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A Compound Representing the D-Glycerate Terminus of the Methylglucose-Containing Polysaccharide of *Mycobacterium smegmatis*†

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ABSTRACT: In order to study the structure of the methylglucose-containing polysaccharide (MGP) of *Mycobacterium smegmatis* by NMR spectroscopy, we have prepared the model compound *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid. This compound, which represents the aglycon-containing terminus of MGP, was made from leucrose [*O*-α-D-glucopyranosyl-(1→5)-D-fructopyranose] by successive treatment with sodium borohydride, lead tetraacetate, and hypobromite. The structure of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid was confirmed by chemical and enzymic methods. ^{13}C and ^{1}H NMR spectra of this compound, together with spectra of several disaccharides, were obtained for future reference in the polysaccharide study. The nine resonances in the ^{13}C spectrum were assigned by comparison with the spectrum of methyl α-D-glucopyranoside. Analysis of the ^{1}H NMR spectrum showed that the two methylene protons on C-3 of the glycerate moiety were less equivalent in the sodium salt than in the acid. This may be attributable to hydrogen bonding between the carboxylate and the hydrogen atom of the glycerate 3-hydroxyl group.

Mycobacterium smegmatis contains two polysaccharides which stimulate fatty acid biosynthesis by interacting with acylcoenzyme A derivatives and long-chain transacylases (Yabusaki & Ballou, 1978; Wood et al., 1978). Ballou (1968) reported that one of these polysaccharides is a methylglucose-containing polysaccharide (MGP) with the structure shown in Figure 1, acylated at six to nine positions (Smith & Ballou, 1973). One of us noted that, contrary to expectations based upon this structure, exhaustive methylation, followed by acid hydrolysis, did not yield 2,4,6-tri-*O*-methylglucose.[1] This ether should have been obtained from the third glucose unit from the glyceric acid end of the chain. We therefore set out to examine the structure of MGP by a number of methods, including NMR spectroscopy. It should be possible to assign resonances of the hexose residues on the basis of comparison with spectra of suitable disaccharides. The as-

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[1] This observation was made by D.J.W. while on a sabbatical leave in the laboratory of Professor C. E. Ballou at the University of California, Berkeley. The structural reinvestigation is proceeding in both laboratories, but the discrepancy between the structure (Figure 1) and the methylation result is still unresolved.

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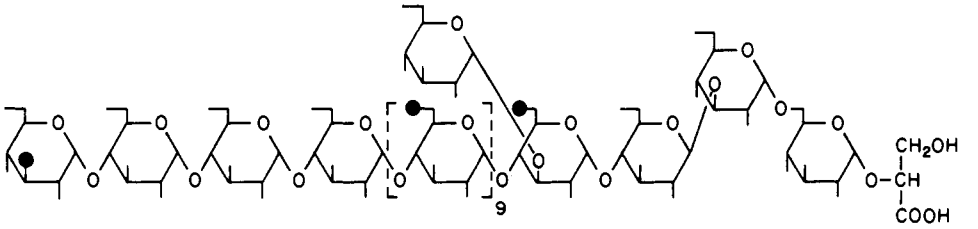

FIGURE 1: Structure of MGP, the methylglucose-containing polysaccharide of *Mycobacterium smegmatis*. Closed circle = OCH_3.

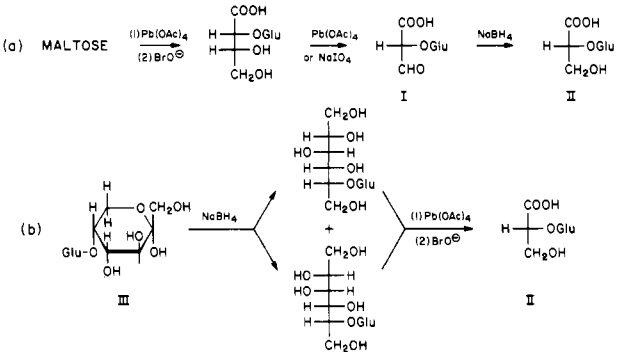

FIGURE 2: Two routes for the preparaton of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (II). Glu = α-D-glucopyranosyl.

signment of resonances of the aglycon-containing end of MGP could then be based upon the spectra of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (II; Figure 2). We wish to report the preparation of this compound and the interpretation of its ^1H and ^{13}C NMR spectra.

2-*O*-Glycosylglyceric acids do not appear to have been synthesized previously, although 2-*O*-α-D-mannopyranosyl-D-glyceric acid has been extracted from red algae (Bouveng et al., 1955) and its acetate has been examined by ^1H NMR spectroscopy (Whyte, 1969). An attempt to prepare *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid from maltose (Figure 2a) failed, due to overoxidation of compound I and subsequent cleavage to form glucose. The successful preparation (Figure 2b) is based upon the degradation of *O*-α-D-glucopyranosyl-(1→5)-D-fructopyranose, or "leucrose" (III), which can be prepared from sucrose by the dextransucrase of *Leuconostoc mesenteroides* (Stodola et al., 1956). Leucrose is reduced with sodium borohydride to give two epimeric 5-*O*-α-D-glucopyranosylhexitols. Lead tetraacetate oxidation of the latter results in removal of carbons 1, 2, and 3 of the hexitol moieties, with formation of *O*-α-D-glucopyranosyl-(1→2)-D-glyceraldehyde, which is then converted into *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (II) by hypobromite oxidation. The configurations of both carbon 1 of the glucopyranosyl ring of leucrose and carbon 5 of the fructose moiety (which becomes carbon 2 of the D-glyceric acid residue of II) are retained throughout the reaction sequence.

