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Fatty acids of human brain cerebrosides*

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SUMMARY

Four regions of two human brains were analyzed for the individual cerebroside acids: cerebral cortex white and gray matter, cerebellum (mainly gray matter), and corpus callosum. The total lipids were extracted from each section and passed through a column of Florisil to remove cholesterol and phosphatides. The crude cerebrosides were next passed through a column of mixed ion exchange resins to remove cerebroside sulfate and then purified by elution from a silicic-acid column. The acids were cleaved from the cerebrosides and separated into four classes: normal saturated, hydroxy saturated, normal unsaturated, and hydroxy unsaturated. The individual acids were then analyzed by gas-liquid chromatography. The cerebroside content of each brain region differed somewhat, as did the relative contents of hydroxy acids. However, the distribution of the acids within each class was rather independent of brain location. The normal saturated acids contained stearic and lignoceric as the major acids, but fairly large amounts of the C_{22}, C_{23}, and C_{25} acids were also present. The hydroxy saturated acids were similar, but contained little hydroxystearic acid. The unsaturated acids of both classes contained the C_{24} acid as the major constituent, together with considerable amounts of the C_{25} and C_{26} acids.

As part of a study of glycolipids, we have analyzed different regions of human brain. Although it has been known for a long time that there is a higher cerebroside concentration in white matter than in gray matter, nothing is known of the content of the individual fatty acids in the cerebrosides of different portions of the brain. A procedure for such analysis had been developed for rat brain (1) and this has been modified for use with human brain. The main modification involved replacement of the saponification step by the use of silicic-acid chromatography.

EXPERIMENTAL

Samples of frozen, unfixed human brain weighing 7 to 50 g were extracted and the lipid extracts purified with Florisil and ion exchange resins as previously described (1). The moderately pure cerebrosides were then prepared for application to a column of silicic acid[1] by making an intimate mixture with a small portion of the adsorbent. This was done by dissolving the lipids in chloroform-methanol 2:1 (v/v), adding the silicic acid (1.2 g/100 mg lipids), and removing most of the solvent with a rotary vacuum evaporator. The remaining solvent was removed by drying in air overnight and then for several hours at high vacuum (3).

A slurry of additional silicic acid (30 g/100 mg cerebrosides) was prepared in Skellysolve B (mainly hexane). The slurry was swirled 30 minutes to remove trapped air and poured in portions, with tapping, into a long glass column (1.8 or 2.2 mm inside diameter). The column was fitted at the bottom with glass wool, sand, and a very fine needle valve. The preparation of the packing was speeded by applying about 5 psi air pressure to the top of the column. The length of the packed region ranged between 70 and 95 cm. The silicic acid was conditioned in the column after the method of Weiss (4) by passing through chloroform-methanol 2:1 (v/v), 5.5 ml/gram silicic acid, then chloroform, 4 ml/gram silicic acid. The solvents were pumped through by means of a precision plunger-type pump, after the method of Spackman *et al.* (5). The means of connecting are shown in Figure 1.

The lipid sample, which had been dried in silicic acid, was then added as a slurry in chloroform and pumping was begun with chloroform-methanol 92:8 (v/v) at a flow rate of about 25 ml per hour. Fractions of 15 ml

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[1] Bio-Rad Laboratories, Richmond, Calif. Prepared according to the method of Hirsch and Ahrens (2) and used without further treatment.

each were collected and evaporated to dryness. The fractions comprising the major peak were pooled and analyzed for galactose content by the phosphoric acid-anthrone method (6).

The cerebrosides thus obtained (141 to 242 mg) were split by refluxing under a very slow stream of nitrogen with 10 ml of concentrated HCl and 50 ml of methanol. After 4 hours of heating, 50 ml of water was added and the mixture of fatty acids and esters was extracted with five 40-ml portions of ether. The ether extract was washed with water, evaporated to dryness under vacuum with the aid of toluene (to prevent splashing), and the residue esterified with dimethoxypropane, HCl, and methanol (7).

