6-Acyl galactosyl ceramides of pig brain: structure and fatty acid composition

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ABSTRACT Two glycolipids were isolated from pig brain and were shown to be the fatty acid esters of kerasin and cerebron in which the second fatty acid moiety is attached to the 6-position of the galactose. The point of attachment was shown in two ways: by permethylation and by cleavage with periodate. Methanolysis of the permethylated cerebroside esters yielded *O*-methyl sphingosines, methyl esters of nonhydroxy or 2-methoxy acids, and methyl 2,3,4-trimethyl galactoside. Cleavage of the cerebron ester with periodate, followed by treatment with sodium borohydride and dilute HCl, yielded ceramide plus 1-monoglyceride. The ester-linked fatty acids were primarily 16:0, 18:0, and 18:1, while the amide-linked fatty acids showed the wide assortment of chain lengths typical of brain cerebrosides. The methylation step, with silver oxide and methyl iodide, yielded two derivatives with the cerebroside esters, but the structural explanation for the difference was not elucidated. The galactose in the cerebron ester was shown to exist in the β-pyranoside form.

KEY WORDS 6-acyl cerebrosides · ester-type glycolipids · structure determination · sphingosine derivatives

THE isolation of fatty acyl esters of cerebroside from brain was first reported by Norton and Brotz (1). They obtained a mixture of compounds, among which were fatty acyl derivatives of cerebron. The additional fatty acid was attached in ester linkage at an indeterminate position. Quite recently Klenk and Doss (2) have shown that the analogous nonhydroxy compound, fatty acyl kerasin, is also present. This paper reports further characterization of the acyl cerebrosides and evidence for the attachment of the ester group at the 6-position of the galactose.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DEGS, poly(diethylene glycol succinate). Solvents: C, chloroform; M, methanol; W, water. Fatty acids are identified by number of carbon atoms: number of double bonds (e.g. 18:0 = stearic acid). The term *kerasin* is used here to represent the cerebrosides containing nonhydroxy fatty acids and *cerebron* to represent those containing 2-hydroxy fatty acids. The lipids isolated are designated KE (kerasin esters), CE (cerebron esters), UE (unknown esters), and GG (galactosyl glycerides). *Ceramides* are the *N*-acyl sphingosines; the term *hydroxy ceramide* is used to designate those ceramides in which the fatty acid is a 2-hydroxy acid. *Galactosyl ceramides* are cerebrosides in which the sugar residue is galactose.

Pending an international ruling on the designation of the position of acyl substituents in cerebrosides, we have arbitrarily adopted a convention whereby the unprimed number in "6-acyl cerebrosides" refers to the 6-position of the glycosyl moiety. This is in accordance with the present name "cerebroside 3-sulfate." We suggest that any acyl derivatives of the sphingosine moiety be numbered with a prime, e.g., "3′-acylcerebroside" for one in which the 3-hydroxyl of sphingosine is esterified.

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MATERIALS AND METHODS

Isolation

Since an investigation of the subcellular distribution of brain lipids (3) showed that the acyl cerebrosides were not detectable in myelin, we started with brain from which much of the white matter had been removed. The cerebral cortex and cerebellum from 12 fresh pig brains were washed to remove blood, the outer membrane was discarded, and the tissue (715 g) was extracted with four volumes (2860 ml) of C–M 50:50. After filtration, the residue was extracted again with two volumes of C–M 2:1. The pooled extracts were evaporated to dryness by lyophilization from benzene, taken up in C–M 8:1, and loaded onto a column of Florisil (deactivated with 8 ml of water per 100 g of Florisil). The loading ratio was 25 mg of lipid per g of Florisil. Elution with C–M 8:1 (2 ml/g of adsorbent) removed most of the cholesterol, and an additional 0.4 ml/g yielded all the glycolipid esters. The effluent was collected in fractions that were analyzed by TLC, which

readily separates the esters from the faster-moving cholesterol and slower-moving cerebroside.

The concentrate was purified further by chromatography on silica gel (Unisil, Clarkson Chemical Company, Inc., Williamsport, Pa.). The loading ratio was 110 mg/g of adsorbent and the first solvent was chloroform (10 ml/g), which removed most of the cholesterol. Elution with C–M 96:4 (12 ml/g) yielded the cerebroside esters, now free of kerasin.

