This C-16 hydroxy ester and methyl ricinoleate have nmr and infrared spectra which are identical except for differences attributable to unequal chain lengths. The mass spectra of the unsaturated ester and its hydrogenated derivative indicate[7] that the hydroxyl group is at the C-12 position and that the double bond is between C-1 and C-12.

Permanganate–Periodate Oxidation of the C-16:1 Hydroxy Ester.—A 53-mg portion of the ester was mixed with 480 mg of sodium iodate, 10 mg of potassium permanganate, 210 mg of potassium carbonate, 40 ml of water, and 10 ml of methanol[12] and stirred for 15 hr at room temperature. Products were isolated, esterified, and chromatographed as described in the preceding section. Pooling of consecutive eluate portions yielded four fractions totaling 47 mg. By gas chromatographic analysis, fraction 2 (33.2 mg) was 85% dimethyl azelate and 15% unknowns, fraction 3 (5.7 mg) was 70% dimethyl azelate, 20% unknowns and 10% methyl hydroxyheptanoate, and fraction 4 (6.7 mg) was 90% methyl hydroxyheptanoate. Mass spectral analysis confirmed the presence of dimethyl azelate in fraction 2 and indicated a CH_3OOCCH_2CHOH fragment from methyl 3-hydroxyheptanoate in fraction 4.

Optical Rotation of the C-16:1 Hydroxy Ester.—Methyl 12-hydroxy-*cis*-9-hexadecenoate, $[\alpha]^{26}D$ +6.2° (*c* 0.036 g/ml, methanol), shows a positive background curve which becomes less positive at low wavelengths: $[\alpha]^{27}_{500}$ +8.3°, $[\alpha]^{27}_{400}$ +13.9°, $[\alpha]^{27}_{300}$ +19.6°, $[\alpha]^{27}_{275}$ +20.8°, $[\alpha]^{27}_{250}$ +4.5° (*c* 0.775). Hydrogenation with platinum oxide catalyst in acetic acid gave methyl 12-hydroxyhexadecanoate, mp 45.6–46.0° after crystallization from petroleum ether, which had a plain negative ORD curve. *Anal.* Calcd for $C_{17}H_{34}O_3$: C, 71.28; H, 11.96. Found: C, 71.3; H, 11.9.

Optical Rotation of Methyl Densipolate.—Methyl densipolate (preparation described elsewhere)[13] was found to be levorotatory, $[\alpha]^{26}D$ −0.58° (*c* 0.131 g/ml, methanol). Smith, *et al.*,[3] reported $[\alpha]^{27}D$ 0 ± 1° (*c* 3.7, methanol).

Fatty Acid Composition of *L. densipila* Seed Oil.—*L. densipila* seeds were minced in a Brabender sample chopper, then steeped in petroleum ether at room temperature. From 64.4 g of seed, 16.9 g of oil was obtained. The oil had no selective ultraviolet adsorption between 220 and 350 mμ. An 11.2-g sample was saponified at room temperature by 4.0 g of potassium hydroxide in 20 ml of 50% ethanol. Eleven portions of ether extracted the nonsaponifiables (450 mg). After acidification of the alkaline solution, ether extracted 10.47 g of fatty acids. Methyl esters were prepared with methanolic hydrochloric acid at 60°, and a 650-mg sample was separated into nonhydroxy esters (40%) and hydroxy esters (60%) by chromatography on silicic acid.[11] Peaks in gas chromatograms were identified by comparison to standards and by use of equivalent chain length[14] data. Integrated peak areas were corrected by relative detector response factors determined for nonhydroxy esters on a purchased standard mixture and for hydroxy esters on a synthetic mixture of the pure esters. Chromatograms of hydrogenated and nonhydrogenated esters were compared to determine the relative amounts of C-18:3, C-20:0, and C-20:1 esters.

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Studies on the Fine Structure of Clam Glycogen[1]

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The fine structure of glycogen isolated from six species of fresh-water clam has been determined by periodate oxidation, methylation, and β-amylolysis. Periodate oxidation of the glycogen, followed by sodium borohydride reduction, yielded a polyalcohol which on methylation and degradation yielded methoxyacetaldehyde dimethylacetal, 1,3-di-*O*-methylglyceritol, 1,4-di-*O*-methylerythritol and 1-*O*-methyl-D-erythritol. The results obtained indicate that the specimens of glycogen from the different clam species possess a similar structure composed of linear chains of 1,4-α-linked glucose units with 1,6-α-linkages at the branch points. The average size of the repeating unit varies from 11 to 13 glucose units. All the clam glycogens show an identical behavior toward concanavalin-A and, therefore, can not be differentiated on the basis of this reaction.

Since the isolation of glycogen from dog liver by Claud Bernard in 1857,[3] its extraction from other animals[4] has been reported. Certain plants and microorganisms have been found to contain glycogen-like materials.[4] The structural investigations of glycogen from these sources have been carried out by the use of techniques based on methylation,[5] periodate oxidation,[6] and enzymic[7–9] and chemical degradation.[10] Although the periodate oxidation has been widely employed in the structural studies, limited work has been reported on glycogen polyaldehyde particularly, from the point of view of its application to the study of the fine structure of glycogen. This paper deals with the isolation and characterization of glycogen from the fresh-water clam and a detailed chemical account of glycogen polyaldehyde.