Following removal of the solvents under vacuum, the esters were separated into normal and hydroxy esters by chromatography with Florisil. Each fraction was then further separated into saturated and unsaturated groups with the aid of mercuric acetate (1). The mercuric acetate reaction was modified by allowing the reaction mixture to sit overnight at room temperature instead of heating. The hydroxy esters were converted to methyl ethers and all four fractions were analyzed by gas-liquid chromatography (1).

DISCUSSION OF THE METHODS

Isolation of the Purified Cerebrosides. Our previous work with rat brain cerebrosides had disclosed the presence of an impurity characterized by a carbonyl group, as shown by infrared spectra. The impurity could be removed by treatment with alkali and then ion exchange resins. However, this method failed in the case of human cerebrosides and we turned to silicic acid, which had recently become available in a much improved form. The silicic acid satisfactorily removed the carbonyl impurity and gave cerebrosides of reasonably good galactose and fatty acid content (Table 1). The sphingosine values were high, particularly in the second group of analyses (see below).

The silicic-acid column yielded some minor fractions, as well as a small amount of lipid trailing from the cerebroside peak. The trailing portion, which was discarded, contained only about 1% of the cerebroside hexose.

It was found that bubbles appeared in the silicic-acid column unless several pounds of pressure were maintained throughout the packing. This pressure was maintained by constricting the outlet with the needle valve at the bottom. In our earlier work we used a piece of Neoprene tubing and a screw clamp to adjust the resistance, but the swelling action of the solvent made it necessary to adjust the clamp frequently. The needle valve also needs one or two adjustments, but this is very easily done. It should be pointed out that this type of column restrictor withstands pressure much better and gives flow adjustments much more easily than the Teflon® stopcocks we have tried, and is in effect cheaper than these stopcocks because it can be removed readily and placed on the outlet of any other column fitted with quarter-inch glass capillary tubing.

The other metering valve (Fig. 1) was included in the

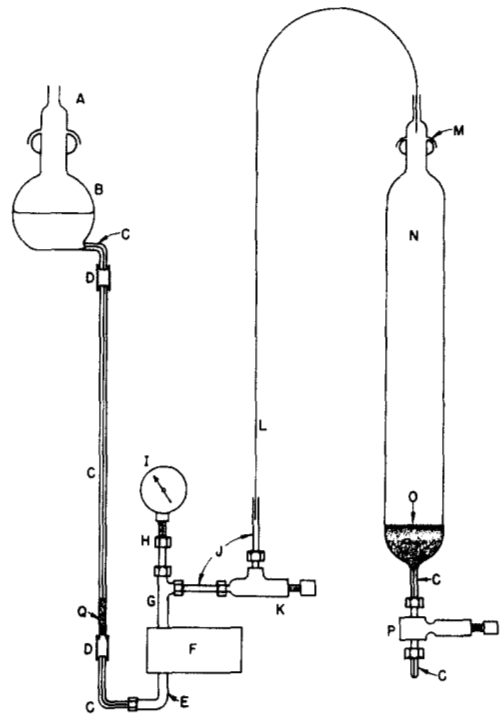

FIG. 1. Pumping system for lipid chromatography. *A*. Connection to compressed air, for priming the pump. *B*. Reservoir flask for solvent. Capillary tube, *C*, is offset to allow placing magnetic stirrer under flask when gradient elution is desired. *C*. Capillary glass tubing, nominally 6 to 7 mm diameter, chosen to fit through 1/4″ Teflon Swagelok ferrules. *D*. Neoprene tubing, previously extracted with chloroform-ethanol in a Soxhlet extractor. *E*. Swagelok elbow, stainless steel, #400-2-2-316, with Teflon ferrules, #403-1 and 404-1 (Crawford Fitting Co., Cleveland 10, Ohio). *F*. Chromatographic miniPump, 1/8″ plunger, 29 rpm, maximum capacity 124 ml/hour (Milton Roy Co., Philadelphia 18, Pa.). *G*. Swagelok tee, #200-3TMT-316. *H*. Swagelok adapter, #201-A-2F-316. *I*. Small pressure gage, 1/8″ pipe connection. *J*. Short piece of 1/8″ outside diameter stainless steel tubing. *K*. Metering valve, Nupro SS-2MA (Nuclear Products Co., Cleveland 10, Ohio). *L*. Stainless steel hypodermic needle tubing, 20 gauge. One end is soldered into the 1/8″ steel tubing; the other end is cemented into a piece of capillary glass tubing with epoxy cement. The capillary tubing is previously sealed to an 18/7 socket. *M*. Neoprene sheet rubber gasket, 1/64″ thick, made with two cork borers. The 18/7 joint is held together with two or three spring clamps. *N*. Chromatographic column, made of standard or heavy wall tubing. *O*. Glass wool and sand; base for packing. *P*. Metering valve, Nupro SS-4M, with 1/4″ Teflon Swagelok ferrules. *Q*. Glass wool filter, to keep dirt out of pump.