The third purification step was carried out on another Unisil column, 1 × 44 cm, with a much lighter load (10 mg/g). Elution with chloroform (4 ml/g) and C–M 98:2 (5 ml/g) removed traces of cholesterol and of what appeared to be nonhydroxy ceramide. Further elution with C–M 98:2 and 96:4 (15 ml/g) only partially separated cerebroside esters from galactosyl glycerides [also found in this fraction by Norton and Brotz (1)]. While this purification step was thus not completely effective, it was essential for removing ceramide, which migrates on TLC together with the cerebroside esters.

The pooled esters were applied to 0.5 mm layers of Silica Gel HR with a streak applicator (Radin-Pelick Streaker, Applied Science Laboratories Inc., State College, Pa.) and chromatographed in C–M 90:10. The weight of esters applied to each 20 cm wide plate was 20 mg. The bands were made visible by means of a spray of M–W 1:1, which is superior to water alone.[1] The plate was dried and the powder from each major zone was placed in a small column and eluted with C–M–W 7:7:1 (4). A half volume of water was added to each eluate to extract water-soluble impurities eluted from the powder.

Permethylation

The three lipids with the highest R_f values were transferred to jacketed test tubes, then refluxed for 5 hr with redistilled methyl iodide (0.05 ml/mg of lipid) and silver oxide (200 mg/ml). The mixture was stirred magnetically during the refluxing and for an additional 16 hr at room temperature. A stream of nitrogen removed the excess methyl iodide, and the residue was extracted by stirring with C–M 2:1 for 10 min. The supernatant solution was removed and the residue extracted similarly three more times.

The permethylated lipids were then purified as above by TLC, with a loading ratio of 5 mg per 20 cm plate. C–M 150:2 was the developing solvent and alkaline bromothymol blue (5) the detecting agent. The zones were extracted as before, but with chloroform as the eluent. With this solvent the indicator stays in the silica gel.

The permethylated lipids were purified further on a Unisil column, first with benzene to remove a nonpolar impurity; chloroform (35 ml/g of adsorbent) was used to elute the permethylated esters. The column was washed with chloroform and benzene before use.

In addition, samples of purified kerasin and brain cerebroside were methylated as above, but with longer refluxing (23 hr).

Periodate Oxidation

Carter, Rothfus, and Gigg's method for cleaving cerebrosides with periodate (6) was modified slightly because of the lower solubility of cerebroside ester in the oxidation medium. Only the CE preparation was cleaved. 1.45 mg of this preparation were dissolved in 0.1 ml of 95% ethanol–chloroform 51:14 by slight warming. Addition of 0.04 ml of 0.1 M periodic acid yielded a slight precipitate so the suspension was sonicated briefly. The suspension was stored for 5 hr in the dark, cleared by the addition of 0.09 ml of ethanol–chloroform 2:1, and stored overnight.

To the oxidized ester we added 0.08 ml of chloroform and 0.3 ml of water and the excess periodic acid was determined by the microtitration method of Schiffman, Kabat, and Leskowitz (7) as modified by Distler, Kaufman, and Roseman (8). A 250 µl Hamilton microsyringe was used as a microburet.

After the titration, 0.5 ml of chloroform was added and the inorganic components were removed in the upper phase. The oxidized lipid was washed further with three portions of M–W 50:50, the lower-phase solvent was removed with nitrogen, and the residue was reduced with sodium borohydride (6). This was done by dissolving the lipid in 0.28 ml of 95% ethanol–chloroform 56:13 and adding 0.12 ml of $NaBH_4$ solution (2.5 mg/ml of water). Since some precipitation occurred, the mixture was sonicated and then stirred for 6 hr at room temperature. The product was hydrolyzed by adding 10 µl of 6 N HCl and leaving the mixture for 40 hr. The inorganic products were removed by adding 1 ml of C–M 50:50 and discarding the upper layer. Two washings as above yielded a lipid mixture, which was examined by TLC.

Other Materials and Methods

2,3,4- and 3,4,6-trimethyl galactose were the gifts of Dr. Pierre Stoffyn. 2,4,6-Trimethyl glucose was a gift from Dr. Irwin Goldstein. The methyl glycosides of 2,3,4,6-tetramethyl galactose and 2,4,6-trimethyl galactose were prepared by methanolysis of permethylated cerebroside and cerebroside sulfate. 1-Monopalmitin was purchased from The Hormel Foundation (Austin, Minn.). Dr. Benjamin Weiss donated generous samples of methylated sphingosines.

