A Glycerol Galactofuranoside from the Lipid of an Anaerobe*

RICHARD E. REEVES, NELDA G. LATOUR, AND RAY J. LOUSTEAU

From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans

Received March 30, 1964

The lipid from an anaerobic organism provisionally designated *Bacteroides symbiosus* was found to contain D-galactose. Alkaline hydrolysis of the lipid yielded a new glycerol galactoside, specific rotation −73°, with a crystalline hexabenzoate, mp 133–134°, specific rotation −6°. The properties of the new galactoside indicate it to be a 1-glycerol β-D-galactofuranoside. This appears to be the first recorded recognition of a naturally occurring galactofuranoside.

An anaerobic organism provisionally designated *Bacteroides symbiosus* is consumed in quantity by *Entamoeba histolytica* during amebal growth in the Shaffer-Frye culture systems (Shaffer, 1952; Reeves *et al.*, 1957). Our interest in determining the lipid requirements for amebal growth led to a study of the composition of the bacterial lipid. This report deals with the finding of a new glycerol galactoside in the lipid.

EXPERIMENTAL

The Organism and Culture Methods.—The organism is maintained in a parent culture and in several different growth-type subcultures which were isolated from the parent culture (Reeves, 1962). The parent culture has been deposited in the American Type Culture Collection, Washington, D.C., under the acquisition number 14940.

For the lipid studies the organism was grown on a medium containing the following substances, quantities in g/liter: trypticase (Baltimore Biological Laboratories), 20; glucose, 10; sodium chloride, 2.5; dipotassium phosphate, 2; yeast extract (Difco), 2; mercaptosuccinic acid (Eastman), 1.5; and sodium hydroxide to *p*H 7.0. The methods of cultivating and harvesting the bacteria were those described by Reeves *et al.* (1957), except that large lots of cells were collected in a continuous-flow refrigerated centrifuge, and the washing was done with 0.9% saline.

Galactose Analyses.—When not otherwise indicated galactose determinations were made with the phenol–sulfuric acid method of Montgomery (1957) using a D-galactose standard.

Paper Chromatography.—Three solvent systems were employed: the organic phase of 1-butanol–acetic acid–water (25:25:3), 1-butanol-acetone-ammonia-water (40:50:3:15), and 1-butanol-pyridine-water (3:2:1.5). The last was capable of resolving galactose from added glucose. Visualization of spots was accomplished with silver nitrate–NaOH, periodate-benzidene, or galactose oxidase sprays.

Phosphorus Analyses.—The method of Bartlett (1959) was employed for phosphorus analyses.

Galactose Oxidase.—The galactose oxidase reagent was prepared by diluting to 25 ml the contents of a Galactostat Kit obtained from Worthington Biochemical Corp.

RESULTS

Preparation of Bacterial Lipid.—In a typical experiment an innoculum consisting of 250 ml of an 8-hour culture of the type *c* organism was added to 12 liters of medium which was then incubated for 16 hours at 36°. The cells were collected by centrifugation and washed twice with saline, and the moist cells were combined with 75 ml of methanol. Chloroform (150 ml) was added and the slurry was stirred at room temperature for 2 hours. The cells were then filtered, washed with acetone and ether, and dried *in vacuo*, wt = 10.05 g.

To the methanol-chloroform filtrate was added 0.2 volume of water and the organic layer was separated and evaporated to dryness leaving 0.555 g of a yellow lipid, containing 1.15% nitrogen (Kjeldahl), 2.27% phosphorus, and 7.82% carbohydrate (as galactose). Similar lipids containing 7–10% galactose were obtained from the parent culture of the organism and also from growth types *a* and *e*. D-Galactose and glycerol were identified among the water-soluble products of acid hydrolyses of the bacterial lipids.