The glycogen was isolated from six species of fresh-water clams by extraction with alkali.[11] All the samples of glycogen showed similar physical and chemical behavior. Attempts to differentiate them by concanavalin-A[12] were unsuccessful as indicated by their glycogen values given in Table I.

Oxidation with periodate[6] was carried out on all six samples of glycogen. The consumption of the oxidant[13]

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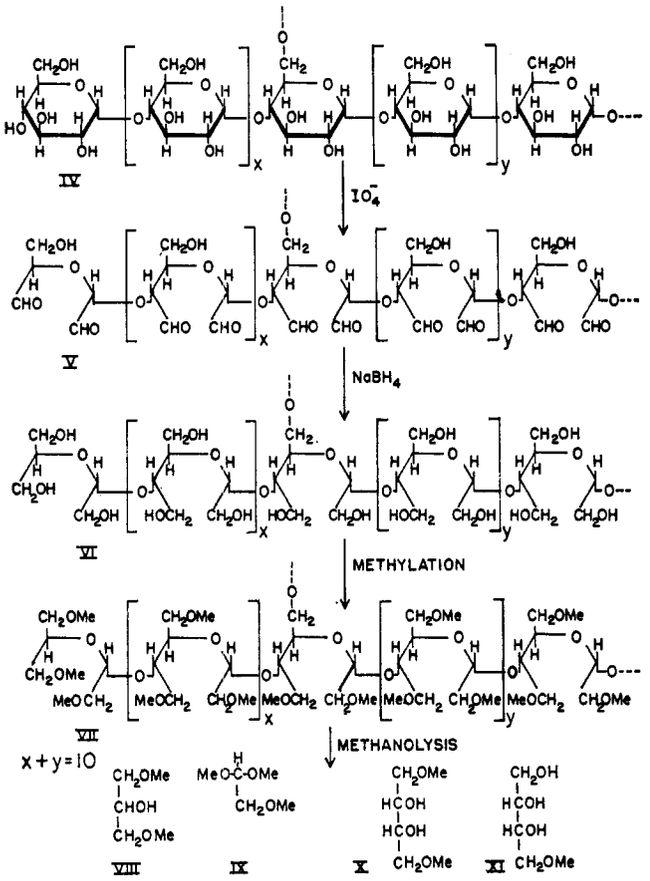

Figure 1.—Preparation and methylation studies on glycogen polyaldehyde.

TABLE I

EXTRACTION OF GLYCOGEN FROM SIX SPECIES OF FRESH-WATER CLAM AND THEIR REACTION WITH CONCANAVALIN-A

Source	Yield,[a] %	$[\alpha]^{24}D$, deg, water	gv^b
Lampsillis siliquoidea	1.76	+194	1.08
Amblema costata	2.20	+195	1.12
Obovaria obliquaria	2.20	+193	1.16
Fusconaia undata	2.20	+201	1.16
Elliptio dilatatus	1.40	+202	1.18
Anodonta grandis	...	+193	1.18

[a] Calculated on the wet tissue weight basis. [b] Glycogen value: optical density of a glycogen solution with concanavalin-A at 420 mμ with respect to human liver glycogen as 1.

and the production of formic acid[14] were ascertained by the methods of Fleury and Lange. The molar proportion of formic acid produced during oxidation corresponded to an average chain length ($\bar{c}l$) of 12 to 13 glucose units, a value similar to that reported earlier for glycogens derived from other sources.[6] Table II summarizes the results of the periodate oxidation.

Because of the similar chemical behavior of these glycogen samples, extensive structural studies were conducted on only one specimen obtained from *Anodonta grandis*. This particular sample of glycogen, after further purification by acetylation and deacetylation, showed $[\alpha]^{24}D$ +193.4° and yielded only glucose on acid hydrolysis. Glass-fiber paper electrophoresis[15] and ultracentrifugation indicated that the material was essentially homogeneous.

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TABLE II

DETERMINATION OF THE AVERAGE CHAIN LENGTH ($\bar{c}l$) OF CLAM GLYCOGEN BY PERIODATE OXIDATION (TIME, 10 DAYS)

Source of glycogen	Moles of IO_4/glucose unit	$\bar{c}l$
Lampsillis siliquoidea	1.12	12.8
Obovaria obliquaria	1.08	13.4
Amblema costata	1.12	12.6
Fusconaia undata	1.08	12.7
Elliptio dilatatus	1.14	13.1
Anodonta grandis	1.05	13.4