[1] We are indebted to Dr. Liselotte Hof for this observation.

GLC was performed on a Barber-Colman instrument, model 5000, with a stationary phase of DEGS, and an F & M model 700 with Apiezon packing as described before (9). A mixture of fatty acid methyl esters, 19:0 and 21:0, was used as internal standard for quantification (10). For the identification of the methylated sugars, the DEGS column was operated at 190°C and the Apiezon column at 130°C.

IR spectra were made by examination of KBr pellets in a Perkin-Elmer model 237. The pellets were pressed with the die-holder combination of W. R. Hewitt (St. Louis, Mo.).

RESULTS

Isolation of the Cerebroside Esters

The yield of crude lipids from 715 g of tissue was 69 g, and the yield of crude esters from the Florisil column was 2.2 g. The next two columns yielded 350 mg and then 301 mg of glycolipid esters. Fig. 1 is a thin-layer chromatogram of the ester mixture from the last column-chromatographic step and of the esters separated by preparative TLC. It is evident that the R_f values did not differ greatly and that some cross-contamination is to be expected. The length of the "unknown esters" (UE) spot suggests that this fraction is a mixture. Based on the yields from preparative TLC, the composition of the group of glycolipid esters was 5% KE, 24% CE, 16% UE, and 55% GG. All were colorless solids.

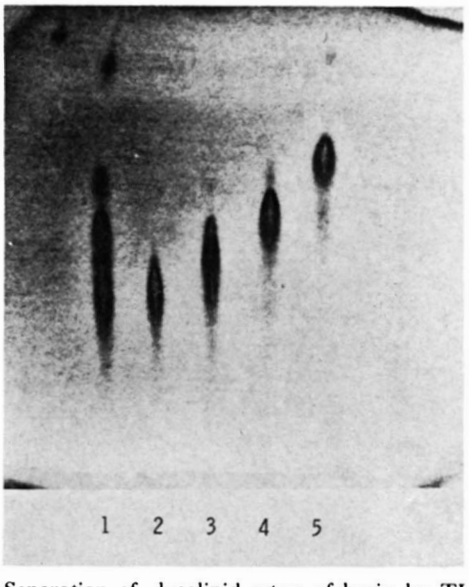

FIG. 1. Separation of glycolipid esters of brain by TLC. Conditions: 0.25 mm thick Silica Gel G developed with C–M 90:10 and sprayed with bromothymol blue. Spot *1* is the natural mixture of glycolipid esters, isolated via three column chromatographic steps (see text). The remaining spots are from the esters isolated by preparative TLC. Spot *2* is GG (galactosyl glycerides), spot *3* is UE (unidentified esters), spot *4* is CE (cerebron esters), and spot *5* is KE (kerasin esters).

Infrared Spectra

The spectra of KE and CE were very similar. They differed from the spectrum of brain cerebrosides in several respects: (*a*) the esters showed a strong peak at 5.75 μ (about as strong as the amide carbonyl peak), due to the ester linkage; (*b*) the amide carbonyl peak was shifted from 6.15 μ to about 6.05 μ; and (*c*) the absorption of the *trans* double bond of sphingosine at 10.3 μ was quite weak. Norton and Brotz noted the same peak at 6.05 μ but did not comment on the shift (1). The spectrum of UE was somewhat more complicated.

The presence of a peak at 12.8 μ in KE, CE, and UE showed that the galactose existed in pyranose form (11). A peak at 11.2 μ, characteristic of β-galactosides (12), was seen clearly only in the spectrum of UE. None of the samples showed a peak at 11.85 μ, which would be expected if the galactose were present in the α-form.

Alkaline Methanolysis Products

Treatment of the four esters with NaOH–C–M for 5 min at room temperature (13) followed by washing and TLC yielded methyl esters and cerebrosides. KE gave primarily kerasin, with a little cerebron, and CE the reverse. UE gave both cerebroside spots, but primarily cerebron. GG gave a faint spot for cerebron together with a strong spot just below, which evidently represents the alkyl ether of galactosyl glycerol. Norton and Brotz obtained this compound from glycolipid B (1) by saponification. Like the lysophosphatides, this lyso compound appeared as a white spot on TLC plates sprayed with bromothymol blue. Acid hydrolysis of crude acyl galactosides gave rise to 1-alkyl glycerol, identified by comparison on TLC with batyl alcohol and the use of periodate–benzidine sprays, which reveal glycols (14).