Experimental Section

Materials

Dextransucrase was prepared from a 2-L culture of *L. mesenteroides* NRRL B-512 (American Type Culture Collection, 10830) as described by Tsuchiya et al. (1955), except that the medium contained yeast extract, tryptone, and tomato juice (Gibbs et al., 1963) rather than corn steep liquor. This afforded 46 mg of dextransucrase with 12 000 units of activity as defined by Tsuchiya et al. (1952).

Leucrose was prepared by incubation of sucrose with dextransucrase and subsequent removal of dextran, sucrose, fructose, and glucose according to Stodola et al. (1956). Removal of substances migrating at R_G = 0.02, 0.09, 0.15, and 0.31 (paper chromatography; see below) was achieved by chromatography on a column of charcoal–Celite, as described by Whistler & Durso (1950). Elution with water (600 mL) gave a 50% recovery of chromatographically pure leucrose, R_G = 0.47, which gave a red color with the urea–phosphoric acid reagent of Wise et al. (1955). Acid hydrolysis gave only glucose and fructose.

Table 1: Lead Tetraacetate Oxidation of Glucosylalditols[a]

compd	concn (mM)	$Pb(OAc)_4$ uptake in 1.5 h[b] (mol/mol)	HCHO released in 1.5 h[b] (mol/mol)
O-α-D-glucopyranosyl-(1→2)-erythritol	45.5	0.84[c]	0.85[c]
substance X[d]	39.5	0.26	<0.05

[a] Initial concentration of lead tetraacetate was 50 mM (see Methods). [b] Corrected for consumption of lead tetraacetate by the glucose ring (see Methods). [c] Lead tetraacetate consumption and formaldehyde release leveled off at 1.5 h. [d] The glucosylglycerol resulting from reduction of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid methyl ester.

Yeast α-glucosidase (Type III), almond β-glucosidase, and *Eschericia coli* glycerokinase were obtained from Sigma Chemical Co. DL-Glyceric acid (Sigma; calcium salt) and *rac*-glycerol 1-phosphate (Sigma; sodium salt) were converted into the corresponding free acids with Dowex 50 (H^+). Laminaribiose was a gift from Dr. G. O. Aspinall, York University, Toronto.

O-α-D-Glucopyranosyl-(1→2)-erythritol, mp 144–146 °C, and *O*-α-D-glucopyranosyl-(1→2)-glycerol were prepared by degradation of maltose with lead tetraacetate according to Charlson & Perlin (1956) and Charlson et al. (1956).

NaB^3H_4 (870 Ci/mol) and [U-^{14}C]glycerol (8 Ci/mol) were purchased from the Amersham Corp. $^2H_2SO_4$ was obtained from Merck Sharp & Dohme, Montreal. NaO^2H was made by reacting sodium with deuterium oxide.

Methods

Paper chromatograms were irrigated with ethyl acetate–acetic acid–formic acid–water (18:3:1:4 v/v) and visualized with periodate–starch–iodide (Metzenberg & Mitchell, 1954), alkaline silver nitrate (Trevelyan et al., 1950), or bromophenol blue (Hais & Macek, 1963). R_G refers to the rate of migration relative to that of D-glucose. Whatman 3MM paper was washed with 1 N HCl before use in preparative chromatography.

Gas–liquid chromatography (GLC) was performed on a Hewlett-Packard gas chromatograph (Model 5720A) fitted with a flame ionization detector. The column used (8 ft × 1/8 in.) was 3% OV 210 on Chromosorb W (80–100 mesh), with a nitrogen flow rate of 20 mL/min.

Glucosides were estimated by the phenol–sulfuric acid reaction (Dubois et al., 1956). The elemental analysis was performed by the Schwarzkopf Laboratory, New York.

Oxidations of glucosylalditols (Table I) were performed with 50 mM lead tetraacetate in acetic acid containing 1.65% water.

Table II: Enzymic Hydrolysis of Glycosides

compd	D-glucose liberated (mol/mol of substrate)	
	α-glucosidase	β-glucosidase
methyl α-D-glucopyranoside	0.81	0.04
methyl β-D-glucopyranoside	0	0.43
O-α-D-glucopyranosyl-(1→2)-D-glyceric acid	0	0
substance X[a]	0.92	0.04

[a] The glucosylglycerol resulting from reduction of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid methyl ester.

Lead tetraacetate was estimated according to Criegee (1931), using 0.02 M sodium thiosulfate. Liberated formaldehyde was estimated with chromotropic acid (Lambert & Neish, 1952) after removal of lead as its oxalate (Perlin, 1955). Since the object was to study the oxidation of alditol moieties of glucosylalditols, lead tetraacetate consumption was always corrected by the uptake of methyl α-D-glucopyranoside (43.5 mM) occurring in the same time. The latter consumed lead tetraacetate at 0.18 (mol/mol)/h for the first 2 h and did not produce formaldehyde.

The susceptibility of glucosides toward glucosidases (Table II) was examined by incubation of each compound (10 mM) for 22 h at 37 °C and estimation of liberated glucose with the Glucostat reagent (Worthington). For α-glucosidase the buffer was 0.05 M sodium phosphate, pH 6.8. The initial enzyme concentration was 12.5 μg (1.12 units) per mL. At 2 h it was increased to 37.5 μg (3.36 units) per mL. For β-glucosidase the buffer was 0.025 M sodium acetate, pH 5.25. The enzyme concentration, initially 0.17 mg (0.68 unit) per mL, was raised to 0.29 (1.2 units) per mL after 2 h.

Acid hydrolyses of glucosides were performed in 2 M trifluoroacetic acid for 2 h at 110 °C.

For infrared spectroscopy, an aqueous solution of the sample (4 mg) was spread on a thallium bromoiodide disk (KRS-5; Harshaw Chemical Co.) and dried in vacuo over P_2O_5 for 3 days. The spectrum was measured on a Perkin-Elmer 180 instrument.