A complete methylation of the glycogen was brought about by the successive application of the Haworth,[5] Purdie,[16] and Kuhn methods.[17] The methylated glycogen (OCH_3, 45.3%), after hydrolysis and fractionation of the hydrolysate on a hydrocellulose–cellulose column,[18] yielded 2,3,4,6-tetra-*O*-methyl-D-glucose (I), 2,3,6-tri-*O*-methyl-D-glucose (II), and 2,3-di-*O*-methyl-D-glucose (III). Traces of other isomers of di-*O*-methyl-D-glucose and mono-*O*-methyl-D-glucose resulting from the incomplete methylation and/or demethylation during hydrolysis[19] were also detected, but it is believed that they are of no structural significance. Components I–III, which arise respectively from 1-, 1,4-di-, and 1,4,6-tri-*O*-substituted glucose units, were produced in a molar ratio of 1:9:1, thus giving $\bar{c}l$ 11. This value is in close agreement with that previously found for other specimens of glycogen.[20]

The *A. grandis* glycogen (IV) was then subjected to prolonged periodate oxidation (Figure 1) and the resulting polyaldehyde (V) was isolated by the freezing and thawing technique.[21] The polyaldehyde was reduced to the corresponding polyalcohol (VI) which in turn was methylated in the usual manner. The polyalcohol was methylated much more readily than the parent polysaccharide probably because of its acyclic nature and the presence of primary alcohol groups. Methanolysis of the methylated glycogen polyalcohol (VII) afforded a mixture of 1,3-di-*O*-methylglyceritol (VIII), methoxyacetaldehyde dimethylacetal (IX), 1,4-di-*O*-methylerythritol (X), and 1-*O*-methyl-D-erythritol (XI). Component X arises from the 1,4-linked residues and was identified as its 2,3-di-*p*-toluenesulfonate. Component XI which is derived from those glucose residues at which branching occurs, was identified as crystalline 2,3,4-tri-*p*-nitrobenzoate. Component IX which arises from C-1 and C-2 of all the glucose residues was characterized as crystalline *p*-nitrophenylhydrazone of methoxyacetaldehyde.

Since X arises from the nonterminal glucose units and XI from the glucose residues at which branching occurs, it has been pointed out[22] that the determination of their molecular ratio should provide a method for the end-group assay.

Dische and Borenfreund[23] reported the use of diphenylamine as a specific colorimetric reagent for

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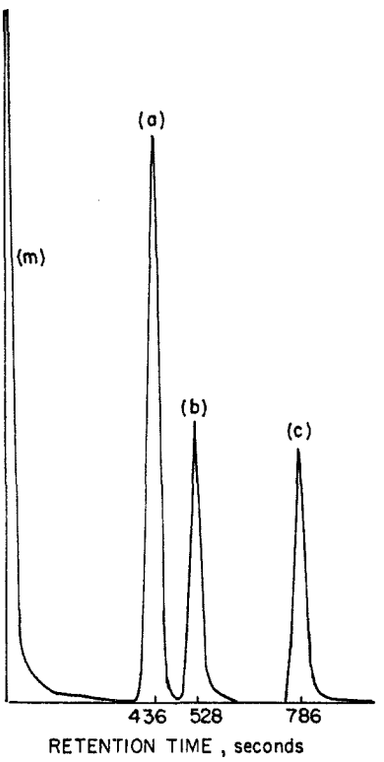

Figure 2.—Gas chromatogram of the acid degradation products of methylated glycogen polyalcohol. Peaks represent (a) methoxyacetaldehyde dimethylacetal, attenuation 8×; (b) 1,3-di-*O*-methylglyceritol; and (c) 1,4-di-*O*-methylerythritol and 1-*O*-methyl-D-erythritol, attenuation 8×; peaks m and w represent methanol and water, respectively. Operating conditions: detector, thermoconductivity cell; column packing, 20% silicone oil DC 500 on Teflon; column dimensions, 6 × 0.25 in.; column temperature 65–225°; program speed, 11°/min; block temperature, 300°; carrier gas flow rate, 40 ml/min; carrier gas reference, 12 ml/min.

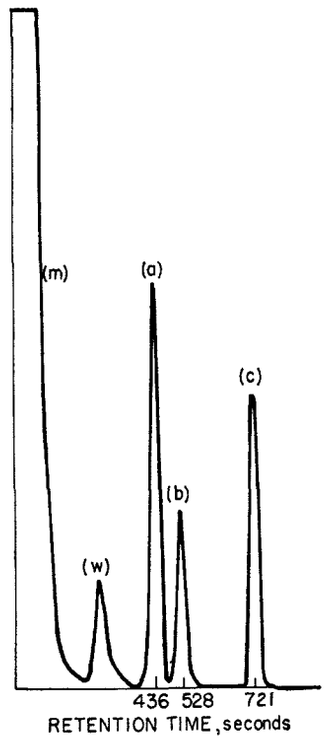

Figure 3.—Gas chromatogram of the acid hydrolysis products of methylated hexahydric alcohol. Peaks represent (a) methoxyacetaldehyde dimethylacetal; (b) 1,3-di-*O*-methylglyceritol; and (c) 1,4-di-*O*-methylerythritol and (m) methanol. For operating conditions, see legend under Figure 2.

glycolic aldehyde. Inasmuch as this reagent gives a similar color reaction with methoxyacetaldehyde, it was concluded that the diphenylamine reagent could be employed for the determination of X and XI subsequent to their oxidation with periodate.

