NEW GALACTOLIPIDS OF BRAIN: A MONOALKYL-MONOACYL-GLYCERYL GALACTOSIDE AND CEREBROSIDE FATTY ACID ESTERS *

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A glycolipid fraction of brain of lower polarity than the cerebrosides has been isolated and resolved into four previously unreported crystalline galactolipids. The structures of three of these have been determined in part.

Methods and Materials

Total lipids were extracted from fresh bovine cerebral white matter by the method of Folch, et al. (1957). Analytical methods were as follows: phosphorus, Chen, et al. (1956); nitrogen, Jacobs (1960); galactose, Svennerholm (1956); ester, Snyder and Stephens (1959) and plasmalogen, Wittenberg, et al. (1956). Carbon-hydrogen analyses were performed by Schwarzkopf laboratories, Woodside, New York.

Paper chromatography of sugars was carried out both in EtOAc:pyridine: H_2O:HOAc, 5:5:3:1 (Fischer and Nebel, 1955) and in n-BuOH:pyridine:H_2O, 6:4:3 (Chargaff, et al.,1948). Spots were detected by the $AgNO_3$-NaOH technique of Trevelyan, et al. (1950). Thin layer chromatography (T.L.C.) of fatty acids was done on Silica-gel G (Brinkmann Instruments) using $CHCl_3$:HOAc, 96:4 (Jatzkewitz and Mehl, 1960) and hexane:ether:HOAc, 70:30:1 (Malins and Mangold, 1960). For analytical work with T.L.C., spots were visualized by H_2SO_4 charring; for preparative work, the zones were located under U.V. light after spraying with 2,7-dichlorofluorescein (Mangold and Malins, 1960), and the compounds were eluted with hot 2:1, $CHCl_3$:CH_3OH. All column chromatography was performed on

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Unisil silicic acid (Clarkson Chemical Co.) using discontinuous gradients of methanol in chloroform.

Results

Isolation: Typically, 5.0 g. of total lipids was applied to a column of 200 g. of silicic acid and five fractions collected by the following scheme.

Fraction	Volume	$CHCl_3$:CH_3OH,v/v	Wt.,g.	Composition
1	2 liters	100:0	1.2	mostly cholesterol
2	1 "	98:2	0.060	uncharacterized, hexose-free
3	1 "	97:3	0.033	new glycolipids
4	1 "	96:4	0.030	new glycolipids
5	2 "	90:10	1.1	crude cerebrosides

In this fashion, from 66 g. of lipid we obtained 458 mg. of fraction 3 and 374 mg. of fraction 4, totalling 1.26% of the lipid weight and containing 2.8% of the lipid galactose.

T.L.C. in 9:1, $CHCl_3$:CH_3OH showed both fractions 3 and 4 to have at least seven components, with most of the material in four major spots which were present in both fractions. Both fractions had negligible P,plasmalogen and cholesterol. Fraction 3 had 1.1% N, 9.3% hexose and a hexose/ester ratio of 0.68. Fraction 4 had 0.65% N, 16.8% hexose and a hexose/ester ratio of 0.85. The four major compounds were successfully obtained separately by preparative T.L.C. in 9:1, $CHCl_3$:CH_3OH. These four compounds were further purified by column chromatography and crystallization from 95% ethanol to give white crystalline powders. They all ran as separate single spots on T.L.C. in the above system, and were designated glycolipids A (Rf 0.33), B (Rf 0.35), C (Rf 0.44) and D (Rf 0.54) in order of increasing Rf. Paper chromatography of an acid hydrolysate (2N HCl, 20 hrs., 100°C) showed the hexose group of each compound to consist solely of galactose. The properties and structure proof of each of these compounds will be taken up individually.

Glycolipid B: 128 mg. (0.19% of total lipid), sinter point 68°C.