Isolation of the Glycerol Galactoside.—To 36.6 mg of bacterial lipid in a stainless steel tube was added 1.5 ml of 1 N sodium hydroxide. The tube was loosely stoppered and heated in a boiling-water bath for 5 hours. The contents was then cooled, acidified with 1.6 ml of 1 N hydrochloric acid, and the ether-soluble material was extracted and reserved for later study. The aqueous solution was passed through a monobed deionizing resin of sufficient capacity to remove all the chloride ion. The effluent solution contained 3.7 mg of bound galactose. Upon concentration and examination by paper chromatography it was found to yield three periodate-reacting spots, R_F 0.70, 0.54, and 0.37 (butanol-pyridine-water system). The fast-moving spot has not yet been identified. It does not regularly occur in the hydrolysates prepared in the manner described. The second spot was identified as glycerol by its migration in three solvent systems. The slower-moving spot represented the new glycerol galactoside. The free glycerol and the glycerol galactoside were regularly obtained after saponification of lipids obtained from the parent culture of the organism, and from growth types *a*, *c*, and *e*.

A pooled sample of effluents from the deionization process described above, containing 12.4 mg of bound galactose, was evaporated and streaked on a 178-mm (7-in.) section of Whatman 3 mm filter paper. The paper was irrigated with the butanol–acetic acid–water solvent. After guide spots were dried and cut out the slow-moving material was eluted from the remainder of the chromatogram with water. Evaporation left 18.6 mg of a syrup containing 11.7 mg of bound galactose. The specific rotation of this material was −73° (*c* 1, in water at 25°).

The above substance gave only one periodate-reacting spot upon paper chromatography in three solvent systems. It did not react with the galactose oxidase reagent directly, but did so after acid hydrolysis. Following hydrolysis it gave two spots by paper chro-

* This investigation was supported in part by a U. S. Public Health Service grant (E-2951). One of us (R.J.L.) held a Tobacco Research Industries Summer Fellowship.

matography. The faster-moving spot was identified as glycerol. The slower-moving spot had an R_F of 0.25, identical with that of galactose in the butanol-pyridine-water system. This spot could be visualized on the chromatograms with the galactose oxidase spray reagent.

Rate of Hydrolysis.—To assist in determining the type of glycosidic linkage present in the new galactoside its rate of hydrolysis was studied in 0.1 N hydrochloric acid at 100°. The sample employed contained a trace of free glycerol, but no significant amount of reducing sugar. Using the Folin-Wu method for reducing-sugar determination (Folin, 1926), with a D-galactose standard, 0.050, 0.17, 0.5, and 0.65 mg of galactose were liberated in 1, 3, 10, and 30 minutes, respectively. Complete hydrolysis of the sample gave 0.78 mg galactose. The calculated velocity constant for the hydrolysis was 0.09 min^{-1}. Under similar conditions the velocity constants for ethyl β-D-galactofuranoside and methyl β-D-galactopyranoside were found to be 0.18 min^{-1}, and 0.013 min^{-1}, respectively.

Green and Pacsu (1937b) reported the velocity constant for the hydrolysis of ethyl α-D-galactofuranoside to be 0.08 min^{-1} at 100° in 0.01 N hydrochloric acid.

Periodate Oxidation.—Periodate-oxidation studies were made on chromatographically purified glycerol galactoside which did not contain free glycerol. Under standard conditions the substances consumed 2 moles of periodate rapidly (within 15 minutes) and a third mole in a further 3 hours. Under the same conditions ethyl β-D-galactofuranoside consumed 1 mole of periodate rapidly and a second mole over a period of 2 hours. A terminal glycol determination by the method of Reeves (1941) gave 2.08 moles of formaldehyde per mole of glycerol galactoside, indicating two glycol-containing end groups per molecule.

Hexabenzoate.—Benzoylation of 14 mg of the syrupy glycerol galactoside in 2 ml of pyridine with 0.3 ml of benzoyl chloride yielded 21 mg of crystalline hexabenzoate. This product was recrystallized from ether–petroleum ether for analysis; mp 133–34°; specific rotation $-6 \pm 3°$ (*c*, 1 in chloroform at 25°).

Anal. Calcd. for $C_{51}H_{42}O_{14}$ (878.9); C, 69.70; H, 4.83. Found: C, 69.99; H, 4.89.

Aminolysis of the hexabenzoate in ammonia-containing methanol regenerated the original glycerol galactoside.