After separation of X and XI from the methanolysate of methylated glycogen polyalcohol by paper chromatography, the components were treated with sodium periodate, followed by reduction of the residual periodate to iodate with sodium arsenite. The diphenylamine color reaction was then carried out and the absorbance of the resulting green solution was measured at 660 mμ. Since the intensity of the color produced varied with the time of heating, the standards were run with each assay. The average value of the chain length obtained from these experiments was about 13 glucose units, a result in good agreement with the values obtained by other methods.

Inspection of the formulas (Figure 1) shows that the 1,3-di-*O*-methylglyceritol (VIII) produced from the methanolysis of methylated glycogen polyalcohol, should be equivalent to the amount of 1-*O*-methyl-D-erythritol (XI) and, hence, determination of the ratio VIII:X should also provide a value for the average chain length. Another component present in the methanolysate is methoxyacetyldehyde dimethylacetal (IX) which arises from C-1 and C-2 of every glucose unit and, hence, the ratio VIII:IX would also give the average chain length. These cleavage fragments are relatively volatile, undetectable on paper and, hitherto, it has not been possible to determine them quantitatively. However, the technique of gas–liquid partition chromatography (glpc) has enabled this difficulty to be surmounted.

The methylated glycogen polyalcohol after methanolysis was subjected to gas–liquid partition chromatography using a silicone oil–Teflon column. The gas chromatogram showed three peaks (Figure 2) which corresponded to (a) methoxyacetaldehyde dimethylacetal (IX), (b) 1,3-di-*O*-methylglyceritol (VIII), and (c) 1,4-di-*O*-methylerythritol (X) and 1-*O*-methyl-D-erythritol (XI). The molar proportion of each component was computed by dividing the area of the curve corresponding to that component by its molecular weight. The combined molar proportion of X and XI was determined by dividing the area under curve c by the molecular weight of X since X forms the major fraction although curve c represents both these components. A correction was necessary in determining the molar proportion of IX and X. The correction factors were determined by chromatographing (Figure 3) a known mixture of VIII, IX, and X prepared by methanolysis of the methylated hexahydric alcohol[22] (XII) obtained from methyl β-lactoside by periodate oxidation, reduction, and methylation. The results of six experiments are recorded in Table III.

Whereas the methanolysis of the methylated polysaccharide yields a complex mixture of methylated glycosides containing both α- and β-anomers of furanosides and pyranosides,[24] the hydrolysis of the methylated polyalcohol as proposed in the present work results in a simple mixture and, hence, is much easier to analyze by

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TABLE III

CHAIN LENGTH OF CLAM GLYCOGEN BY GLPC

Methoxyacetaldehyde dimethylacetal, moles	Dimethylglyceritol, moles	Dimethylerythritol + methylerythritol, moles	$\bar{c}l^{a,b}$ A	$\bar{c}l^{a,b}$ B
12.6	1	12.8	13.8	12.6
11.7	1	12.8	13.8	11.7
15.0	1	12.8	13.8	15.0
15.2	1	12.8	13.8	15.2
13.3	1	14.1	15.1	13.3
15.2	1	14.1	15.1	15.2

[a] (A) Moles of 1,4-di-*O*-methylerythritol and 1-*O*-methyl-D-erythritol + 1. (B) Moles of methoxyacetaldehyde dimethylacetal per mole of 1,3-di-*O*-methylglyceritol.

the gas–liquid portion chromatographic technique. Several other derivatives such as acetates[25] and trimethylsilyl ethers[26] have been employed for the gas chromatography of sugars but so far no satisfactory quantitation has been accomplished.

Finally, the glycogen from *A. grandis* was subjected to exhaustive β-amylolysis with β-amylase by means of which maltose was generated by a stepwise attack which begins at the nonreducing ends. The degradation continues until the hydrolysis reaches within 2–3 units of the branching point. Determination of the maltose, produced during β-amylolysis, by Somogyi–Nelson method[27] showed that 40.7% hydrolysis occurred. Since the repeating unit is about 12, the number of glucose residues removed as maltose by β-amylase from the exterior chains is approximately 5. Thus, the number of residues in the exterior chains is 7–8 and in the interior chains 3–4 glucose units.[28]

Experimental Section

Solutions were concentrated under reduced pressure below 40° using a rotary evaporator. Paper chromatography was carried out by the descending method at room temperature on Whatman No. 1 or 3MM papers. The solvent systems were (v/v) (A) 1-butanol–ethanol–water (4:1:5 upper layer);[29] (B) pyridine–ethyl acetate–water (1:2.5:3.5 upper layer);[30] (C) butanone–water azeotrope,[31] bp 72.8; (D) benzene–ethanol–water–ammonium hydroxide (200:47:17:1 upper layer).[32] The spray reagents were (E) ammonical silver nitrate;[33] (F) *p*-anisidine trichloroacetate.[34]

Isolation and Purification of Glycogen.—Whole clam tissue (1000 g.) from *Lampsilis siliquoidea* was dissolved by heating in 30% sodium hydroxide (2 l.). Glycogen was precipitated from the alkaline extract by methanol (2.5 volumes). The resulting crude material was freed of protein contaminants by treating with 4% trichloroacetic acid. Further purification was achieved by precipitating glycogen several times from its aqueous solution with ethanol (2.5 volumes). Finally, the sample was washed with 95% ethanol, ether, and petroleum ether (bp 60–80°) and was dried *in vacuo:* yield, 17.6 g. A similar procedure was applied for the extraction of glycogen from other clam species.