pumping system to allow raising the resistance further, thereby increasing the accuracy of the pump (5). We adjusted this valve to keep the pump pressure at about 20 psi.

There is an appreciable volume of liquid in the pressure gauge; this tends to diffuse into the main stream of solvent rather slowly when a change is made to a solvent of higher density. For this reason we evacuated the pumping line for 15 minutes before changing from chloroform-methanol to chloroform.

The sample of cerebrosides was added to the silicic-acid column as a dried mixture in a little silicic acid because the lipid was too insoluble to be dislodged from the glassware by chloroform. Addition as a solution in chloroform-methanol (4) and elution with chloroform (of increasing methanol content) gives two cerebroside peaks instead of one. It is evident that this technique causes part of the cerebrosides to move with the initial portion of chloroform-methanol, and the remainder of the cerebrosides remain adsorbed until the methanol content of the eluting liquid is high enough to move them as an equilibrating sample. The finding by Weiss (4) that lignoceric acid could be isolated from the first peak and cerebronic acid from the second suggests that there is a preferential removal of the less polar cerebrosides in the solvent used for addition. This explanation was confirmed[2] by analyzing two such cerebroside fractions with the Florisil method. Both fractions contained normal acids, the content being somewhat lower in the second fraction.

TABLE 1. ANALYTICAL DATA FOR PURIFIED CEREBROSIDES

	1	2	3
	Galactose	Fatty Acids*	Sphingosine
	per cent	*per cent*	*per cent*
Cerebral cortex, white	20.9†	46.6	
	21.1	44.5	40.7
Cerebral cortex, gray	21.6	46.1	39.6
	21.4	45.2	41.7
Cerebellum	21.4	46.2	38.0
	21.5	44.9	44.4
Corpus callosum	23.6	48.5	39.8
	21.6	44.2	43.1
Theoretical values‡	21.6	46.5	35.9

* As methyl esters.

† In paired figures, top figure = brain from 70-year-old man; bottom figure = brain from 44-year-old man.

‡ Calculated from typical fatty acid analyses, assuming cerebrosides are monohydrate; MW = 835.

Splitting of the Cerebrosides. Ethanol-aqueous HCl has been used by Skipski *et al.* (8) for splitting off the acids of cerebrosides, and such a system is far more convenient than dry HCl mixtures. However, the formation of ethyl esters would make gas-liquid chromatography more difficult, so we substituted methanol for ethanol and raised the reflux period to 4 hours. Several trials had indicated this period was sufficient, but the low yields of esters from our second brain (Table 1, column 2) suggest that this period is too short. Sweeley and Moscatelli (9) have used 5 to 6 hours with methanol-HCl.

The crude esters obtained by ether extraction of the hydrolyzates weigh more than expected from theory and evidently contain some sphingosine, extracted possibly as the fatty acid salt. When the esters are separated with Florisil, the sphingosine is held back on the column and can be eluted with acetic acid-chloroform-methanol 3:4:4 (v/v). In the case of our second brain, the slightly low yields of esters were accompanied by a high yield of this sphingosine fraction. It seems likely that this fraction included ceramides. Although it is possible that incomplete hydrolysis may cause preferential splitting of one class of cerebrosides, the data for the fatty acid analyses do not indicate that this is an important effect.

The values in Table 1 for sphingosine were obtained by combining the weights of the two long-chain base fractions obtained by (*a*) taking the residual water-HCl-methanol solution after removal of esters, making it alkaline (Tropaeolin O indicator), and extracting with ether; and (*b*) eluting the Florisil column with acetic acid-chloroform-methanol and washing the chloroform with water, alkali, and water. The pooled sphingosine fractions were analyzed by Dr. Charles C. Sweeley, of the University of Pittsburgh, by gas-liquid chromatography (9).