It is evident that all the isolated preparations were slightly contaminated with adjacent lipids. It is also apparent that UE is a mixture of esters of kerasin and cerebron that differ in structure from KE and CE as defined here.

Examination of the IR spectrum of deacylated CE showed good agreement with published spectra for cerebron (15, 16). Of particular interest was the distinct peak at 11.2 μ, which indicates that the galactose in CE is in β-linkage.

Permethylation Products

Examination by TLC of the crude permethylated KE and CE revealed the presence of two major spots of similar intensities, together with a few slower minor spots. Fig. 2 shows the TLC of the ethers after purification by preparative TLC; *a* refers to the faster compound and *b* refers to the slower one. The yields of methylated KE*a* and KE*b* were 2.3 mg and 1.3 mg from 7 mg of KE.

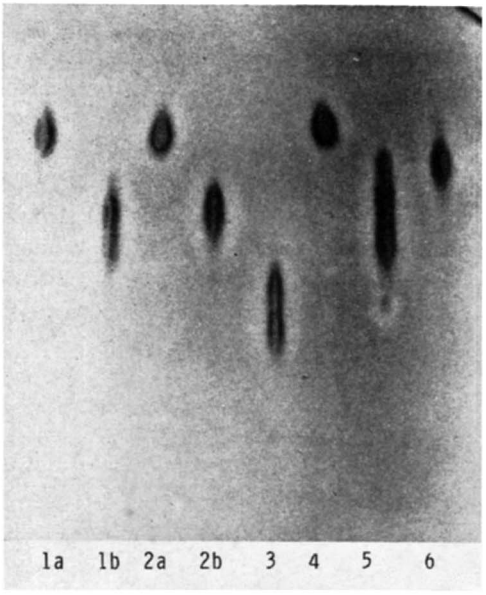

FIG. 2. Separation of permethylated cerebroside and permethylated cerebroside esters by TLC. TLC conditions as in Fig. 1 except that the solvent was C–M 75:1. Spots *1a* and *1b* are derived from KE; *2a* and *2b* are from CE; *3* is from total brain cerebroside (kerasin + cerebron); *4*, *5*, and *6* are from UE.

From 25 mg of CE, 5.0 mg of CE*a* and 5.2 mg of CE*b* were obtained. Lipid UE yielded several products which were isolated by TLC as three fractions.

IR spectra of the four ethers from KE and CE closely resembled one another. Their ester band at 5.75 μ was unchanged from that of the original esters (showing that the methylation procedure did not destroy the ester bond) and a moderately strong band at 9.1 μ due to the methyl ether linkages was now visible. The *trans* peak at 10.3 μ was now strong and clear, as in brain cerebroside, and a small peak at 7.3 μ became larger in methylated CE. A distinct shift could be seen in the permethylated CE compounds: the amide peaks that were initially at 6.08 and 6.50 μ moved to 5.94 and 6.60 μ in CE*a* and 6.00 and 6.57 μ in CE*b*.

TLC of permethylated kerasin revealed only one spot, with an R_f equal to that of the faster of the pair of spots given by permethylated brain cerebroside (Fig. 2). Since brain cerebroside contains both kerasin and cerebron, which separate readily on TLC plates, it seems that the permethylated compounds are also separated to some extent in the system used here. It is interesting that permethylated cerebrosides exhibited distinctly lower R_f values than permethylated cerebroside esters.

The Fatty Acids of the Permethylated Esters

Unlike the original cerebroside esters, the methylated derivatives were rather stable to alkaline methanolysis. Exposure to the same reagent (NaOH–C–M), but this time for 24–48 hr, followed by TLC, showed almost complete disappearance of the glycolipid esters, formation of methyl esters, and a new spot with an R_f lower than that of permethylated brain cerebrosides. The new spot was presumably that of kerasin or cerebron in which all hydroxyl groups were methylated except for the position previously occupied by a fatty acid moiety. The deacylated ethers from the *a* forms of permethylated KE and CE exhibited slightly higher R_f values than the *b* forms. KE*b* also yielded a small spot that moved faster than the glycolipid spot from KE*a*.