I. Periodate Oxidation Studies on Clam Glycogens. End-Group Analysis.—The procedure of Abdel-Akher and Smith[6] was used for the end-group analysis of glycogen from various species of clam. An experiment with a sample of glycogen from one of the species, *A. grandis*, is described below.

Glycogen (592 mg), $[\alpha]^{24}D$ +193.4° in water (*c* 1.0), was oxidized with 0.1 *N* sodium periodate at 4–5° in the dark. A blank experiment was carried out concurrently under the same conditions. At suitable intervals known aliquots from the reaction mixture as well as from the blank were taken to determine the periodate consumption[13] and the amount of formic acid formed during oxidation.[14]

II. Methylation Studies.—A sample of glycogen (4 g) from *A. grandis*, $[\alpha]^{24}D$ +193.4° in water (*c*, 1.0) was treated with 30% sodium hydroxide (200 ml) and methyl sulfate[5] (60 ml) twice, successively. The methylated glycogen which separated out from the solution, was recovered by filtration. After washing the residue with hot water to remove salts, it was dried. To ensure complete methylation, the residue was dissolved in acetone (20 ml) and further methylated with methyl iodide (30 ml) and silver oxide[15] (5 g). This operation was repeated once more. Finally, two treatments of Kuhn methylation[17] were given using N,N-dimethylformamide as solvent.

The methylated product from the Kuhn methylation was dissolved in acetone (50 ml) and the solution was poured, with stirring, into petroleum ether (1.5 l.). The precipitate of the methylated glycogen was recovered by filtration and washed with petroleum ether and dried *in vacuo:* yield 2.65 g, $[\alpha]^{24}D$ +209° in chloroform (*c* 0.5). *Anal.* Calcd for $C_9H_{16}O_5$: OCH_3, 45.6. Found: 45.3.

Hydrolysis of Methylated Glycogen.—The methylated glycogen (572.4 mg) was hydrolyzed[35] by refluxing with 2.5% methanolic hydrogen chloride for 12 hr followed by heating with 1 *N* sulfuric acid at 100° for 22 hr. The hydrolysate was examined by paper chromatography using solvent C and spray F. Three major spots corresponding to 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose were detected.

Separation and Identification of the Methylated Glucose Derivatives.—The syrupy hydrolysate (556.8 mg) from the preceding experiment was dissolved in butanone–water azeotrope (2 ml) and transferred to a cellulose–hydrocellulose column[18] (38 × 3.5 cm). Resolution of the mixture of the methylated sugars was effected with the same solvent and the following fractions were obtained: A, 63.6; B, 412.3; C, 46.2; D, 5.9; and E, 1.2 mg.

Fraction A was shown by acid hydrolysis and paper chromatography of the hydrolysate to be a mixture of 2,3,4,6-tetra-*O*-methyl-D-glucose (50.2 mg) and methyl 2,3,6-tri-*O*-methyl-D-glucose (10.0 mg). Fractions B and C were identified as 2,3,6-tri-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose, respectively. Fractions D and E were found to be di-*O*- and mono-*O*-methyl-D-glucose, respectively, and were not further identified.

(1) 2,3,4,6-Tetra-*O*-methyl-D-glucose crystallized spontaneously and after recrystallization from petroleum ether, the 2,3,4,6-tetra-*O*-methyl-D-glucose had mp and mmp 95–96°, $[\alpha]^{24}D$ +84.4° in water (*c* 0.5).[36]

(2) After recrystallization from ether, 2,3,6-tri-*O*-methyl-D-glucose had mp and mmp 117–118°, $[\alpha]^{24}D$ +70° in water (*c* 0.5).[36]

(3) 2,3-Di-*O*-methyl-D-glucose was chromatographically identical with 2,3-di-*O*-methyl-D-glucose: $[\alpha]^{24}D$ +50.4° in methanol (*c* 0.5).

A portion (10 mg) of the 2,3-di-*O*-methyl-D-glucose in ethanol (5 ml) containing aniline (0.5 ml) was refluxed for 4–5 hr. After removal of the solvent and excess of aniline, a crystalline product was obtained. Recrystallization of the product from ethyl acetate gave *N*-phenyl 2,3-di-*O*-methyl-D-glucosylamine:[37] mp and mmp 132–134°; lit. 134°.

β-Amylolysis of Glycogen.—Glycogen (42.7 mg) was dissolved in water (5 ml) and β-amylase (15 mg, Wallerstein) in 1 *M* sodium acetate buffer (15 ml) at pH 4.8 was added. The reaction mixture was incubated at 37°. After temperature equilibration for 0.5 hr, the volume was adjusted to 25 ml. An aliquot (1 ml) was taken after 56 hr, diluted to 10 ml with water, and maltose was determined by the Somogyi–Nelson method.[27] The total maltose thus produced was 18.25 mg, corresponding to a value of 40.7% for the β-amylolysis limit. In a duplicate experiment, a

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value of 41% was obtained after incubation period of 86 hr using twice as much enzyme.

