SULFOGALACTOSYL DIACYLGLYCEROL: OCCURRENCE AND BIOSYNTHESIS OF A NOVEL LIPID IN RAT BRAIN

Thomas J. Flynn, D.S. Deshmukh†, G. Subba Rao, and Ronald A. Pieringer

Department of Biochemistry
Temple University School of Medicine
Philadelphia, Pennsylvania 19140

Received May 12,1975

Summary

Intracerebral injection of $^{35}SO_4^=$ into rats produced a ^{35}S-labeled lipid which was characterized by mild alkaline deacylation, acid hydrolysis, dioxane solvolysis, periodate oxidation, chromatography and infrared spectrophotometry to be 3'-sulfogalactosyl diacylglycerol. A detergent-solubilized enzyme from brain microsomes catalyzed the synthesis of this lipid from (^{35}S)-3'-phosphoadenosine-5'-phosphosulfate and monogalactosyl diacylglycerol.

The relatively recent finding of significant amounts of a sulfogalactosyl monoalkylmonoacylglycerol (but not the diacylglycerol derivative) in testis and spermatozoa (1,2) suggested that the galactosyl diacylglycerol of myelin (3,4) might also exist in a sulfated form. Our initial investigations on the isolation and biosynthesis of sulfogalactosyl diacylglycerol of brain are reported here.

EXPERIMENTAL PROCEDURE AND RESULTS: Each of 25 rats (22 days old) received an intracerebral injection of 125 μCi (^{35}S) Na_2SO_4 (364 mCi/mmole). After 18 hours the brains were excised and homogenized in 2 ml H_2O per gm wet weight. The homogenate was then extracted with 19 volumes of chloroform: methanol (2:1 v/v)(5). The chloroform phase was washed (5), dried, and then chromatographed on a column (23 x 4 cm) of silicic acid (150 gms)(6). The first solvent, 1.5 l. of chloroform, eluted no radioactive compounds; the second solvent, 2 l. of acetone, eluted 5 x 10^6 CPM of ^{35}S-compounds; and the third 500 ml of methanol

†Present address: N.Y. Institute for Research in Mental Retardation, Staten Island, N.Y. 10314.

removed 390,000 CPM from the column. The acetone fraction contained 138.5 mg lipid (dry weight), and was further purified on thin layers of silicic acid ($CHCl_3$:CH_3OH:H_2O-65:25:4)(Fig. 1-lane A). Of five lipids detected by the carbohydrate sensitive α-naphthol-H_2SO_4 stain, two lipids (1 and 2, lane A, fig. 1) contained radioactivity as determined by counting in a scintillation spectrometer. In a typical experiment lipid-1 had 87,000 CPM and lipid-2 contained 17,000 CPM. ^{35}S-lipid-1 is probably cerebroside sulfate because it has the same R_f as cerebroside sulfatide standard, it stains positively with α-naphthol (carbohydrate specific)(7) and benzidene (secondary amide specific)(8), and it is not degraded by mild alkaline hydrolysis (9)(lane B, fig. 1).

Lipid-2 had an R_f unlike any of the standards and reacted positively to α-naphthol but negatively to benzidene stains. On mild alkaline hydrolysis (deacylating conditions)(9) lipid-2 could no longer be detected in significant amounts as a lipid (compare lane A with lane B, fig. 1). Approximately 90% of its radioactivity was recoverable as a water-soluble compound.

The water-soluble ^{35}S-labeled deacylation product of lipid-2 traveled 7 cm, and inorganic sulfate standard ran 35 cm on paper electrophoresis (pyridine: acetic acid: water- 1:10:89, pH 3.6; 50 volts per cm for 40 min). On treatment with 0.8 N HCL in methanol for 4 hrs in a sealed tube at 100°, water-soluble ^{35}S-compound yielded (100%) inorganic ^{35}S-sulfate (35 cm on paper electrophoresis) and nonradioactive products one of which was identified as galactose by gas chromatography (as described in 10) of trimethyl silyl ether derivates (α- and β- galactose have retention times of 11.8 and 13 min respectively). The complete release of inorganic sulfate indicates the lipid does not contain a sulfonate group, which is very resistant to acid hydrolysis (11). Removal of a sulfate from sulfated lipids also can be achieved by the relatively specific and mild technique of solvolysis with dioxane (12). Dioxane solvolysis of ^{35}S-lipid-2 produced (in 63% yield) inorganic ^{35}S-sulfate (identified by paper electrophoresis) and a nonradio-