The methanolysis products were purified on a silica gel column, benzene yielding the methyl esters (from the ester-linked fatty acids) and C–M 2:1 yielding the deacylated lipid. The *N*-acyl moiety of the deacylated lipid was methanolyzed with 5% HCl in methanol and the resultant methyl esters were extracted with four portions of hexane (17).

The esters derived from amide linkage of permethylated KE*a* and KE*b* were shown by TLC to be primarily of the nonhydroxy type, although methyl ethers of the 2-hydroxy esters were also present. The reverse was found for CE*a* and CE*b*. The presence of the minor components was expected from the earlier finding of cross-contamination in KE and CE (shown by methanolysis of the *un*methylated lipids). Not more than a trace of unmethylated hydroxy acid was found, which showed that the esterified fatty acid was not linked to the hydroxyl of the amide-bound fatty acid of CE. (Of course this could not be the site of linkage in KE.)

The chain-length distribution within the two types of fatty acids and the *a* and *b* derivatives is shown in Table 1. As shown by Klenk and Doss with human brain (2) the ester-linked acids are primarily 16:0, 18:0, and 18:1. We also found some 16:1 and our distribution was somewhat different (they reported 65%, 27.5%, and 7%, respectively).

The nonhydroxy amide-linked acids in the KE and CE preparations differed in their chain-length distributions. Those of KE showed primary emphasis on 24:0 and 24:1, with the usual wide distribution seen in brain kerasin (17). Those of CE showed the same assortment of acids, but 16:0, 18:0, and 18:1 were relatively dominant. This difference is evidently due to the ability of silica gel TLC plates to achieve partial separation of lipid families on the basis of fatty acid chain length, those members with shorter chain lengths migrating more slowly. This phenomenon is observed frequently with sphingomyelin; see also the TLC separations of Gaucher spleen cerebrosides (19). Since the KE and CE preparations were isolated by means of preparative TLC, the phenomenon explains why there was some cross-contamination in the final materials.

The same partial separation by chain length is seen

TABLE 1 FATTY ACID COMPOSITION OF PERMETHYLATED CEREBROSIDE ESTERS (DATA IN PERCENTAGE OF TOTAL FATTY ACIDS IN EACH CATEGORY)

	Ester-Linked Acids				Amide-Linked Nonhydroxy Acids				Amide-Linked Hydroxy Acids			
Fatty Acid	KE*a*	KE*b*	CE*a*	CE*b*	KE*a*	KE*b*	CE*a*	CE*b*	KE*a*	KE*b*	CE*a*	CE*b*
16:0	42	38	42	39	3	5	20	17				
16:1	3	3	3	3								
18:0	33	35	28	30	8	9	18	16				
18:1	22	24	26	28	1	3	11	9				
20:0					3	3	3	4				
22:0					10	12	7	10	16	15	19	25
22:1					2	2					2	2
23:0					4	4	4	4			8	8
23:1					1	1					1	1
24:0					21	23	13	13	40	38	31	34
24:1					37	28	25	28	28	28	23	20
25:0					2	2					3	2
25:1					3	2					3	2
26:0					1	2					3	2
26:1					5	4			17	18	8	6

KE, kerasin esters. CE, cerebron esters. *a* and *b* denote two forms of permethylation products, faster- and slower-moving, respectively, on TLC (see Fig. 2).

in the acyl cerebrosides containing hydroxy acids (Table 1). The CE contaminating the KE preparation was enriched in hydroxy acids of higher chain lengths. The distribution of chain lengths in CE hydroxy acids was typical of brain cerebron (18).

Comparison of the fatty acid composition of the *a* and *b* forms of the permethylated lipids shows no distinct difference in chain length distributions. Thus the differences in their R_f values must be attributed to some other characteristic.

The molar ratios of the ester-linked and amide-linked fatty acids of the different preparations are shown in Table 2. These ratios are close to 1 in both KE and CE. Comparison of the two types of amide-linked acids shows that roughly 10% cross-contamination between KE and CE occurred.

