. Biophys. Res. Commun.* **7**: 128, 1962.
27. Kishimoto, Y., and N. S. Radin. *J. Lipid Res.* **1**: 79, 1959.
28. Davison, A. N., and N. A. Gregson. *Biochem. J.* **85**: 558, 1962.
29. Seminario, L. M., N. Hren, and C. J. Gómez. *J. Neurochem.* **11**: 197, 1964.
30. O'Brien, J. S. *Science* **147**: 1099, 1965.
31. Mohrhauer, H., and R. T. Holman. *J. Lipid Res.* **4**: 151, 1963.