Proton NMR spectra were obtained at 60 MHz on a Bruker HX-60 spectrometer. The instrument was operated in the Fourier transform mode using a BNC-12 computer (Bruker/Nicolet), a Bruker pulse controller, and radio-frequency power amplifiers. The 100-MHz spectra of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid and its salt (at 25 °C) were obtained with a Varian HA-100 spectrometer. Samples were deuterated by dissolution in deuterium oxide, followed by lyophilization. This process was repeated twice, and deuterium oxide samples of 40–50 mg/mL were then made, containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard. Solutions of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid and its sodium salt were at pD 3.5 and 9, respectively. A solution of methyl α-D-glucopyranoside was adjusted to pD 2.5, 5, 7, and 9 by acidifying with $^2H_2SO_4$ to pD 2.5 and increasing the pD stepwise with NaO^2H. A spectrum was obtained at each pD value. Each 60-MHz spectrum was taken at 30 and 90 °C, since (a) the spectra were intended as references for the MGP spectrum at 90 °C and (b) the HO^2H peak position changed with temperature, allowing the whole spectrum of each compound to be obtained.

The glyceric acid resonances of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, and its salt, were analyzed by using a Nicolet Model 1180 computer and the ITRCAL program. ITRCAL is a Nicolet Instrument Corp. adaptation of LAOCN3 which was originally written by Castellano & Bothner-By (1964). The H-2, H-3, and H-3″ region of the spectrum was considered as an ABC system, in that all chemical shifts and all coupling constants were allowed to vary independently. The ITRCAL program permitted a manual variation of chemical shifts to obtain a reasonable spectrum before the iterative fit was attempted.

^{13}C NMR spectra were obtained at 15.09 MHz on the Bruker HX-60 spectrometer. Wide-band proton decoupling was effected with a solid-state modification of a Bruker BSV-2 decoupler. Aqueous solutions of samples, 30–60 mg/mL, were contained in 10-mm tubes, with external deuterium oxide in a coaxial 4-mm tube. The deuterium oxide served as a field-frequency lock reference. Each spectrum was calibrated against external dioxane by taking the spectrum of the latter (in aqueous solution) with the same deuterium oxide lock sample, using identical frequency settings on the spectrometer. A chemical-shift difference of 68.25 ppm was observed between external dioxane and external tetramethylsilane. This value was used to express chemical shifts in parts per million downfield from tetramethylsilane. The solution of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid was at pH 3. To make the salt, we adjusted the solution to pH 8.5 with ammonia. To test the effect of pH upon the spectrum, we brought solutions of methyl α-D-glucopyranoside to pH 3 or pH 8.5 with HCl or ammonia, respectively.

Radioactive samples were dissolved in 10 mL of Aquasol scintillator solution (New England Nuclear). The total volume of water in each sample was adjusted to 1.0 mL, and radioactivity was measured with a Beckman LS 3100 liquid scintillation counter.

O-α-D-Glucopyranosyl-(1→2)-D-glyceric Acid. Sodium borohydride (434 mg) was added to a solution of leucrose (320 mg, 0.94 mmol) in water (20 mL). After 20 h at room temperature, the borohydride was destroyed with Dowex 50 (H^+), and the resulting boric acid was removed by repeated evaporation with methanol. The product, dissolved in 1.4 mL of water, was stirred with a solution of lead tetraacetate (4.08 mmol) in acetic acid (80 mL). After 2.5 h the consumption of lead tetraacetate had almost stopped and was 3.4 mol/mol of leucrose, correlated for uptake by the pyranose ring, as described above (theory, 3.0 mol/mol). A solution of oxalic acid dihydrate (0.51 g) in acetic acid was added, and the mixture was stirred for 30 min at room temperature and for 18 h at 3 °C. The resulting lead oxalate was removed by filtration through Celite, and acetic acid was removed by evaporation with several changes of water. An aqueous solution of the residue was passed through Dowex 50 (H^+) to complete the removal of lead and concentrated to 10 mL. The phenol–sulfuric acid assay of the solution gave the equivalent of 0.80 mmol of glucose.

The solution (10 mL) was then stirred with barium carbonate (1.0 g) while 220 μL (4 mmol) of bromine was added over 30 min. The mixture was stirred for a further 3 h, filtered through Celite, and aerated for 45 min to remove excess bromine. Bromide was removed by stirring for 30 min with silver carbonate, freshly prepared from 1 g of $AgNO_3$ and 0.5 g of $NaHCO_3$. The mixture was filtered through Celite to give filtrate Z, which contained the equivalent of 0.49 mmol of glucose. Paper chromatograms treated with periodate–starch–iodide showed substances at R_G = 0.05, 0.27, 0.95, 1.14, and 2.90. All components, except for the one having a R_G = 0.95, could be visualized with bromophenol blue. Samples of the components of R_G = 0.95 and R_G = 1.14 were purified by preparative chromatography on Whatman 3MM paper and

hydrolyzed separately, with the following result: R_G = 0.95 → R_G = 1.0 and 1.53; R_G = 1.14 → R_G = 1.0 and 2.7 (position of glyceric acid). The R_G 1.14 component was subsequently shown to be the title compound.

The component of R_G = 0.95 was removed as follows. Filtrate Z was concentrated to 9 mL and mixed with an equal volume of 2 N NaOH. The resulting solution was heated on the steam bath for 30 min, passed through Dowex 50 (H^+), and concentrated to a small volume. A chromatogram now showed spots at R_G = 0.05, 0.27, 1.14, and 2.90. Final purification was effected by chromatography on a column (1.8 × 34 cm) of cellulose (Whatman CF 11) irrigated with ethyl acetate–acetic acid–water (18:3:2 v/v). The peak emerging at 1.44–1.68 L was concentrated to a low volume, and acetic acid was removed by repeated addition and evaporation of water. This gave an aqueous solution of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (102 mg, 40% yield from leucrose). Chromatography (periodate–starch–iodide or silver nitrate) showed a main component at R_G = 1.14 (product) and a trace substance, R_G = 0.47 (not identified). Analysis of a sample dried to a glass over P_2O_5 in vacuo for 3 days: Calcd for $C_9H_{16}O_9 \cdot 2H_2O$: C, 35.53; H, 6.63. Found: C, 35.66; H, 6.52.