TABLE 2 RELATIVE PROPORTIONS OF THE DIFFERENT GROUPS OF FATTY ACIDS IN THE PERMETHYLATED ACYL CEREBROSIDES (DATA ARE REPORTED IN MOLAR RATIOS VS. ESTER-LINKED ACIDS)

Permethylated Ester	Ester-Linked Fatty Acids	Amide-Linked Nonhydroxy Acids	Amide-Linked Hydroxy Acids
KE*a*	1	0.92	0.06
KE*b*	1	0.92	0.09
CE*a*	1	0.13	0.83
CE*b*	1	0.14	0.82

The Sphingosines of the Permethylated Lipids

After acid methanolysis of the methylation products from KE, CE, and UE, and extraction of the methyl esters, the methanol layer was evaporated in a stream of nitrogen. The residue was dissolved in methanol and examined by TLC with C–M–W–ammonia (20). Bromothymol blue gave blue spots for the amines and ninhydrin (after bromothymol blue) gave pink spots (21).

The main product in each case, as detected by bromothymol blue, gave a pair of closely overlapping spots corresponding to *erythro-* and *threo*-3-*O*-methylsphingosines. The faster spot appeared to be isomer I, the slower, isomer II, as named by Carter, Norris, Glick, Phillips, and Harris (22). Weiss (23) has shown that these isomers (as obtained from cerebrosides by refluxing with methanolic sulfuric acid) also contain 5-*O*-methylsphingosines, which form by allylic rearrangement during methanolysis. The solvent system used here did not separate the 3- and 5-isomers, so we cannot tell whether our conditions of methanolysis also formed the 5-isomers.

The reaction of the *O*-methylsphingosines with ninhydrin was poor compared to that with the pH indicator.

All samples gave rise to a bromothymol blue-reactive spot above the methylsphingosines, which reacted more intensely with ninhydrin than did the latter. Perhaps this is a degradation product formed from the *O*-methyl ether during methanolysis, such as dehydrosphingine. Traces of sphingosine and dihydrosphingosine could be seen in all samples, particularly the latter in CE*b*. It is likely that a small portion of CE*b* was actually incompletely methylated CE*a*. We had noticed in trial runs with cerebroside that the 3-positions in the dihydrosphingosine and galactose moieties were methylated rather slowly. The trace amounts of sphingosine that were observed could have come from 3-*O*-methylsphingosine by hydrolysis during the methanolysis procedure. Methanolic HCl forms water during acid

methanolysis (17), and it is known that allylic derivatives are acid-labile.

The virtual absence of unmethylated base shows that the 3-position was not the site of the ester linkage in any of the isomers.

The nature of the amine in KE and CE was examined further by GLC. The unmethylated acyl cerebrosides were cleaved with M–W–HCl (24), the amine portion was oxidized with periodate (25), and the aldehydes were purified by silica gel chromatography to remove the methoxy aldehydes (derived from the methanolic HCl). Gas chromatography of the unsubstituted aldehydes showed the expected two peaks from C_{18}-sphingosine and C_{18}-dehydrosphingosine. Small amounts of lower homologues were seen in the GLC recordings, but no higher homologues. Evidently there is no relation between the acyl cerebrosides and gangliosides, which have both C_{18}- and C_{20}-amines.

TLC of the sphingosines prior to periodate oxidation showed no difference between CE, KE, UE, and brain cerebroside, especially in the ratios of *threo* to *erythro* isomers (19).

The Sugars of the Permethylated Esters

Other portions of the methanolysis mixtures, after removal of the methyl esters, were passed through a small column of mixed ion exchange resins (Amberlite MB-3) to remove amines and HCl. Elution with methanol yielded the methylated sugar, which was studied by GLC. On DEGS, only one major peak was found for KE*a*, CE*a*, and CE*b;* its retention time matched that of methyl 2,3,4-trimethyl galactoside. Its retention time relative to that of methyl 2,3,4,6-tetramethyl galactoside was 4.0. The sugar portion of KE*b* showed in addition to the trimethyl galactoside peak three more small peaks with relative retention times (compared with tetramethyl galactoside) of 1.9, 2.5, and 2.7. The total area of these three peaks amounted to $^2/_3$ the area of the major peak.

Examination of the sugar derivatives with the Apiezon GLC column confirmed the identification of the 2,3,4-trimethyl galactoside. This column readily distinguished the galactoside from the 2,4,6- and 3,4,6-trimethyl galactosides.

Additional confirmation that the 2,3,4-derivative was the predominant isomer was made by hydrolyzing the galactoside samples to the free sugar ether and examining the products by TLC (26).

It is thus evident that the esterified fatty acid residue in KE and CE must reside on the 6-position of the galactose.

