Relation to Absorption and Metabolic Fate of Certain Polyols, Presented before Society of Toxicology, Wiliamsburg, Va., March 9, 1964.

7. Wick, A. N., Morita, T. N., Joseph, L., Proc. Soc. Exp. Biol. and Med., 1954, v85, 188.

8. Krantz, J. C., Jr., Carr, C. J., ibid., 1938, v39, 577.

9. Carr, C. J., Krantz, J. C., Jr., Metabolism of the Sugar Alcohols and Their Derivatives, Advances in Carbohydrate Chemistry, Academic Press, New York, 1945, v1, p175.

10. Krantz, J. C., Jr., U. S. Patent No. 2,143,324, Jan. 10, 1939.

11. Litchfield, J. T., Jr., Wilcoxon, F., J. Pharmacol. Exp. Therap., 1947, v96, 99.

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Brain Sphingolipids in Experimental "Allergic" Encephalomyelitis.* (30093)

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Previous work of Edgar(1) showed that the ratios of sphingomyelin to lipid-galactose of brain specimens from 2 cases of multiple sclerosis were considerably lower than the respective ratios found in 7 brains from cases without neurological disease. Since this relatively small amount of material hardly allowed definite conclusions it was thought to be of interest to study these sphingolipid constituents in experimental "allergic" encephalomyelitis (E.A.E.), even more so as they have been implicated recently to be possibly involved in the auto-immunization mechanism underlying that disease(2,3). It thus was decided to examine the ratios of sphingomyelin to lipid-galactose in brains and spinal cords of normal rabbits and rabbits with E.A.E.

Material and methods. Disease-free female albino rabbits (Huntingdon Farm, Philadelphia) of about 2.5 to 3 kg weight were used. E.A.E. was produced by intradermally injecting into the foot pad of each paw 0.2 ml aliquots of an emulsion prepared from 10 g fresh, normal spinal cord of rabbits, 750 mg dry *Mycobacterium butyricum* (from American Type Culture Collection grown in our laboratory), 5 ml Freund's adjuvant, 1 ml Arlacel (Falba), and 5 ml Bayol F. Approximately 2 to 6 weeks following the injection 60 to 80% of the animals developed severe paresis starting at the hind legs. The paretic rabbits and controls were sacrificed by intravenous injection of air. The brains and spinal cords then were removed, freed of blood and meninges, weighed, and kept frozen until lipid extractions could be carried out shortly afterwards. Some brains and spinal cords were placed into formalin for neuropathological examination.

Chemical procedures. The lipids of the brains or spinal cords were extracted with chloroform-methanol 2:1 (v/v) and then washed according to Folch *et al*(4). Thus purified lipid extracts were stored under nitrogen in the cold room until the analysis could be performed. The sphingomyelin analysis utilized the resistance of the component to mild alkaline hydrolysis(5,6). The results presented here were based particularly on the determination of the differences between the values of the total choline and the choline released from the "alkali labile" phospholipids by the hydrolysis. Sphingomyelin was calculated by multiplication of that difference, representing the value of sphingomyelin-choline, by a factor of 6.4. The choline determinations were carried out essentially by the procedure of Smits(7). The accuracy of the sphingomyelin determinations was checked by analysis of pure sphingomyelins prepared in this laboratory(8). A series of earlier ex-

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TABLE I. Sphingomyelin and Lipid-Galactose of Brain and Spinal Cord of Normal Rabbits and Rabbits with Experimental Allergic Encephalomyelitis (E.A.E.).*

No. and organ	Normal rabbits				E.A.E. rabbits			
	Wet wt (g)	Sphingomyelin % wet wt	Lipid-galactose % wet wt	*Sphingomyelin* lipid-galactose	Wet wt (g)	Sphingomyelin % wet wt	Lipid-galactose % wet wt	*Sphingomyelin* lipid-galactose
Brains 4 normal 5 E.A.E.	7.82 ± .45	.71 ± .09	.21 ± .03	3.37 ± .13	9.19 ± .19	.6 ± .03	.32 ± .03	1.89 ± .2
Spinal cords 6 normal 6 E.A.E.	3.6 ± .1	1.65 ± .11	.52 ± .02	3.18 ± .23	4.73 ± .21	1.27 ± .10	.62 ± .06	1.99 ± .09

* Values express arithmetic means and standard errors. Significant P values are given in text.

periments used the simpler procedure of determining sphingomyelin from the difference between total phospholipid- and "alkali labile" phospholipid-phosphorus(6). Although essentially similar conclusions could be drawn from these experiments, they were not included here because somewhat higher sphingomyelin values were found by that simpler technique in this laboratory and elsewhere (9,10,11,12).

Lipid-galactose was determined essentially by the method of Brand and Sperry(13), which has been used most frequently for analysis of cerebrosides up to recently. The term lipid-galactose(1) rather than cerebroside-galactose was applied since Radin(14) and others showed that not only cerebrosides, but also other glycolipids such as gangliosides might be determined by that or similar methods. It is pointed out here, however, that the water soluble gangliosides were removed completely during the washing procedure(4) applied for purification of our lipid extracts. It thus may be assumed that the galactose values reported here were derived almost entirely from glycolipids of the cerebroside class; *viz.*, cerebrosides and sulfatides. To convert such values into "cerebroside" figures, the molecular weight of cerebroside is usually taken at 820 requiring multiplication by a factor of 4.55 for the conversion. However, in view of the inferred complexity of these glycolipids such figures should be used with caution as an arbitrary reference only.