Phenol–Sulfuric Acid Color. A solution of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, lyophilized and stored over P_2O_5 in vacuo for 6 days (to constant weight), was assumed to be water free. On a molar basis, the absorbance in the phenol–sulfuric acid assay was equal to that of glucose. Subsequent estimations of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid were therefore based upon this assay, using a standard curve for glucose.

Physical Constants. $[\alpha]^{22}_D$ +140° (*c* 2.5 in H_2O; no mutarotation). A sample of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (2.7 mg, based on a phenol–sulfuric acid assay), on titration to pH 8.6 with 0.01 N NaOH, behaved as a free acid of equivalent weight 270 (theory, 268).

Hydrolysis. *O*-α-D-Glucopyranosyl-(1→2)-D-glyceric acid was nonreducing. On hydrolysis it gave substances migrating at R_G = 1.0 and R_G = 2.7 (glyceric acid position). The yield of D-glucose was 0.95 mol/mol, based upon the Glucostat assay (Worthington Biochemical Corp.).

O-α-D-Glucopyranosyl-(1→2)-glycerol. This preparation involves the reduction of the methyl ester of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid with sodium borohydride by a method previously applied to a methyl uronate by Wolfrom & Anno (1952). The direction of addition (ester to borohydride) is critical. Thirty-two milligrams of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid was dissolved in 5 mL of 5% methanolic HCl. After 80 min at room temperature, the soluton was diluted with 5 mL of water and deacidified with Bio-Rad AG 3 (OH^-), made up in methanol–water (1:1). Solvents were removed, and the methyl ester (27 mg) was dissolved in water (0.5 mL). The ester solution was added, over 5 min, to a solution of $NaBH_4$ (100 mg) in 1 mL of water and left at room temperature overnight. Excess $NaBH_4$ was destroyed with acetic acid. The solution was heated on a steam bath for 30 min with an equal volume of 2 N NaOH, in order to saponify unreduced ester. The solution was passed through a column of Dowex 50 (H^+), and boric acid was removed by evaporation from methanol. The solution was then passed through Dowex 1 (OH^-) and concentrated to afford 28 mg of product, termed substance X [ultimately shown to be *O*-α-D-glucopyranosyl-(1→2)-glycerol], $[\alpha]^{22}_D$ +131° (*c* 1.4 in H_2O).

On paper chromatography it migrated at R_G = 1.24, the same rate as that of a sample of *O*-α-D-glucopyranosyl-(1→2)-glycerol prepared from maltose according to Charlson et al. (1956). Hydrolysis of substance X gave compounds of R_G = 1.0 and 2.63 (position of glycerol). Four milligrams of hydrolysate was successively reduced with $NaBH_4$, acetylated with Ac_2O–NaOAc, and extracted with chloroform. GLC gave two peaks with the same retention times as D-glucitol acetate (16.80 min) and glycerol acetate (0.92 min). The relative weights, obtained by correcting peak areas with the response factors of Sweet et al. (1975), were, glucitol acetate/glycerol acetate, 0.9:1.0.

O-α-D-Glucopyranosyl-(1→2)-[^{3}H]glycerol. The configuration of the glyceric acid moiety of *O*-α-D-glucopyranosyl-(1→2)-glyceric acid was examined as follows. A solution of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid methyl ester (prepared as described above; 3.5 mg, 12.4 μmol) was added, over 5 min, to a stirred solution of 40 mg of $NaBH_4$ and ca. 1 mCi of NaB^3H_4 in 0.4 mL of water. The solution was treated similarly to the nonradioactive compound, to afford 12.2 μmol of *O*-α-D-glucopyranosyl-(1→2)-[^{3}H]glycerol containing 53 × 10^6 dpm. A scan of a paper chromatogram showed that all of the radioactivity was located at R_G = 1.24.

[^{3}H]Glycerol. *O*-α-D-Glucopyranosyl-(1→2)-[^{3}H]glycerol (26.5 × 10^6 dpm) was hydrolyzed to give 24.6 × 10^6 dpm [^{3}H]glycerol, which was isolated by preparative chromatography on Whatman 3MM paper. [U-^{14}C]Glycerol (4.73 × 10^6 dpm) was then added to give a ratio of dpm of ^{3}H/dpm of ^{14}C of 5.2. The mixture of ^{14}C- and ^{3}H-labeled compounds was called [^{14}C,^{3}H]glycerol.

sn-[^{14}C,^{3}H]Glycerol 3-Phosphate. A solution (final volume 1 mL) was made, containing the following substances dissolved in 0.05 M triethanolamine hydrochloride buffer, pH 7.5: glycerol (1 μmol), [^{14}C,^{3}H]glycerol (0.4 μmol; 1.81 × 10^6 dpm of ^{3}H; 3.48 × 10^5 dpm of ^{14}C), ATP (sodium salt; 8 μmol), $MgCl_2$ (10 μmol), and *E. coli* glycerokinase (3 mg, 120 units). The solution was kept at 37 °C for 30 min, adjusted to pH 8 with NaOH, and applied to a column (1 × 18 cm) of Dowex 1 (Cl^-). The column was washed with 100 mL of water and eluted with a gradient made from 250 mL of water (mixer) and 250 mL of 0.04 M HCl (reservoir). The product appeared at 96–224 mL, the same position as *rac*-glycerol 1-phosphate. The solution was concentrated to 80 mL and lyophilized to give *sn*-[^{14}C,^{3}H]glycerol 3-phosphate (2.64 × 10^5 dpm of ^{14}C; ^{3}H/^{14}C dpm ratio, 5.3).