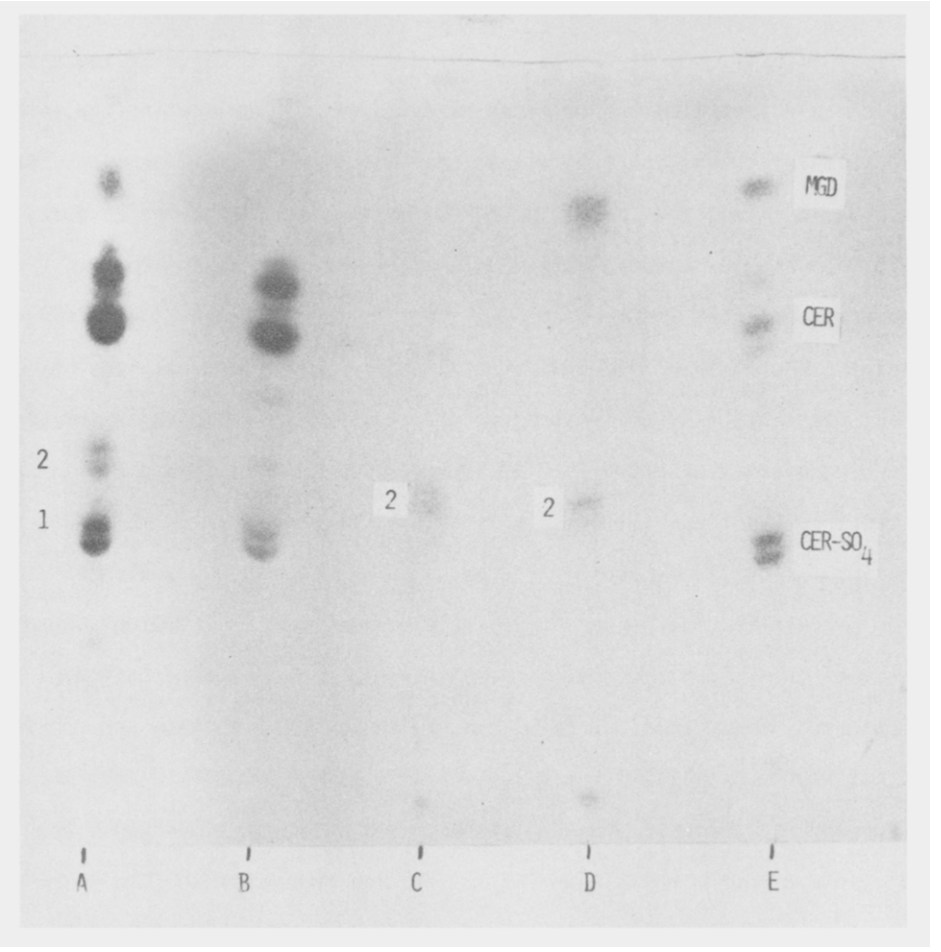

Figure 1: Thin layer silicic acid chromatogram developed with chloroform: methanol: water (65:25:4) and stained with α-napthol-H_2SO_4 to reveal carbohydrate-containing lipids. Lane A, a portion of a lipid mixture from an acetone fraction of a silicic acid column; radioactivity at lipid-2 was 1091 DPM and at lipid-1 was 6398 DPM (ratio of 0.17). Lane B, a somewhat smaller amount of the lipid mixture seen in lane A was deacylated with a 0.2 N NaOH in methanol (15 min. at 37°) and the remaining lipid-soluble compounds chromatographed; radioactivity at lipid-2 was 22 DPM and at lipid -1 was 3488 DPM (ratio of 0.0063). Lane C, purified lipid-2. Lane D, 63% desulfated (as measured by release of ^{35}S to water-soluble SO_4) of purified lipid-2 by dioxane solvolysis to MGD. Lane E, standard cerebroside sulfate ($Cer-SO_4$), cerebroside (Cer), and monogalactosyl diacylglycerol (MGD) all purchased from commercial sources.

active lipid that chromatographed with standard monogalactosyl diacylglycerol on thin layers of silicic acid (compare lane C and lane D of fig. 1).