Periodate Degradation of Acyl Cerebron

Since the possibility must be considered that the methylation step somehow caused an acyl transfer from another position, we degraded an additional sample of CE with periodate. The oxidation of 1.46 mg of CE consumed 2.36 μmoles of periodate. From the fatty acid distribution of CE its molecular weight may be calculated to be 1100, so 1.8 moles of periodate were consumed per mole of CE. TLC of the oxidation product showed that a small amount of the original lipid remained, consistent with the expected value of 2.0 moles/mole. The poor solubility of CE in the reaction mixture probably slowed the reaction, which goes much faster with cerebroside (which consumed 2.1 moles/mole). This value is consistent with the methylation data, as the 2,3,4-positions of galactose should react with 2 moles of periodate.

Reduction of the oxidation product with borohydride, followed by mild acid hydrolysis and TLC on boric acid-impregnated Silica Gel G (27) with C–M 98:2, showed the presence of spots corresponding to *N*-hydroxyacyl sphingosine ($R_f = 0$) and 1-monopalmitin ($R_f = 0.32$). The latter compound gave a distinctive whitish spot with bromothymol blue; this is further evidence for the identification. In addition, there was a spot just below the monoglyceride that was identical in R_f and color with the spot given by nonhydroxy ceramide; it presumably came from the KE that contaminated the original CE. Parallel experiments with 1-monopalmitin showed that the reduction and hydrolysis steps did not hydrolyze or isomerize the monoglyceride to a serious extent.

Further confirmation of the identification was made by spraying the same plate with sodium metaperiodate and then benzidine, which yielded a white spot on blue background for both the standard monoglyceride and the CE-derived monoglyceride.

Similar identifications were made by means of Silica Gel G plates developed with hexane–ether–acetic acid 30:70:1. This system, unlike the boric acid system, does not separate 1-monoglycerides from 2-monoglycerides. However, the benzidine spray is specific for the former. It is thus evident by an independent series of reactions that the fatty acyl substituent in CE is attached to the 6-position of cerebron.

DISCUSSION

The experiments described clearly demonstrate the presence in pig brain of two types of 6-fatty acyl cerebrosides, in which the amide-bound fatty acids are of the nonhydroxy and 2-hydroxy types. The cerebrosides were shown to have the structure and fatty acid distribution of typical mammalian brain cerebrosides. Because of the presence of small extra peaks and spots in the various chromatograms, one cannot rule out the presence of small amounts of isomers. Some of the spots and peaks

are undoubtedly from impurities appearing as the result of using small amounts of material, as with KE*b*, and some are undoubtedly side reaction products. The mixture of crude glycolipid esters (prior to separation by TLC) seemed to react completely with periodate, which makes it unlikely that any 3-acyl cerebroside exists in brain.

It might be thought that the 1-monoglyceride formed in the periodate degradation scheme would isomerize to the 2-isomer. However, this could not be seen with the standard monoglyceride when it was exposed to the same conditions. The reverse isomerization, from the 2- to the 1-position, would not be expected since Noda and Fujiwara (28) showed that this does not occur even under more severe conditions.

The initial report by Norton and Brotz (1) is not entirely consistent with ours. Their separated lipids C and D showed only cerebron on saponification, no kerasin being noted. These could correspond to our CE and UE, but we found UE to yield both types of cerebrosides on alkaline methanolysis. The slowest of our ester lipids, GG, proved to be a mixture of fatty galactosyl glycerol compounds, corresponding well to their *second*-slowest spot, lipid B.

The methylated sugar moiety of lipid UE yielded a complex pattern of peaks with GLC and further work is needed to characterize this mixture. It does appear that the ester-linked acid in UE is not attached to the sphingosine moiety and that the galactose is in the β-pyranose form. Since UE migrates on silica gel more slowly than KE and CE, it may be concluded that only one fatty acid residue is attached in ester linkage.

A compound similar to our KE has been isolated from normal and Gaucher spleen (29). On the basis of incomplete examination it was proposed that the structure is 6-fatty acyl glucosyl ceramide, making it the glucosyl analogue of the brain lipid.

Like Klenk and Doss (2), we did not encounter the sphingoplasmalogens described by Kochetkov, Zhukova, and Glukhoded (30) nor were we able to detect *N*-palmitoyl ethanolamine, reported to be in brain (31).