Periodate Oxidation of [^{3}H,^{14}C]Glycerol. A solution (1.75 mL) of sodium periodate (0.05 M) in acetic acid–water (1:3 v/v) was added to a solution of glycerol (50 μmol) and [^{14}C,^{3}H]glycerol (58 000 dpm of ^{14}C) in 7 mL of water. The resulting solution was stirred in the dark for 1 h, and excess periodate was destroyed by addition of 0.5 M sodium arsenite (1.75 mL). The solution was brought to pH 6.5 with NaOH and distilled. Dimedone solution (12 mg in 3 mL of water) was added to the distillate, and the mixture was kept for 4 h at room temperature and 20 h at 3 °C. The resulting formaldehyde–dimedone adduct was collected on a small sinter (M), washed with water, and dissolved in Aquasol (10 mL). A total of 22 200 dpm of ^{14}C was obtained, and the ratio of dpm of ^{3}H/dpm of ^{14}C was 7.3.

Periodate Oxidation of sn-[^{14}C,^{3}H]Glycerol 3-Phosphate. This was performed the same as that for the labeled glycerol, except that the substrate was a mixture of *rac*-glycerol 1-phosphate (acid form; 50 μmol) and *sn*-[^{14}C,^{3}H]glycerol 3-phosphate (130 000 dpm of ^{14}C). The resulting formaldehyde–dimedone derivative contained 21 100 dpm of ^{14}C.

The ratio of dpm of ^3H/dpm of ^{14}C was 0.38.

Results and Discussion

Properties of O-α-D-Glucopyranosyl-(1→2)-D-glyceric Acid. The product contained a trace impurity, R_G = 0.47. In view of the analytical results given below, the proportion of this substance was considered to be insignificant.

Lyophilization of an aqueous solution, followed by drying to constant weight, appeared to give a dry product, as it reacted with the theoretical amount of base.

The infrared spectrum included a strong carbonyl stretching band at 1715 cm^{-1}, attributable to a carboxyl group.

Acid hydrolysis gave 1 mol of D-glucose, which could be assayed by the Glucostat reagent. The other hydrolysis product chromatographed at the same rate as DL-glyceric acid. Further evidence for a glyceric acid moiety was obtained by conversion of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid into the corresponding glucosylglycerol (see below).

Linkage to Glyceric Acid. Reduction of the methyl ester of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid with borohydride gave a substance X which could be hydrolyzed to glucose and glycerol (paper chromatography and GLC of derived alditol acetates) and which chromatographed with authentic *O*-α-D-glucopyranosyl-(1→2)-glycerol. The position of substitution on the glycerol moiety of substance X was examined by lead tetraacetate oxidation. The glycerol moiety of a (1→1)-linked glucosylglycerol molecule should consume one lead teetraacetate molecule, with the release of one molecule of formaldehyde. That of the corresponding (1→2)-linked molecule should neither consume lead tetraacetate nor produce formaldehyde. In fact, 0.26 mol of lead tetraacetate was consumed and no formaldehyde was released (Table I). [Under the same conditions *O*-α-D-glucopyranosyl-(1→2)-erythritol took up 0.84 mol of lead tetraacetate and released the same quantity of formaldehyde.] The lack of formaldehyde formation clearly indicated that substance X contained a (1→2) linkage. The lead tetraacetate consumption of substance X, while closer to zero than 1 mol/mol, was less definitive.

We were unable to use enzymes to make a direct confirmation of the configuration of the anomeric carbon atom of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, as the latter was not hydrolyzed in the presence of α- or β-glucosidase (Table II). However, the derived glucosylglycerol, substance X, was 92% hydrolyzed by α-glucosidase, demonstrating the presence of an α linkage. [The susceptibility of *O*-α-D-glucopyranosyl-(1→2)-glycerol to α-glucosidase has already been demonstrated by Saier & Ballou (1968).] A small degree of hydrolysis occurred in the presence of β-glucosidase. This was attributable to incomplete specificity of the sample of β-glucosidase, as it hydrolyzed methyl α-D-glucopyranoside to the same extent (Table II). These results showed that substance X was *O*-α-D-glucopyranosyl-(1→2)-glycerol, thus confirming the α-(1→2) linkage in *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid. Further evidence for the α linkage was obtained by proton NMR spectroscopy (see below).

Configuration of the Glyceric Acid Moiety. Since we had developed an efficient method of conversion of *O*-α-D-glucopyranosyl-D-glyceric acid into glucosylglycerol (above), we decided to use this, rather than the direct enzymic assay of D-glyceric acid, to examine the configuration of the glyceric acid part of our sample of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid. The rationale is as follows (Figure 3).

Reduction of the methyl ester of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (II) or *O*-α-D-glucopyranosyl-(1→2)-L-glyceric acid (VI) with NaB^3H_3, followed by hydrolysis

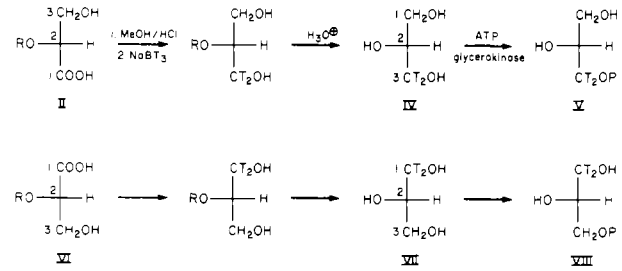

FIGURE 3: Assignment of configuration of the glyceric acid moieties of the diastereomeric *O*-α-D-glucopyranosyl-(1→2)-glyceric acids. R = α-D-glucopyranosyl; T = ^3H-labeled hydrogen; P = phosphate. The same reagents are used for the conversion of II into V as for that of VI into VIII.