The relative resistance of the sulfate moiety to mild alkaline hydrolysis

suggests that it is not bonded to the primary alcohol function of the sixth carbon of galactose (13). A more definitive approach to locating the position of the sulfate in the lipid was attempted through periodate oxidation. Approximately 1 to 2 mg of standard bovine cerebroside, standard bovine cerebroside sulfate, and smaller amounts of (^{35}S)-lipid-2 were each dissolved in 0.5 ml chloroform: methanol (1:1 v/v) and 0.075 ml aqueous $NaIO_4$ (0.33 gm/2 ml). Control tubes containing 0.075 ml water in place of $NaIO_4$ were also run. After 45 hrs in the dark at room temperature the reaction was stopped by the addition of one drop of ethylene glycol. Addition of 1 ml chloroform and 2 ml of water resulted in a two phase system. An aliquot of the water phase was found to contain no radioactivity above the control indicating no conversion of lipid-2 from an organic-soluble to an aqueous-soluble compound. Chromatography of aliquots of the chloroform phase in the thin layer system used in fig. 1 showed that compared to controls the $NaIO_4$-treated cerebroside sulfate and ^{35}S-lipid-2 did not change mobilities (R_f remained at 0.39 for cerebroside sulfate and 0.49 for lipid-2); however, $NaIO_4$-treated cerebroside traveled considerably faster (into a region where less polar compounds run) than the control (R_f changed from 0.65 to 0.91). The sulfate of cerebroside sulfate is bonded to the secondary alcohol at the third carbon of the galactose moiety (13) making cerebroside sulfate very resistant to periodate oxidation because it lacks adjacent vicinal hydroxyl groups. Cerebroside has no blocking group and on complete oxidation with periodate the alcohol function at position 3 should be oxidized to water soluble formic acid. Thus compared to untreated cerebroside periodate-treated cerebroside should be a less polar lipid. The fact that ^{35}S-lipid-2 did not change mobility on exposure to periodate indicates a resistance to periodate (possibly because of the sulfate group at the 3 position) or that the chromatography system could not separate oxidized and unoxidized lipid-2. In order to confirm that the galactose moiety of ^{35}S-lipid-2 had not been attacked by periodate, equal amounts of (^{35}S)-lipid-2 from $NaIO_4$-treated and control tubes were deacylated under mild alkaline conditions (9). The

water-soluble ^{35}S-deacylated compounds were then reacted with 0.8 N HCl in methanol at 100° for 4 hrs in sealed tubes. After removing solvent and drying, trimethylsilyl ether derivatives were prepared and chromatographed in the gas liquid system (10). From the periodate-treated material 75 nmoles of galactose and 32 nmoles of monogalactosyl glycerol (retention time 24.5 min) or a total of 107 nmoles of galactose equivalents were recovered. From the control tube 99 nmoles of galactose and 21 nmoles of monogalactosyl glycerol or a total of 120 nmoles of galactose equivalents were recovered. The almost complete recovery of undegraded galactose shows that periodate did not attack ^{35}S-lipid 2. The sulfate is therefore esterified to the alcohol group at the third carbon of galactose. The finding of monogalactosyl glycerol (variable amounts were produced in each acid hydrolysis) as a partial degradation product of lipid-2 supports the data obtained with dioxane solvolysis in showing that monogalactosyl diacylglycerol is the lipid to which the sulfate is bonded.

Although the change in mobility of bovine cerebroside from R_f 0.65 to 0.91 demonstrates that the conditions of periodate oxidation were capable of oxidizing a glycolipid with vicinal hydroxyl groups, the periodate system was further checked with (^{14}C-ul) monoglucosyl diacylglycerol from Streptococcus faecalis (faecium) (14) and phosphatidyl (^{14}C-ul-)-monoglucosyl diacylglycerol from Pseudomonas diminuta[1]. Since each carbon of the glucose moiety is equally labeled, periodate oxidation of each lipid should reduce the lipid-soluble counts by 1/6 since carbon-3 is released as water-soluble formic acid. Starting with equal amounts of (^{14}C-ul)-monoglucosyl diacylglycerol, 14,017 CPM were recovered in chloroform phase of the control and 10,811 CPM were found in the chloroform phase of the periodate-treated sample (or slightly more than 1/6 of the total radioactivity is lost). When the system was tested with phosphatidyl (^{14}C-ul)-monoglucosyl diacylglycerol (a lipid also capable of releasing formic

[1]The ^{14}C-lipid from P. diminuta was prepared by J.M. Shaw using broken cell preparations (15) and has the same structure as the lipid isolated by Wilkinson (16) from this organism.

acid since the phosphatidyl group is bonded to the 6 position of the glucose) (16), 6874 CPM were recovered as lipid-soluble radioactivity from the control and 5794 CPM were recovered as lipid-soluble radioactivity from the periodate treated (again representing a loss of 1/6 of the CPM).