Anal.	%C	%H	%N	%P	%Gal.	ester μmole/mg.	mole ratio Gal./ester
Calc'd for 80% $C_{41}H_{80}O_9$ and 20% $C_{41}H_{78}O_{10}$	68.4	11.2	0.0	0.0	25.0	1.67	0.83
Found	68.1	11.2	0.1	0.06	24.7	1.63	0.84

(The calculated analysis is based on a mixture of 80% monoalkyl-monoacyl-glyceryl galactoside and 20% galactosyl diglyceride having long chain residues averaging 16 carbons.) Infrared absorption in Nujol mull: 3460(s), 1737(s), 1667(w), 1290(w), 1268(w), 1249(w), 1225(w), 1202(w), 1177(s), 1118(m), 1093(s), 1077(s), 1042(s), 1020(m), 985(m), 943(w), 925(w), 895(w), 877(w), 855(w) and 785(m) cm^{-1}.

Saponification of 10 mg. of glycolipid B in 7 ml. of 0.1N NaOH in 2:4:1, $CHCl_3$:CH_3OH:H_2O at 37°C for 20 hr. gave two major products separated by base extraction. One was a mixture of normal (non-hydroxy) fatty acids, identified by T.L.C. and infrared spectroscopy; the other was a new galactose-containing lipid (E), having an Rf (0.54) less than phrenosine (Rf 0.66) on T.L.C. in 70:30:4, $CHCl_3$:CH_3OH:H_2O. E was purified by silicic acid chromatography, yield 4 mg. (79% assuming E is a galactoside of a long chain glyceryl ether). Infrared absorption of E in CCl_4 suspension: 3660(w), 3550(s), 3551(s), 3360(s), 2923(s), 2853(s), 1645(w), 1470(s), 1374(m), 1352(w), 1330(w), 1288(w), 1149(m), 1124(s), 1098(s), 1053(s), 1025(s), 939(m) and 877(m) cm^{-1}.

The products of acid hydrolysis of E confirmed the assumption that E was a glyceryl ether galactoside. Reflux of 1.5 mg. of E in 2N HCl in 50% ethanol for 5 hrs., followed by the usual workup, gave an aqueous layer containing only galactose (paper chromatography), estimated to be about 0.4 mg. (75%) by comparison with reference standards. The $CHCl_3$ layer contained only one compound (1.02 mg., 100%), the infrared spectrum of which was, except for a trace of carbonyl absorption, identical with synthetic d,l-α-octadecyl glyceryl ether (batyl alcohol) (Kornblum and Holmes, 1942), and which co-chromatographed with it in two T.L.C. systems (70:30:1, hexane:ether:HOAc, Rf 0.25 and 9:1, $CHCl_3$:CH_3OH, Rf 0.52). The infrared spectrum of this glyceryl ether was not similar to that of β-alkyl glyceryl ethers (Debuch, 1959).

The nature of the hydrolysis products and the analysis indicate the major component of glycolipid B is a fatty acid ester of an α-alkyl glyceryl ether galactoside. The infrared spectra of both glycolipid B and its hydrolysis product, E, show the type 2b absorption and the absence of the type 2a absorption, indicating the galactosidic linkage is of the β-configuration (Barker, et al, 1956). By analogy with the plant galactosyl diglycerides (Carter, et al., 1956; Benson, et al., 1958; Carter,et al., 1961) we can assign the galactose group to the α-position of the glyceryl ether and the acyl group to the β-position. It is probable that glycolipid B is admixed with about 20% of galactosyl diglyceride since the analysis shows a molar excess of ester groups over galactose, and since the latter compound has recently been shown to be present in brain (Steim and Benson, 1963). Galactosyl diglyceride would co-chromatograph with the monoether analog, since it has been noted many times that replacement of a long chain fatty ester with a long chain ether group does not appreciably alter the chromatographic behavior of lipids (Gilbertson and Karnovsky, 1963; Svennerholm and Thorin, 1960; Renkonen, 1962; Carter, et al., 1958; Hanahan and Watts, 1961). The probable structure of the major component of glycolipid B is thus 3'-O-alkyl-2'-O-acyl-glyceryl-1'-β-D-galactopyranoside.

Glycolipid C: 50 mg. (0.076% of total lipids), sinter point 105°C.

Anal.