of the resulting glucosylglycerol, would give [3-^3H]glycerol[2] (IV) or [1-^3H]glycerol (VII), respectively. The ratio of IV to VII can be determined by measuring the distribution of tritium on C-3 and C-1 of the labeled glycerol. This method resembles that described by Bublitz & Kennedy (1954) but depends upon the determination of $^3H/^{14}C$ isotope ratios of formaldehyde–dimedone adducts rather than measurement of their specific radioactivities. Our double-isotope labeling procedure has the advantage that yields and recoveries of the formaldehyde–dimedone adduct are not included in the calculation of tritium distribution. The resulting [^3H]glycerol, mixed with [U-^{14}C]glycerol, is phosphorylated specifically at position 3 (Karnovsky et al., 1957) with ATP in the presence of glycerokinase. The resulting double-labeled *sn*-glycerol 3-phosphate (V and/or VIII) and the original double-labeled glycerol (IV and/or VII) are separately oxidized with periodate, and the ratios of dpm of ^3H/dpm of ^{14}C of the liberated formaldehyde are determined in each case. It is assumed that equal weights of formaldehyde are derived from C-1 and C-3 of glycerol and that the formaldehyde obtained from *sn*-glycerol 3-phosphate originates from C-1.

The procedure was applied to *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, with the following results: formaldehyde from glycerol, dpm of ^3H/dpm of ^{14}C = 7.3; formaldehyde from *sn*-glycerol 3-phosphate, dpm of ^3H/dpm of ^{14}C = 0.38. The fraction of tritium on C-1 is estimated to be (0.38/7.3) × 50 = 3%. Taking into account the error expected for this type of experiment (Bublitz & Kennedy, 1954; Walton, 1973), it was concluded that the [^3H]glycerol contained 97 ± 3% of compound IV, labeled at C-3. Hence, our sample of glucosylglyceric acid was predominantly diastereomer II, in which the glyceric acid moiety has the D configuration.

NMR Spectroscopy. NMR spectroscopy of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid helped to confirm its structure. The spectra were taken together with those of four reference disaccharides under similar conditions for future use in the interpretation of polysaccharide spectra.

Since the ^{13}C NMR spectrum of methyl α-D-glucopyranoside remained unchanged in the range of pH 3–8.5 (Table III), it seemed legitimate to compare spectra of aqueous solutions of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (pH 3) and its salt (pH 8.5) with those of neutral solutions of disaccharides. Similar arguments apply to ^1H NMR spectroscopy, where it was shown that, for maltose, the chemical

[2] Stereochemical numbering of glycerol (IUPAC–IUB Commission, 1967) is used here. The carbons of the *pro-S* and *pro-R* carbinol groups are numbered 1 and 3, respectively, as shown in Figure 3. On reduction of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, the carboxyl carbon, C-1, becomes C-3 of the glycerol moiety.

Table III: Carbon-13 Chemical Shifts of *O*-α-D-Glucopyranosyl-(1→2)-D-glyceric Acid, Methyl α-D-Glucoside, and Some Disaccharides, at 25 °C[a]

compd	chemical shift (ppm) C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	C-1	C-2	C-3
methyl α-D-glucopyranoside[b]	100.7 (100.5)	73.0 (73.1)	74.7 (74.8)	71.1 (71.4)	72.7 (72.8)	62.1 (62.3)			
O-α-D-glucopyranosyl-(1→2)-D-glyceric acid	99.0	73.8[c]	74.4	70.9	72.9[c]	62.0	175.5	77.6	64.1
O-α-D-glucopyranosyl-(1→2)-D-glyceric acid, NH_4^+ salt	99.0	73.7[c]	74.9	71.0	73.2[c]	62.1	178.5	80.6	64.7
laminaribiose	104.1 (103.9)								
isomaltose	99.7 (99.4)								
maltose	101.1 (101.0)								
cellobiose	104.1 (103.9)								

[a] In parts per million downfield from external tetramethylsilane. Assignments of resonances of methyl glucoside and the disaccharides are those of Usui et al. (1973). The chemical shifts quoted in their paper are included in parentheses in this table. [b] Identical shifts were observed at pH 3 and pH 8.5. [c] Ascriptions which might be reversed.

Table IV: Proton NMR Data for *O*-α-D-Glucopyranosyl-(1→2)-D-glyceric Acid and Four Disaccharides

compd	chemical shift (ppm)[a,b] H-1′ 30 °C	H-1′ 90 °C	H-2	H-3	H-3″	coupling constant (Hz)[b] $J_{1',2'}$ 30 °C	$J_{1',2'}$ 90 °C	$J_{2,3}$	$J_{2,3''}$	$J_{3,3''}$
O-α-D-glucopyranosyl-(1→2)-D-glyceric acid	5.08		4.45	3.95	3.95	3.4		3.1	4.2	−10
O-α-D-glucopyranosyl-(1→2)-D-glyceric acid, Na^+ salt	5.00		4.18	3.88	3.80	3.4		3.5	5.8	−11
maltose[c]	5.40	5.34				3.2	3.2			
isomaltose	4.94	4.95				3.2	3.6			
laminaribiose	*d*	4.70				*d*	6.5			
cellobiose	4.52	4.52				7.4	7.5			

[a] Downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. All samples were run at 30 °C and at 90 °C. Only one set of figures is given for glucosylglyceric acid and glucosylglycerate, as line positions were not altered by heating. [b] First-order analysis for the H-1′ shift and $J_{1',2'}$; computer analysis for H-2 and H-3 shifts, $J_{2,3}$, and $J_{2,3'}$. [c] Maltose gave identical values at pD 2.5, 5, 7, and 9. [d] Value unobtainable at 30 °C, due to overlap by HO^2H peak.

shifts of H-1′ and $J_{1',2'}$ were constant in the range pD 2.5–9.5 (Table IV).