It should be noted that the isolation method described here could be used equally well with much smaller amounts of brain, since no crystallization steps are involved. The large amounts were used mainly to help in validating the individual steps.

DISCUSSION OF THE DATA

The data obtained from the steps prior to gas chromatography are shown in Table 2.

Total Lipids and Cerebrosides. Columns 1 and 2, in

[2] Y. Kishimoto and N. S. Radin. Unpublished work.

TABLE 2. ANALYTICAL DATA FOR LIPIDS AND FATTY ACID CLASSES*

	1	2	3	4	5	6	7
	Total Lipids	Total Cerebrosides	Normal Acids	Hydroxy Acids	Hydroxy/ Normal	Saturated Normal/ Total Normal	Saturated Hydroxy/ Total Hydroxy
	per cent of wet brain		*per cent of cerebrosides*				
Cerebral cortex, white	21.2	3.36	15.6	31.0	1.99	0.58	0.57
	18.9	2.99	13.8	30.7	2.23	0.52	0.71
Cerebral cortex, gray	8.4	0.49	12.7	33.4	2.63	0.79	0.59
	8.5	0.47	5.4	39.8	7.37	0.70	0.71
Cerebellum	9.0		7.9	38.3	4.85	0.52	0.56
	8.6	0.67	12.3	32.6	2.65	0.47	0.69
Corpus callosum	21.4	3.52	20.7	27.8	1.34	0.51	0.64
	17.7	2.66	15.4	28.8	1.87	0.53	0.76

* See footnotes to Table 1 for explanatory notes.

agreement with the work of many others, show that white matter contains much more lipid and cerebrosides than gray matter. The difference is greater in the case of cerebrosides. Cerebellum resembles gray matter in this respect. The two brains differ in that the lipid and cerebroside concentrations are greater in the older brain, especially in the corpus callosum. Our figures for cerebroside concentrations are about one-third lower than those found by other workers (10, 11, 12), due mainly to the inclusion of cerebroside sulfate in the earlier analyses.

Hydroxy Versus Normal Acids. The values found for these two classes of acids are shown in columns 3 and 4, and the ratios of the two classes in column 5. As in rat brain cerebrosides (13), the hydroxy acids predominate. Cerebellum again resembles gray cortex, both showing somewhat greater predominance of hydroxy acids. Large individual differences are apparent in column 5, but we cannot state now whether these can be correlated with age.

Saturated Versus Unsaturated Acids. To conserve space, we present only the ratios of the concentrations. In column 6 we see that the degree of unsaturation in the normal acids is quite similar in both brains in all brain regions except gray cortex, where the saturated acids predominate. The degree of unsaturation is higher than that found in rat cerebrosides (13).

In the case of the hydroxy acids (column 7), there is marked similarity in all brain regions of any one brain. However, the younger brain consistently shows less unsaturation.