The infrared absorption spectrum of purified lipid-2 taken on a Perkin Elmer model 457 grating spectrophotometer was the same as that published by Ishizuka et al (3) for sulfogalactosyl monoacylmonoalkylglycerol of testis except that the relative ester absorption (1720 cm^{-1}) of lipid-2 is twice as strong as that produced by the testis lipid.

A ^{35}S-lipid having the same chromatographic properties of lipid-2 could be produced in vitro from 3'-phosphoadenosine-5'-phosphosulfate-(^{35}S)(^{35}S-PAPS) and monogalactosyl diacylglycerol in the presence of a 1% triton-X-100 soluble extract of microsomes from brain of 21 day old rats. The incubation system contained 75 µgm plant monogalactosyl diacylglycerol, 1 µmole ethylenediamine tetraacetic acid, 5 µmoles imidazole buffer, pH 7.0, 77 µgm of enzyme protein, 1.7% triton-X-100, and 150,000 CPM (123,348 CPM/nmole) ^{35}S-PAPS in a final volume of 0.175 ml. The reaction (carried out at 37° for 1 hr) was dependent on presence of exogenous monogalactosyl diacylglycerol (MGD)(630 CPM per mg protein without MGD and 5799 CPM per mg protein with MGD), and on enzyme (no ^{35}S-lipid without enzyme). The ^{35}S-product had the same migration relative to cerebroside sulfate as ^{35}S-lipid-2 generated in vivo on the silicic acid thin layer system used in fig. 1. It yielded on mild alkaline deacylation (9) a water-soluble ^{35}S-product that had the same mobility on paper electrophoresis as the deacylated ^{35}S-lipid-2. The in vitro synthesized lipid also released inorganic $^{35}SO_4$ (identified by electrophoresis) on acid hydrolysis of its deacylated water-soluble ^{35}S-derivative.

We conclude from these initial studies that 3'-sulfomonogalactosyl diacylglycerol is a constituent of rat brain and that it is enzymatically formed from monogalactosyl diacylglycerol and PAPS.

ACKNOWLEDGEMENT: Expert technical assistance was provided by Mr. Barry Gordon.

This work supported by research grants AI-05730 and NS-10221 from the U.S. Public Health Service. R.A. Pieringer gratefully acknowledges the receipt of Research Career Development Award NS-12776.

REFERENCES

1. Ishizuka, I., Suzuki, M., and Yamakawa, T. (1973) J. Biochem. (Toyko) 73, 77-87.
2. Kornblatt, M.J., Knapp, A., Levine, M., Schachter, H., and Murray, R.K. (1974) Can. J. Biochem. 52, 689-697.
3. Deshmukh, D.S., Flynn, T.J. and Pieringer, R.A. (1974) J. Neurochem. 22, 479-485.
4. Pieringer, R.A., Deshmukh, D.S., and Flynn, T.J. (1973) Prog. Brain Res. 40, 397-405.
5. Folch, J., Lees, M. and Sloan-Stanley, G.H. (1957) J. Biol. Chem., 226, 497-509.
6. Rouser, G., Kritchevsky, G., and Yamamoto, A., (1967) Lipid Chromatographic Analysis, vol. 1, G. Marinetti ed., pp. 116-120, Marcel Dekker, New York.
7. Tacin, H. and Mishkin, A.R. (1965) J. Chromatog. 18, 170-173.
8. Bischel, M.D. and Austin, J.H. (1963) Biochim. Biophys. Acta 70, 598-600.
9. Ambron, R.T., and Pieringer, R.A. (1971) J. Biol. Chem. 246, 4216-4225.
10. Wenger, D.A., Petitpas, J.W., and Pieringer, R.A., (1968) Biochemistry, 7, 3700-3707.
11. Wolfersberger, M.G. and Pieringer, R.A. (1974) J. Lipid Res. 15, 1-10.
12. Meyers, G.L., Pousada, M., and Haines, T.H. (1969) Biochemistry, 8, 2981-2986.
13. Haines, T.H. (1971) Progress in the Chemistry of Fats and Other Lipids, vol. 11, R.T. Holman, ed., Pergamon Press, p. 315.
14. Pieringer, R.A., (1968) J. Biol. Chem. 243, 4894-4903.
15. Shaw, J.M. and Pieringer, R.A. (1975) Federation Proc., 34, 525.
16. Wilkinson, S.G. (1969) Biochim. Biophys. Acta 187, 492-500.