Reaction of Glycogens from Fresh-Water Clams with Concanavalin-A.—Glycogen from the six species of fresh-water clams was dissolved in water at four different concentrations (0.1–1 mg/ml). Reaction with concanavalin-A was carried out on a 1-ml aliquot using the procedure of Cifonelli and Smith.[12] Curves for the above samples as well as for human liver glycogen as a standard were obtained by plotting absorbancies at 420 mμ *vs.* concentrations. The ratio of the absorbancies produced by an equal weight of clam glycogens and the standard human liver glycogen were calculated and are recorded as glycogen values in Table I.

Studies on the Glycogen Polyaldehyde. Periodate Oxidation of Clam Glycogen (*A. grandis*).—To a solution of glycogen (20 g) in water (900 ml), 0.2 *M* periodic acid (900 ml) was added. After adjusting the volume to 2 l. with water, the reaction mixture was kept at 4–5° in the dark. A blank experiment was carried out under the same conditions. The consumption of periodic acid was determined at suitable intervals by the method already described. After 6 weeks the uptake of periodic acid became constant at 1.12 moles of the oxidant/glucose unit.

Isolation and Reduction of Glycogen Polyaldehyde.[21]—The oxidation mixture was frozen at −10°. On thawing the frozen mixture at room temperature, the glycogen polyaldehyde separated out as an amorphous solid. The polyaldehyde was recovered by centrifuging and washed with water until free from iodic and periodic acids (negative test with acidified potassium iodide). The white amorphous solid was then washed successively with 95% ethanol, ethanol, diethyl ether, petroleum ether, and finally dried at room temperature *in vacuo:* yield 19.6 g or 98%.

The glycogen polyaldehyde (6.3 g) was gradually added, with stirring, to a solution of sodium borohydride (3.5 g) in water (100 ml). The alkaline reaction mixture was allowed to stand at room temperature for 48 hr.

Methylation of Glycogen Polyalcohol.—The solution (100 ml) containing the glycogen polyalcohol was treated with 60% sodium hydroxide (100 ml) and to this solution methyl sulfate (65 ml) was added during 2 hr with vigorous stirring. The partially methylated product was recovered by extraction five times with chloroform. The combined chloroform extracts were washed three times with water, dried over magnesium sulfate, and concentrated to dryness to give methylated glycogen polyalcohol as a yellow syrupy residue (6.5 g).

In order to cleave any borate complexes[38] and to facilitate the completion of the methylation, the partially methylated product was acetylated in pyridine solution (300 ml) with acetic anhydride (10 ml). The resulting partially methylated–acetylated glycogen polyalcohol was dissolved in acetone (50 ml) and subjected to another Haworth methylation. The methylated polyalcohol (5.44 g) recovered in the usual manner, was further methylated in *N,N*-dimethylformamide (30 ml) solution twice with silver oxide (25 g) and methyl iodide (40 ml). The fully methylated product showed no optical activity in chloroform (*c*, 0.4). *Anal.* Calcd for methylated glycogen polyalcohol (see formula in Figure 1): OCH_3, 44.7. Found: OCH_3, 43.8.

Methanolysis of Methylated Glycogen Polyalcohol and Identification of the Degradation Products.—The methylated glycogen polyalcohol (1.048 g) was refluxed with 3.3% methanolic hydrogen chloride (25 ml) for 4–5 hr. The reaction mixture was neutralized with silver carbonate, filtered, and the solvent distilled (atmosphere pressure) to give a distillate (*ca.* 30 ml) and residue (15 ml). Paper chromatographic analysis of the residue using solvent C and spray E showed the presence of 1,4-di-*O*-methylerythritol (R_f, 0.57) and 1-*O*-methyl-D-erythritol (R_f, 0.26).

The distillate (10 ml) was heated with a solution of *p*-nitrophenylhydrazine hydrochloride (0.4 g) in hydrochloric acid (0.4 ml) and water (25 ml). On evaporating the methanol, a yellow crystalline precipitate of methoxyacetaldehyde *p*-nitrophenylhydrazone separated out and was immediately removed by filtration. After recrystallization from aqueous ethanol, the methoxyacetaldehyde *p*-nitrophenylhydrazone[20] showed mp and mmp 115–116°.

The residue consisting of 1,4-di-*O*-methylerythritol (581.2 mg) and 1-*O*-methyl-D-erythritol (82.0 mg) was fractionated on a hydrocellulose–cellulose column using butanone–water azeotrope as eluent. The syrupy 1,4-di-*O*-methylerythritol (50 mg) was treated with *p*-toluenesulfonyl chloride (150 mg). After keeping overnight the reaction mixture was poured, with stirring, into ice-cold water. After recrystallization from ethanol, the 1,4-di-*O*-methyl-2,3-di-*O*-tosylerythritol[22] had mp and mmp 130°.