Results and discussion. The results summarized in Table I show that the mean ratios of sphingomyelin : lipid-galactose of normal rabbit brain or spinal cord were remarkably similar amounting to 3.37 ± 0.13 and 3.18 ± 0.23 respectively. The ratios obtained from brain or spinal cord of E.A.E. rabbits, while also being quite similar to each other, on the other hand, were significantly lower ($P<0.01$) than the normal ones and amounted only to 1.89 ± 0.2 and 1.99 ± 0.09 respectively. Contrasting the described similarity of the ratios were the actual amounts of the two components per 100 gram wet tissue, which were higher in spinal cord than in brain of both normal and E.A.E. rabbits. Comparison of these per cent values of sphingomyelin or lipid-galactose of brain and spinal cord from normal rabbits with those of E.A.E. rabbits showed changes of these 2 components in opposite direction. The mean sphingomyelin percentage on one hand was probably lower in the spinal cord ($P<0.05$) or barely lower in the brain of the E.A.E. rabbits; these lower values were in line with the increase of the weight of the brains or spinal cords from the diseased rabbits. The lipid-galactose percentages, on the other hand, were probably higher in both brain and spinal cord of the E.A.E. rabbits ($P<0.05$) than they were in the controls. It thus must be assumed that the described lowering of the sphingomyelin : lipid-galactose ratios in brains and spinal cords of the E.A.E. rabbits is mostly if not entirely caused by increase of the lipid-galactose and not by decrease of the sphingomyelin content of these tissues. Since the lipid-galactose values presented here were derived mostly from glycolipids of the cerebroside class, it can be suggested, furthermore, that our results indicate a significant increase of those glycolipids in

brain and spinal cord of E.A.E. rabbits. It is believed that further elucidation of these changes and their possible underlying metabolic-enzymatic cause merit continued investigation.

Summary. Determination of the ratios of sphingomyelin : lipid-galactose in brain and spinal cord of a group of E.A.E. rabbits and a group of normal rabbits showed remarkable similarity of the ratios within each of the groups of rabbits. Comparison of the values of the two groups with one another, however, showed that the sphingomyelin : lipid-galactose ratios of both brain and spinal cord from the E.A.E. rabbits were significantly lower than those from the normal ones. The metabolic-enzymatic cause of this change is subject of further investigation.

1. Edgar, E. W. F., Acta Neurol. Scand., 1959, v59/6, 756.

2. Honegger, C. G., Ritzel, G., Rieder, H. P., Z. Immun. Forch., 1964, v126, 49.

3. ———, ibid., 1964, v126, 68.

4. Folch, J., Lees, M., Sloane, St., J. Biol. Chem., 1957, v226, 497.

5. Klenk, E., Rennkamp, F., Hoppe-Seyl. Z., 1940, v267, 145,

6. Schmidt, G., Benotti, J., Hershman, B., Thannhauser, S. G., J. Biol. Chem., 1946, v166, 505.

7. Smits, G., Biochem. Biophys. Acta, 1957, v26, 424.

8. Schwarz, H. P., Dreisbach, L., Barrionuevo, M., Kleschick, A., Kostyk, I., J. Lipid Research, 1961, v2, 208.

9. Brante, G., Acta Physiol. Scand., 1949, v18, suppl. 63.

10. Carter, H. E., Smith, D. B., Jones, D. N., J. Biol. Chem., 1958, v232, 681.

11. Dawson, R. M. C., Biochem. J., 1954, v56, 621.

12. Edgar, G. W. F., Smits, G., J. Neurochem., 1959, v3, 316.

13. Brand, F. C., Sperry, W. M., J. Biol. Chem., 1941, v144, 545.

14. Radin, N. S., Meth. Biochem. Anal., 1958, v6, 1963.

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Monolayer Cultures of Brown Fat Cells.* (30094)

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Classical techniques of explant(1,2) and organ(3) cultures of the brown fat body have been used to study the differentiation of fat cells and the growth of rabies virus in adipose tissue. This paper and a previous abstract(4) report on the growth and hormonal responses of brown fat cells *in vitro* using a technique of trypsin dissociation and reaggregation, to produce monolayer cell cultures.

Interscapular fat pads from one- to three-day-old rats were diced and dissociated by vigorous pipetting in 0.25% trypsin (1:300) in a Ca-Mg-free Hanks' solution(5). Pads from older animals were not used since they have accumulated too much fat and their cells will not settle on glass. Repeated trypsinization of larger fragments was usually necessary. The cellular residues were spun at 1000 rpm in a refrigerated centrifuge at 10°C, and the cell pellets resuspended in Eagle's Medium(6) supplemented with bovine fetal serum. Cell viability was checked by trypan blue ingestion. The cell number was adjusted by dilution to 500,000/ml of medium, and the cells allowed to settle on coverslip surfaces in Leighton tubes. Cultures were incubated at 37°C, and coverslips with attached cells were withdrawn at 12 hour intervals over a 3 day period, fixed in 10% formalin, and stained with Sudan Black in 70% ethanol, and Wright-Giemsa stain. Insulin was prepared as a stock solution of 5 mg/ml by dissolving the crystalline hor-

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