Judging by the great resemblance between cerebroside esters and brain cerebrosides with respect to the chain-length distribution in the fatty acids and fatty amine, the brain probably synthesizes the esters by direct acylation of cerebrosides.

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Note added in proof. We have just learned of a preliminary communication describing the characterization of a cerebron ester in brain which has the fatty acid attached to the 3-position of the sphingosine (Tamai, Y., T. Taketomi, and T. Yamakawa. 1967. *Japan. J. Exptl. Med.* **37:** 79). At the moment there is no way to explain the differences observed.

References

1. Norton, W. T., and M. Brotz. 1963. *Biochem. Biophys. Res. Commun.* **12:** 198.
2. Klenk, E., and M. Doss. 1966. *Z. Physiol. Chem.* **346:** 296.
3. Radin, N. S., Y. Kishimoto, B. W. Agranoff, and R. M. Burton. 1967. *Federation Proc.* **26:** 676.
4. Davison, A. N., and E. Graham-Wolfaard. 1964. *J. Neurochem.* **11:** 147.
5. Jatzkewitz, H., and E. Mehl. 1960. *Z. Physiol. Chem.* **320:** 251.
6. Carter, H. E., J. A. Rothfus, and R. Gigg. 1961. *J. Lipid Res.* **2:** 228.
7. Schiffman, G., E. A. Kabat, and S. Leskowitz. 1962. *J. Am. Chem. Soc.* **84:** 73.
8. Distler, J., B. Kaufman, and S. Roseman. 1966. *Arch. Biochem. Biophys.* **116:** 466.
9. Radin, N. S. 1965. *J. Chromatog.* **20:** 392.
10. Kishimoto, Y., and N. S. Radin. 1966. *J. Lipid Res.* **7:** 141.
11. Verstraeten, L. M. J. 1964. *Anal. Chem.* **36:** 1040.
12. Neely, W. B. 1957. *In* Advances in Carbohydrate Chemistry. M. L. Wolfrom and R. S. Tipson, editors. Academic Press, New York. **12:** 13–33.
13. Kishimoto, Y., W. E. Davies, and N. S. Radin. 1965. *J. Lipid Res.* **6:** 525.
14. Waldi, D. 1965. *In* Thin-Layer Chromatography. E. Stahl, editor. Academic Press, New York. 486.
15. Rosenberg, A., and E. Chargaff. 1958. *J. Biol. Chem.* **233:** 1323.
16. Yokoyama, S., and T. Yamakawa. 1964. *Jap. J. Exptl. Med.* **34:** 29.
17. Kishimoto, Y., and N. S. Radin. 1965. *J. Lipid Res.* **6:** 435.
18. O'Brien, J. S., D. L. Fillerup, and J. F. Mead. 1964. *J. Lipid Res.* **5:** 109.
19. Suomi, W. D., and B. W. Agranoff. 1965. *J. Lipid Res.* **6:** 211.
20. Sambasivarao, K., and R. H. McCluer. 1963. *J. Lipid Res.* **4:** 106.
21. Dittmer, J. C., and R. L. Lester. 1964. *J. Lipid Res.* **5:** 126.
22. Carter, H. E., W. P. Norris, F. J. Glick, G. E. Phillips, and R. Harris. 1947. *J. Biol. Chem.* **170:** 269.
23. Weiss, B. 1964. *Biochemistry.* **3:** 1288.
24. Gaver, R. C., and C. C. Sweeley. 1965. *J. Am. Oil Chemists' Soc.* **42:** 294.
25. Sweeley, C. C., and E. A. Moscatelli. 1959. *J. Lipid Res.* **1:** 40.
26. Malone, M. J., P. Stoffyn, and H. Moser. 1966. *J. Neurochem.* **13:** 1033.
27. Serdarevich, B., and K. K. Carroll. 1966. *J. Lipid Res.* **7:** 277.
28. Noda, M., and N. Fujiwara. 1967. *Biochim. Biophys. Acta.* **137:** 199.
29. Makita, A., C. Suzuki, Z. Yosizawa, and T. Konno. 1966. *Tohoku J. Exptl. Med.* **88:** 277.
30. Kochetkov, N. K., I. G. Zhukova, and I. S. Glukhoded. 1963. *Biochim. Biophys. Acta.* **70:** 716.
31. Bachur, N. R., K. Masek, K. L. Melmon, and S. Udenfriend. 1965. *J. Biol. Chem.* **240:** 1019.