In the ^{13}C spectra of methyl α-D-glucopyranoside and the four disaccharides examined, only small differences from the chemical shifts quoted by Usui et al. (1973) were observed (Table III).

The resonance of the anomeric carbon (C-1′)[3] of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, and its ammonium salt, appeared at higher field (99.0 ppm) than resonances of C-1′ of the other glycosides examined (Table III; spectra are shown in Figure 4). The five signals at 62.1–74.7 ppm formed a similar pattern to the signals attributable to C-2′–C-6′ in the spectrum of methyl α-D-glucopyranoside, as would be expected for a pyranose ring in the C1 conformation. The assignments of resonances of C-3′, C-4′, and C-6′ were based upon the similarity of their chemical shifts to those of the corresponding atoms of methyl α-D-glucopyranoside. Unequivocal assignment of the signals of C-2′ and C-5′ could not be made in this way owing to small differences from the shifts of the corresponding atoms of methyl glucoside.

The three remaining resonances of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid were assigned to the carbons of the glyceric acid moiety (Table III) on the following basis: C-1, carboxyl (low field); C-2 (lower field than C-2′–C-5′ since α to a carboxyl group); C-3, primary alcohol (high field, with different chemical shift from C-6′). A 3-ppm downfield displacement of C-1 and C-2 resonances resulted from salt formation. A similar shift of C-1 and C-2 occurs when propionic acid is converted into its salt (Hagen & Roberts, 1969).

In the ^1H NMR spectra of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid (Figure 5) or its salt (Figure 6), the anomeric proton gave a doublet centered on δ 5.08 or δ 5.00, respectively, in between the positions of the anomeric protons of maltose and isomaltose (Table IV). The small coupling constant ($J_{1',2'}$ = 3.7 Hz) confirmed the α configuration of C-1′.

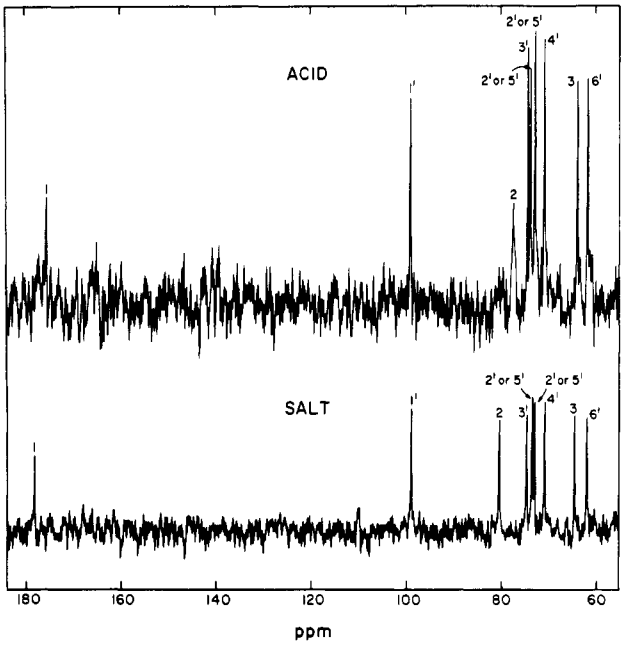

FIGURE 4: ^{13}C NMR spectra (15.09 MHz) of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid and its ammonium salt, at 25 °C. 50 000 transients for each spectrum; sweep width, 4 kHz; pulse width, 5 μs (~30° tip angle); cycle time, 1.0 s. Under these conditions peak intensities were sometimes affected by relative relaxation times of the nuclei concerned.

[3] C-1′ to C-6′ refer to the carbon atoms of the glucopyranosyl ring. C-1 to C-3 refer to the carbons of the glyceric acid moiety.

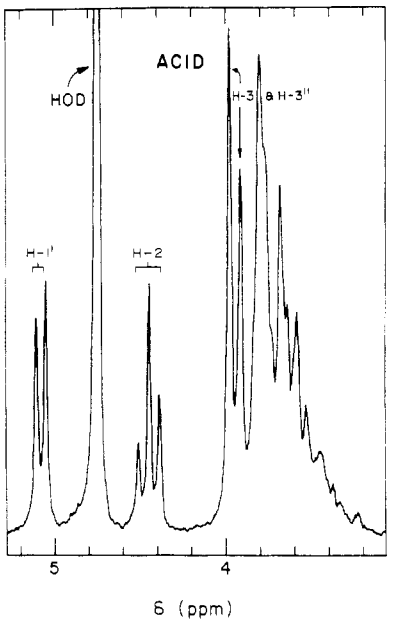

FIGURE 5: Proton NMR spectrum (60 MHz) of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid, at 30 °C. Represents 100 transients.

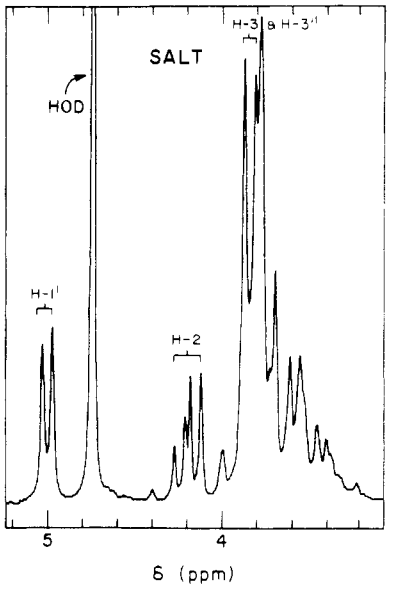

FIGURE 6: Proton NMR spectrum (60 MHz) of sodium *O*-α-D-glucopyranosyl-(1→2)-D-glycerate, at 30 °C. Represents 100 transients.