Gas-Liquid Chromatographic Data. Figures 2 to 5

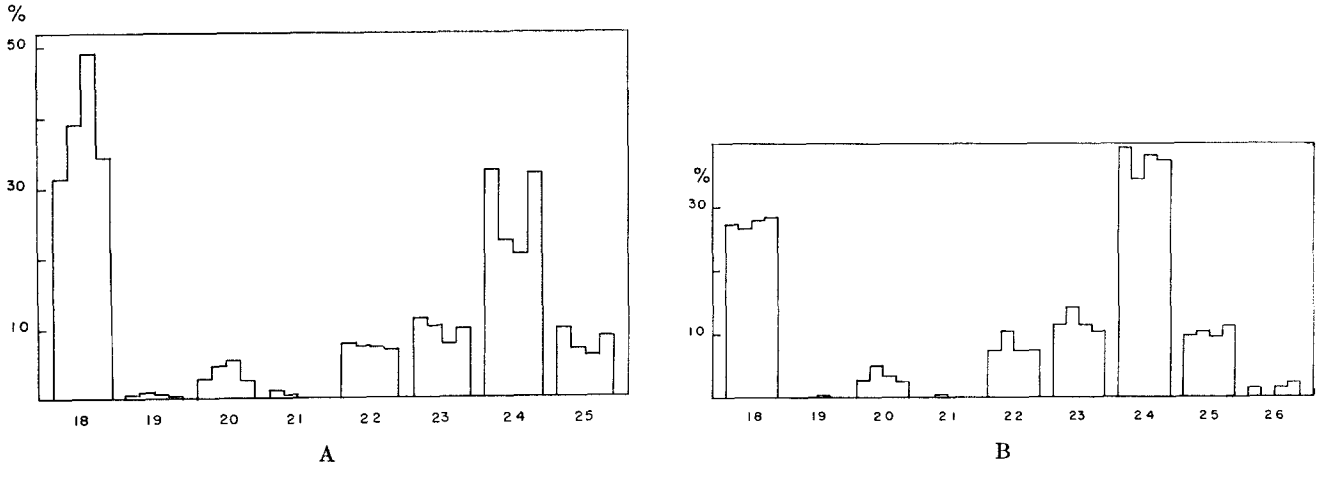

FIG. 2. Distribution of each normal saturated acid in brain cerebrosides, calculated as percentage of the total normal saturated esters. Each bar consists of four sections, corresponding (left to right) to cortex white, cortex gray, cerebellum, and corpus callosum. Figure 2*A* shows the results for the older brain; Figure 2*B*, for the younger brain. The trace of C_{19} acid in Fig. 2*B* is in the cerebellum and the C_{21} acid is in the cortex gray.