	%C	%H	%N	%P	%Gal.	ester, μmole/mg.	mole ratio Gal./ester
Calc'd for $C_{66}H_{127}O_{10}N$	72.4	11.7	1.3	0.0	16.5	0.91	1.00
Found	72.2	11.4	1.4	0.0	16.5	0.86	1.06

(The calculated analysis is for a stearoyl ester of phrenosine.)

Infrared absorption in CCl_4 suspension: 3584(m), 3533(s), 3484(s), 3258(s), 2924(s),2850(s), 1745(m), 1718(s), 1650(s), 1508(s),1466(s), 1391(m), 1366(m), 1348(m), 1332(m), 1312(m), 1285(m), 1266(m), 1221(m), 1202(s), 1188(s), 1149(s), 1124(s), 1082(s), 1048(s), 963(m), 897(m) and 877(m) cm^{-1}.

Glycolipid C (4 mg.) was saponified in 5 ml. 0.1N ethanolic NaOH at 37°C for 2.5 hrs. Chromatographic separation of the total hydrolysis mixture gave

only two main products. One of these was shown to be normal fatty acids (T.L.C. and infrared spectrum). The other was identical with authentic highly purified beef brain phrenosine, both by chromatographic behavior (T.L.C. in 70:30:4, $CHCl_3$:CH_3OH:H_2O) and infrared spectrum.

Glycolipid D: 110 mg. (0.17% of total lipids), sinter point 77°C.

Anal.

	%C	%H	%N	%P	%Gal.	ester, μmole/mg.	mole ratio Gal/ester
Calc'd for $C_{67}H_{127}O_{10}N \cdot 2H_2O$	70.4	11.6	1.2	0.0	15.8	0.88	1.00
Found	70.6	11.4	1.2	0.0	15.8	0.83	1.06

(The calculated analysis is for an average of a $C_{19:1}$ fatty acid ester of phrenosine.) Infrared absorption in CCl_4 solution: 3400(s), 2923(s), 2850(s), 1738(s), 1653(s), 1515(s), 1466(s), 1380(m), 1170(m), 1136(m), 1078(s) and 963(m) cm^{-1}.

Saponification of glycolipid D by the same technique used for glycolipid C also gave only normal fatty acids and phrenosine, identified as described previously.

Glycolipid A: 56 mg. (0.085% of total lipids), sinter point 102°C. Infrared absorption in CCl_4 solution: 3380(s), 2923(s), 2850(s), 1735(s), 1653(s), 1515(s), 1466(s), 1414(w), 1380(m), 1304(w), 1160(s), 1115(sh), 1078(s) and 967(m) cm^{-1}.

This lipid has not been further characterized although its infrared spectrum is very similar to that of glycolipid D. The magnitude of the ester and amide bands indicate these two groups are present in approximately the same molar ratio. The presence of a *trans* double bond (967 cm^{-1}) is indicative of a sphingosine residue. It is reasonable to assume that glycolipid A is also a fatty acid ester of a cerebroside.

Discussion

Kochetkov and co-workers (1961,1962) have reported a group of glycolipids, which they consider minor pure cerebrosides having lower chain length fatty amide residues than the major cerebrosides. It is probable, however, from their

chromatographic data, that this group of lipids corresponds with those reported in this paper.

It is not known yet how glycolipids C and D (and also A, if it proves to be a cerebroside ester) differ from each other. They may be position isomers since there are six free hydroxyl groups in phrenosine available for esterification, or they may differ in the chain length or degree of unsaturation of the fatty acyl group.

Although lipids of many types have been discovered from many sources which have the α-alkyl glyceryl ether structure(Gilbertson and Karnovsky,1963; Svennerholm and Thorin, 1960; Renkonen, 1962; Carter, et al., 1958; Hanahan and Watts, 1961), and galactosyl diglycerides have been found in both plant (Carter, et al., 1956; Benson, et al., 1958; Carter, et al, 1961) and mammalian brain (Steim and Benson, 1963) lipids, no monoether analog of galactosyl diglyceride has been reported previously.

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