A solution of 1-*O*-methyl-D-erythritol (10 mg) in dry pyridine (2 ml) was treated with *p*-nitrobenzoyl chloride (50 mg) and the solution heated for 30 min at 85–90°. The reaction mixture was poured into ice-cold water and the precipitate of 1-*O*-methyl-D-erythritol-2,3,4-tri-*p*-nitrobenzoate[39] was recovered by centrifugation. After washing with water, the recrystallization of the product from ethanol gave fine white needles, mp and mmp 163–65°.

Determination of the Ratio of 1-*O*-Methyl-D-erythritol to 1,4-Di-*O*-methylerythritol by Diphenylamine Reaction. Standard Curves.—Standard curves were prepared by using varying amounts of 1,4-di-*O*-methylerythritol (X) (0.25–1.60 mg) and 1-*O*-methyl-D-erythritol (XI) (0.12–0.80 mg). The following procedure was used: A known volume of solution, not exceeding 6 ml, of X or XI was transferred to a 25-ml volumetric flask and the volume adjusted to 6 ml with water if necessary. The solution was acidified with 1 *N* sulfuric acid (0.3 ml) and to this was added with stirring 0.1 *M* sodium periodate (1.5 ml). After exactly 10 min 1 *M* sodium arsenite (1.5 ml) was added. About 20 sec after the addition of the arsenite solution, iodine appeared momentarily and then faded rapidly. After 5–10 min, the contents of the flasks were adjusted to 25 ml by the addition of water and thoroughly mixed.

To a 2-ml aliquot of the solution in a test tube, 5.7 *M* trichloroacetic acid solution (0.4 ml) and 1% diphenylamine in glacial acetic acid, previously distilled over Cr_2O_3 (4.8 ml), were added successively with mixing and the tubes were heated for 30 min on a boiling-water bath. A blank was carried out simultaneously in a similar manner using water in place of the methylated alcohols. Standard curves were obtained by plotting absorbancies *vs.* concentrations (Table IV).

TABLE IV

STANDARD CURVES FOR 1,4-DI-*O*-METHYLERYTHRITOL AND 1-*O*-METHYL-D-ERYTHRITOL

Component	Concn, μg	Absorbance, 660 mμ
1,4-Di-*O*-methylerythritol	53	0.235
	106	0.430
	159	0.635
1-*O*-Methyl-D-erythritol	25.1	0.245
	50.3	0.502
	75.4	0.77

Determination of the Composition of a Known Mixture of 1-*O*-Methyl-D-erythritol and 1,4-Di-*O*-methylerythritol.—A solution (1 ml) containing 1,4-di-*O*-methylerythritol (2.15 mg) and 1-*O*-methyl-D-erythritol (3.8 mg) in methanol was prepared and the components in 0.1 ml of this solution were separated on Whatman No. 1 paper using solvent D. After developing, the chromatogram was dried in air for a minimum time to avoid any loss of the components due to evaporation. The section of the paper containing 1,4-di-*O*-methylerythritol was eluted with 25 ml of water and that portion of the paper containing 1-*O*-methyl-D-erythritol was eluted with 10 ml of water. Two strips of the same size from another paper irrigated with the same solvent for the same length of time were similarly eluted with water for respective blanks. Each solution (5 ml) was placed in 25-ml flasks and water (1 ml) was added. The determination was carried out as described above. The solutions of known concentrations as standards were treated at the same time.

A 2-ml aliquot was found to contain 15 μg of 1-*O*-methyl-D-erythritol and 33.5 μg of 1,4-di-*O*-methylerythritol giving percentage recoveries of 98.7 and 97.4, respectively.

Determination of the Molar Ratio of 1-*O*-Methyl-D-erythritol to 1,4-Di-*O*-methylerythritol in the Methanolysis Products of the Methylated Glycogen Polyalcohol.—The methylated glycogen polyalcohol (146.7 mg) was refluxed with 3% methanolic hydrogen chloride (*ca.* 2.5 ml) for 4.5 hr. The reaction mixture

(38) M. Abdel-Akher, J. K. Hamilton, and F. Smith, *J. Am. Chem. Soc.*, **73**, 4691 (1951).

(39) I. J. Goldstein, J. K. Hamilton, and F. Smith, *ibid.*, **81**, 6252 (1959).

was neutralized with silver carbonate and filtered without suction to avoid any loss due to evaporation.

A portion (0.1 ml) of the filtrate was fractionated on paper, using solvent D, and a quantitative determination of 1-*O*-methyl-D-erythritol and 1,4-di-*O*-methylerythritol was carried out as described above. A 2-ml aliquot was found, by reference to the standard curves, to contain 13 μg of 1-*O*-methyl-D-erythritol and 60 μg of 1,4-di-*O*-methylerythritol. The molar ratio of mono- to di-*O*-methylerythritol as calculated from these results was 1:10.5 giving a value of 12.5 for the average chain length assuming that for every mole of 1-*O*-methyl-D-erythritol, there is also present 1 mole of 1,3-di-methylglyceritol which arises from the nonreducing ends. In a duplicate experiment, the molar ratio was found to be 1:11.5 thereby giving an average chain length of 13.5. The duplicate was carried out under the same conditionas used for the experiment except that the chromatogram was developed for 16–17 hr in solvent A in place of D. This change of irrigating solvent was necessitated by the fact that the 1-*O*-methyl-D-erythritol showed only a small movement from the origin when solvent D was used and, hence, it was contaminated with any impurities which likewise remained on the starting line of the chromatogram.