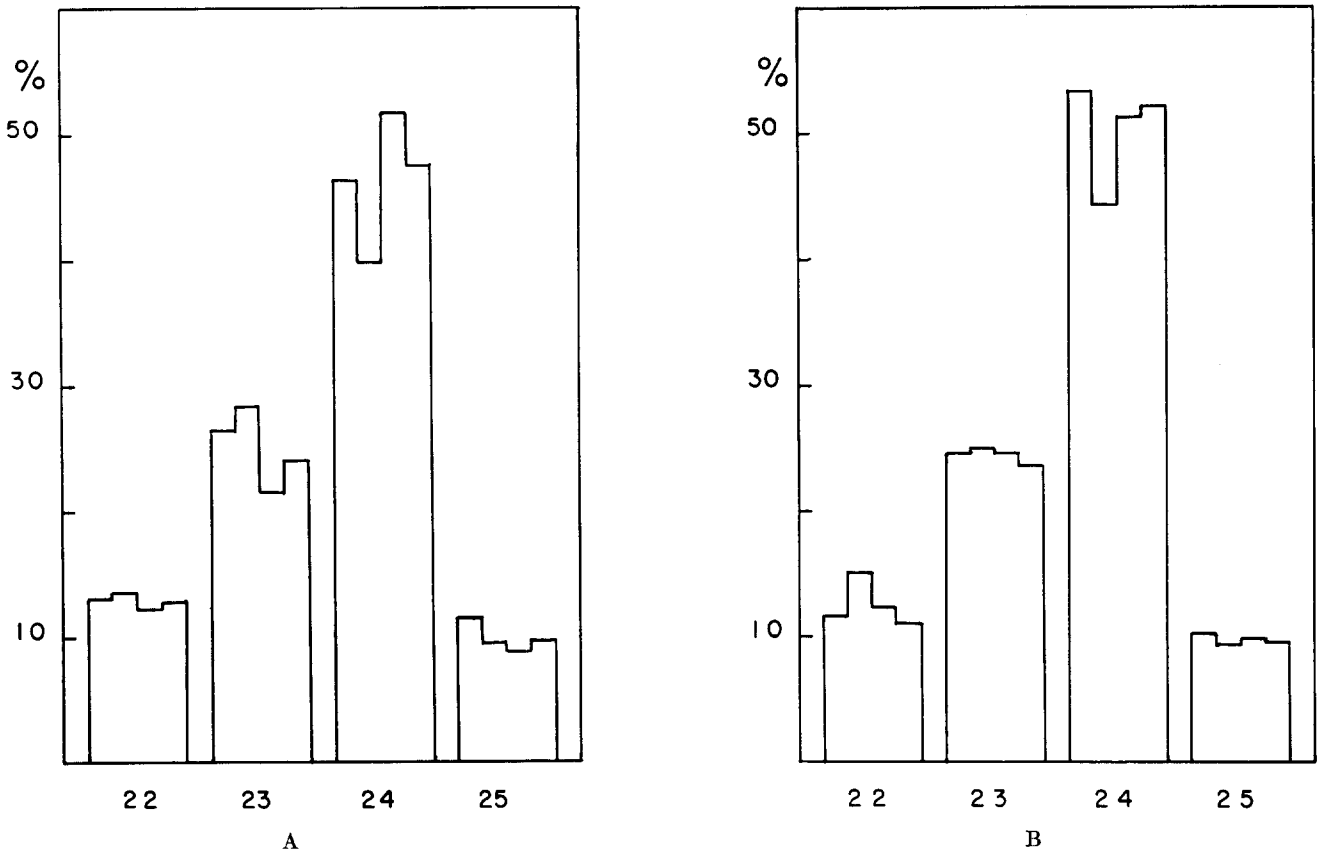

FIG. 3. Distribution of each hydroxy saturated acid. See Figure 2 for further description.

show the results of gas-liquid chromatographic analysis of each fraction. Excluded from the graphs are the longer and shorter acids that were found. Most of these occur in concentrations less than 1%, with a few over 2%. A slight distortion in the percentages arises from the use of esters and ester ethers, which have molecular weights differing from the acids originally present in the cerebrosides.

The normal saturated acids contain stearic and lignoceric acids as their major components, but appreciable amounts of the C_{22}, C_{23}, and C_{25} acids are also present. As in rat cerebrosides (1), the content of odd-numbered acids is unusually high. This high content explains much of the variability in analytical data reported for the C_{24} acids isolated from brain by conventional methods.

The hydroxy saturated acids also show a high content of C_{22}, C_{23}, and C_{25} acids. It has been found in rat brains that the concentrations of the odd-numbered acids increased with age (13), and the finding that the C_{23} hydroxy acid is the second major acid is consistent with the rat data. In contrast to the normal saturated acids, there is only a trace of the C_{18} acid, except in gray cortex. In this region there is a lower value for cerebronic acid that is accompanied by relatively high values (1.1% to 2.3%) for the C_{20}, C_{18}, C_{16}, and C_{14} hydroxy acids. This characteristic of gray cortex was found in both brains.

The unsaturated acids of both classes are notable for their high content of C_{25} and C_{26} acids, especially in comparison with the shorter acids. The C_{24} acids, however, play a much larger role in the unsaturated acids, as noted in our work with rat acids. Klenk and Leupold (14) have isolated the C_{26} normal unsaturated acid.

Trace amounts of unknown acids were observed in the two classes of hydroxy acids, mainly in the C_{19} region.

Particularly interesting is a comparison of the different brain regions with respect to the distribution pattern of the individual fatty acids in each class. This may be done by examining the profile of each bar in the bar graphs. Taking into consideration the variability intrinsic in the analytical method, one can see a marked similarity between the various brain regions. The similarity is even more marked when one reviews the large differences in cerebroside concentrations and ratios of hydroxyl to normal acids (columns 2 and 5, Table 2).