Discussion

Glycerol galactosides have been found in seaweed by Nunn and Von Holdt (1955), in a marine algae by Putman and Hassid (1954), in red algae by Wickberg (1958a) and by Su and Hassid (1962), and in the lipid of wheat flour by Carter *et al.* (1956). These naturally occurring glycerol galactosides have been characterized as α- or β-D-galactopyranosides containing one or more molecules of galactose. All six theoretically possible glycerol galactopyranosides have been synthesized, four by Wickberg (1958b), and two by Charlson *et al.* (1957). The most levorotatory of these had a specific rotation of only −7°, much less than that of the new glycerol galactoside.

In view of chromatographic and enzymatic evidence that the sugar in the new glycoside is D-galactose, its high levorotation (−73°) indicates that it contains a β-galactosidic linkage. That it is a furanoside is probable from the following four lines of evidence: (1) Its molecular rotation (−18,500°) is close to that of ethyl β-D-galactofuranoside (−21,000°) calculated from the specific rotation for this substance reported by Green and Pascu (1937a). (2) Its rate of hydrolysis by acid is seven times greater than that of a β-D-galactopyranoside, and is clearly in the range expected of a galactofuranoside. (3) It fails to react with galactose oxidase, a property shared by ethyl β-D-galactofuranoside, but not by D-galactopyranosides (Rorem and Lewis, 1962). (4) Its reactions with periodate are compatible only with a furanoside structure. The production of 2 moles of formaldehyde, in addition to supporting the furanoside structure, indicates that the glycerol must be linked at position 1.

The structure of a 1-glycerol β-D-galactofuranoside is compatible with all of the recognized properties of the bacterial glycoside, but the anomeric configuration of the glycerol moiety has not yet been clarified.

Glycofuranosides of either galactose or glucose have not hitherto been reported from natural products. The finding of such a substance in a bacterial lipid raises the need for renewed investigation to see how widespread such structures may be in related organisms.

It is suspected that the galactose assays made directly on the total lipid by the phenol–sulfuric acid method err on the low side of the true value. On several occasions more galactose was found following alkaline saponification of the lipid than was indicated by the assays made directly on the lipid. In the lipid for which analyses are reported the galactose-nitrogen-phosphorus ratios were, approximately, 1:1.7:1.5. If the galactose analyses obtained after saponification were employed these ratios would become 1:1.4:1.3. The small divergence from unity exhibited by these latter ratios may indicate that the sugar is a part of the bacterial phospholipid.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Carter, H. E., McCluer, R. H., and Slifer, E. D. (1956), *J. Am. Chem. Soc. 78*, 3735.

Charlson, A. J., Gorin, P. A. J., and Perlin, A. S. (1957), *Can. J. Chem. 35*, 365.

Folin, O. (1926), *J. Biol. Chem. 67*, 357.

Green, J. W., and Pacsu, E. (1937a), *J. Am. Chem. Soc. 59*, 1205.

Green, J. W., and Pacsu, E. (1937b), *J. Am. Chem. Soc. 59*, 2569.

Montgomery, R. (1957), *Arch. Biochem. Biophys. 67*, 378.

Nunn, J. R., and Von Holdt, M. M. (1955), *J. Am. Chem. Soc. 77*, 2551.

Putman, E. W., and Hassid, W. Z. (1954), *J. Am. Chem. Soc. 76*, 2221.

Reeves, R. E. (1941), *J. Am. Chem. Soc. 63*, 1476.

Reeves, R. E. (1962), *J. Bacteriol. 85*, 1197.

Reeves, R. E., Meleney, H. E., and Frye, W. W. (1957), *Am. J. Hyg. 66*, 56.

Rorem, E. S., and Lewis, J. C. (1962), *Anal. Biochem. 3*, 203.

Shaffer, J. G. (1952), *Am. J. Hyg. 56*, 119.

Su, J. C., and Hassid, W. Z. (1962), *Biochemistry 1*, 468.

Wickberg, B. (1958a), *Acta Chem. Scand. 12*, 1183.

Wickberg, B. (1958b), *Acta Chem. Scand. 12*, 1187.