Determination of Chain Length of Clam Glycogen by Gas–Liquid Partition Chromatography.—An F and M gas chromatograph model 500 with thermoconductivity cell detector and a column packed with 20% silicone oil DC 500 (Dow Corning) on Teflon (polytetrafluoroethylene) (w/w) was employed. The operating conditions are described in the legend under Figure 2.

Standards (50–80 μg) of 1,3-di-*O*-methylglyceritol (VIII), methoxyacetaldehyde dimethylacetal (IX), 1,4-di-*O*-methylerythritol (X), and 1-*O*-methyl-D-erythritol (XI) in methanol solution (5–15 μl) were chromatographed individually as well as their synthetic mixtures. The retention time of each compound is recorded in Table V. This is the time in minutes between the point of appearance of methanol curve and the apex of the peak of the compound being tested. A mixture containing components X and XI was not resolved under these conditions.

Determination of the Molar Ratio of VIII, IX, and X in the Methanolysate of Methylated Hexahydric Alcohol from Methyl β-Lactoside.—The methylated hexahydric alcohol (about 10 mg) was methanolyzed by refluxing for 4.5 hr with 2% methanolic hydrogen chloride (1 ml). After neutralizing with silver carbonate, the solution was filtered (no suction), and an aliquot (10 μl) of the filtrate was subjected to gas chromatographic analysis. The gas chromatogram showed three peaks (Figure 3). The molar ratio of methoxyacetaldehyde dimethylacetal (IX), 1,3-di-*O*-methylglyceritol (VIII), and 1,4-di-*O*-methylerythritol (X) thus found was 2:0.95:0.76; theoretical, 2:1:1. Therefore, an appropriate correction for the components VIII and X was applied while computing their molar proportion in the methanolysis products of the methylated glycogen polyalcohol.

TABLE V

RETENTION TIMES OF THE STANDARDS RELATIVE TO METHANOL

Compound	Retention time, Min	Retention time, Sec
Methoxyacetaldehyde dimethylacetal	7	16
1,3-Di-*O*-methylglyceritol	8	48
1,4-Di-*O*-methylerythritol	13	6
1-*O*-Methyl-D-erythritol	14	0
1,4-Di-*O*-methylerythritol + 1-*O*-methyl-D-erythritol	12	12

Determination of the Chain Length of Clam Glycogen.—A solution (10–15 μl) of the methanolysis products of methylated glycogen polyalcohol (0.8–1.0 mg) in methanol (5–15 μl.) was injected into the column. The chain length (*cl*) is given by the number of moles of 1-*O*-methyl-D-erythritol and 1,4-di-*O*-methylerythritol plus 1 or by the number of moles of methoxyacetaldehyde dimethylacetal per mole of 1,3-di-*O*-methylglyceritol.

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Analogs of Firefly Luciferin. III[1]

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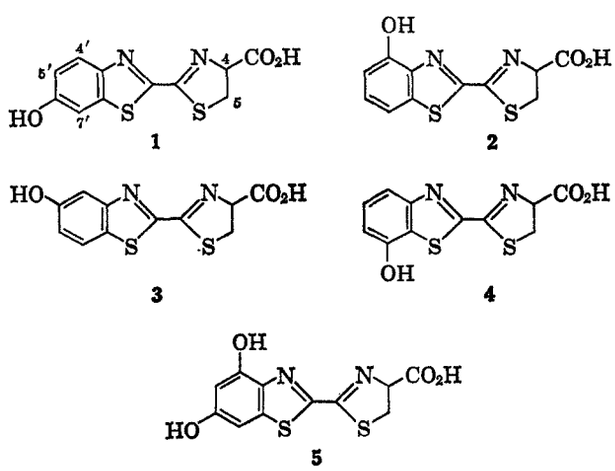
The arylhydroxy isomers of firefly luciferin (compounds **2–4**) and the derivative, 4-hydroxyluciferin (**5**), have been synthesized and tested. Only compound **5** proved active in the enzymatic assay. Physical properties of the luciferins are recorded.

In continuation of our work on firefly luciferin[2] (**1**) and on its analogs,[1] we have prepared the three hydroxy positional isomers of luciferin (**2–4**) and also the dihydroxy analog **5**. (See Chart I.) The 4-hydroxy analog **2** was prepared by the stepwise procedure used in the original synthesis of luciferin,[2] whereas the other analogs were prepared using the modification of Seto, *et al.*[3]

"4-Hydroxyluciferin" (**2**).—Analog **2**, 2-(4-hydroxy-2-benzothiazolyl)-Δ²-thiazoline-4-carboxylic acid, was synthesized from *o*-anisidine. Condensation of this

CHART I



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(3) S. Seto, K. Ogura, and Y. Nishiyama, *Bull. Chem. Soc. Japan*, **36**, 331 (1963). This group of workers [*ibid.*, 173 (1963)] also prepared a tropolone analog of firefly luciferin.