Only one serious exception to this similarity appears,

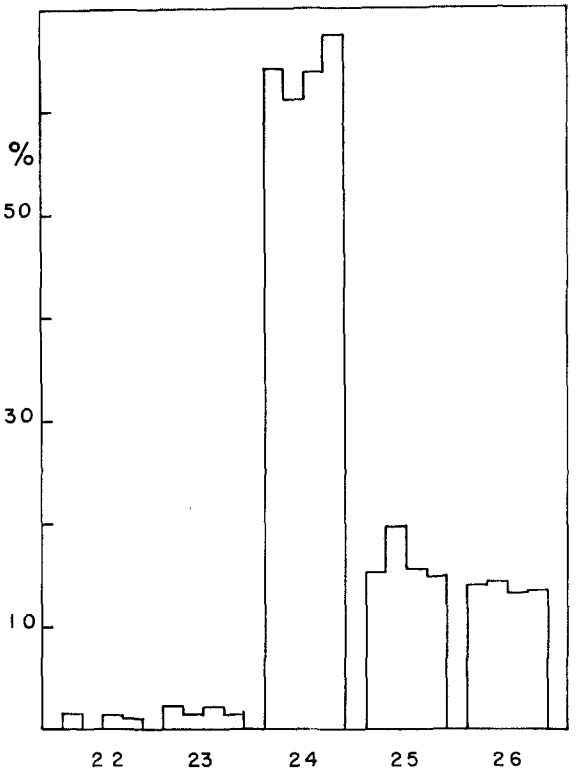

FIG. 4. Distribution of each normal unsaturated acid (younger brain only). See Figure 2 for further description.

in Figure 2*B*, where the stearic and lignoceric acid proportions differ somewhat in the gray cortex and cerebellum. There appears to be a small but consistent difference in all the cerebroside acids of gray cortex, where there is a little less of the C_{24} acids and more of the shorter acids. The effect is most noticeable, as mentioned before, in the hydroxy saturated acid group. Baker (15) has recently reported finding that the shorter acids of the nervous system (ester-linked) become more and more predominant with increasing distance from the center of the brain, a finding consistent with the above. Similarly consistent is the finding[3] that hydroxystearic acid is an important component of the spinal cord cerebrosides (8).

Although much more work is needed to clarify the significance of our data, we would like to offer a tentative hypothesis to explain the similarity in acid distribution between brain sections. If two or more types of cells synthesize the cerebroside acids, one would expect that each type would yield a different distribution pattern, that the two types of cells would occur in ratios differing with their location in brain, and that the distribution pattern would therefore differ from region to region. That the distribution of cell types does vary greatly with brain location is well known. It would appear, then, that only one cell type synthesizes the cerebroside acids (and presumably the cerebrosides too). The concept of a single cell type as the source of myelin has been proposed by Korey (16). This hypothesis also serves to explain the similarity in degree of saturation in the normal and hydroxy acids of the four brain areas (gray cortex again being the exception). However, one must then assume that the relative production of hydroxy acids is influenced by the location of the cells.

[3] A. K. Hajra and N. S. Radin. Unpublished work.

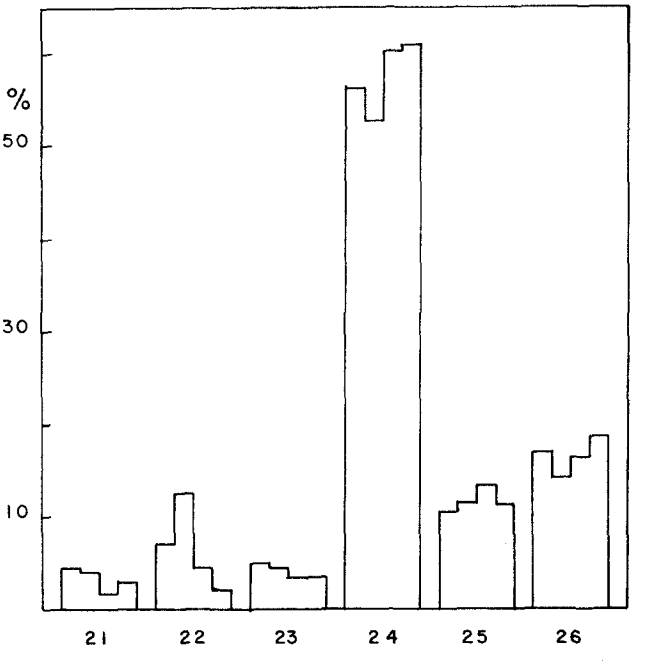

FIG. 5. Distribution of each hydroxy unsaturated acid (younger brain only). See Figure 2 for further description.

Analysis of the four sphingosine samples from one of the brains, kindly carried out by Dr. Sweeley, showed that 95% or more of the bases was sphingosine, about 2% was dihydrosphingosine, and a trace consisted of an unknown long-chain base. Here too the four brain areas were very similar and our postulate of a single cell type may be extended to cover the synthesis of the long-chain bases.

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