The assignments of resonances to protons on C-2 or C-3 of the glyceric acid moiety were made on the basis of spin decoupling. The chemical shifts and coupling constants of these protons (H-2, H-3, and H-3″; Table IV) are the results of computer fits of the 60-MHz spectra, for which the errors are less than the spectral line widths. For the 100-MHz spectra the computed coupling constants of these protons were the same as those in Table IV, but computed chemical shifts differed slightly (<0.04 ppm) from those of the 60-MHz spectra, presumably due to differences in the conditions (temperature and concentration) of the samples in the two spectrometers. Attempts to find other sets of data for the three protons lead either to the same values or to rapid divergence in the iterative procedure.

H-2, H-3, and H-3″ resonate downfield from H-2′ to H-6′ of the glucosyl ring, due to deshielding by the carboxyl group. For the acid, values of $J_{2,3}$ and $J_{2,3''}$ were 3.1 and 4.2 Hz, respectively, intermediate between corresponding values for

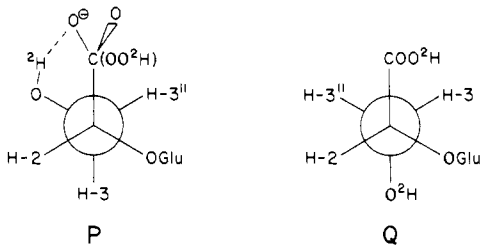

FIGURE 7: Rotamers resulting from torsion about the C-2–C-3 bond of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid or its anion. Rotamer P is shown as the acid or the anion. The dotted line represents a hydrogen bond connecting the 3-hydroxyl hydrogen to the carboxylate group of the anion. Glu = α-D-glucopyranosyl.

the salt ($J_{2,3}$ = 3.5 Hz; $J_{2,3''}$ = 5.8 Hz). All values were within the range 2.5–6.2 Hz observed by Lemieux & Martin (1970) for $J_{5,6}$ and $J_{5,6'}$ in a series of hexopyranoside acetates. The coupling constants of the latter were rationalized in terms of the ratios of two relatively stable C-5, C-6 rotamers. The corresponding C-2, C-3 rotamers for α-D-glucopyranosyl-(1→2)-D-glyceric acid would be P and Q (Figure 7).[4] For the acid, the similarity of the time-averaged values of $J_{2,3}$ and $J_{2,3''}$ is probably attributable to the existence of roughly equal populations of P and Q. For the salt, the greater inequality of $J_{2,3}$ and $J_{2,3''}$ probably reflects a higher proportion of rotamer P, for which $J_{2,3''}$ is much larger than $J_{2,3}$. A relatively strain-free six-membered ring, resulting from hydrogen bonding between the carboxylate and the 3-hydroxyl hydrogen atom, can be formed with rotamer P, but not with Q, and may account for the preponderance of P. We therefore suggest that the 2,3 and 2,3″ coupling constants reflect the influence of intramolecular hydrogen bonding (which is greater for the salt than for the acid) upon the relative populations of C-2, C-3 rotamers. Hydrogen bonds involving carboxylates have recently been demonstrated in glycosides of sialic acid (Jennings & Bhattacharjee, 1977) and of 3-deoxy-*manno*-octulosonic acid (Bhattacharjee et al., 1978). In naturally occurring MGP the 3-hydroxyl group of the aglycon glycerate is acylated. Obviously, the type of hydrogen bond proposed for our model compound cannot exist in this case, and the glycerate carboxylate is available for the type of metal chelation suggested by Smith & Ballou (1973).

Acknowledgments

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[4] For the argument which follows, it is necessary to assume that the H-3″ proton, which gives the greater coupling constant toward H-2, is *pro-S*, while H-3 is *pro-R*. Rigorous assignments have not been made. In a Dreiding model of the anion of II, which includes the proposed hydrogen-bonded ring, the *pro-S* and *pro-R* hydrogens adopt axial and equatorial positions, respectively. The assumption that the axial proton resonates upfield from the equatorial proton would lead to the same assignment as before, i.e., H-3″ of the anion, which resonates upfield from H-3, is *pro-S*.

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Biochemical Properties of Acetylcholine Receptor Subunits from *Torpedo californica*†

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ABSTRACT: Four polypeptide chains composing acetylcholine receptors from the electric organ of *Torpedo californica* were purified by preparative electrophoresis in sodium dodecyl sulfate. Their apparent mole ratio $\alpha/\beta/\gamma/\delta$ is 2:1:1:1. These chains are not readily distinguished by amino acid or carbohydrate composition but are distinguished by apparent molecular weight and polypeptide maps. By peptide maps, no extensive homology is evident between these chains or between any of these chains and higher molecular weight chains found in receptor-enriched membrane fragments.

Acetylcholine receptor (AcChR)[1] solubilized from the electric organ of *Torpedo californica* by Triton X-100 and purified by affinity chromatography is composed of four polypeptide chains termed α, β, γ, and δ. The apparent molecular weights of these chains depend on the electrophoresis system and standards employed but approximate 38 000, 50 000, 57 000, and 64 000 for α, β, γ, and δ, respectively (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1978; Chang & Bock, 1977; Lindstrom et al., 1978; Froehner & Rafto, 1979). α chains compose at least part of the acetylcholine binding site (Weill et al., 1974; Damle et al., 1978). δ chains are located near the acetylcholine binding site (Hamilton et al., 1978; Witzemann & Raftery, 1978). *Torpedo* AcChR's are dimerized through a disulfide bond between δ chains of adjacent monomers (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977; Hamilton et al., 1977). However, the function of the β, γ, or δ chains is unknown. If the ion conductance channel regulated by acetylcholine binding is an integral component of the AcChR macromolecule, then some or all of these chains may be components of this channel.

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[1] Abbreviations used: AcChR, acetylcholine receptor; [^{125}I]-α-BGT, ^{125}I-labeled α-bungarotoxin; $NaDodSO_4$, sodium dodecyl sulfate.

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