Studies on the Chemical Constitution of Agar-agar. XIX. Pyruvic Acid as a Constituent of Agar-agar (Part 3)[1]. Structure of the Pyruvic Acid-linking Disaccharide Derivative Isolated from the Methanolysis Products of Agar

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In the previous work[1], a crystalline acid having the formula $C_{14}H_{21}O_9(OCH_3)_2$-COOH was isolated from the partial methanolysis products of agar. The compound was shown to be a disaccharide derivative consisting of 3,6-anhydro-L-galactose, D-galactose and pyruvic acid, the pyruvic acid residue being linked through its carbonyl group with the D-galactose residue. The structure of the compound has been further investigated, and has been proved to be 4-O-4′,6′-O-1″-carboxyethylidene-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal (I).

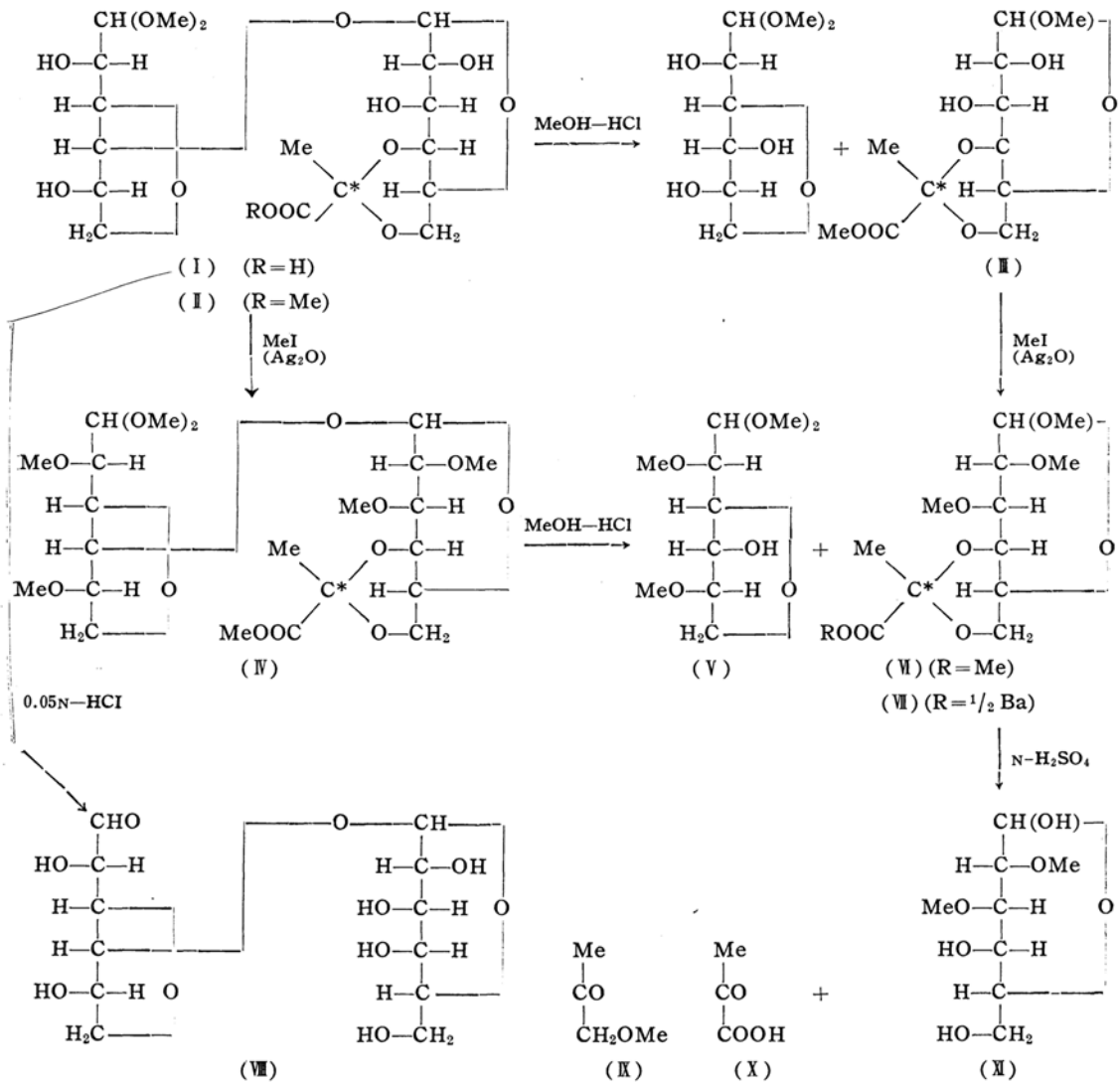
First, the manner in which the pyruvic acid is linked with the D-galactose residue has been studied by methylation of the methyl ester methyl galactoside (III), obtained by methanolysis of I in the previous work. The methylation was carried out by the use of Purdie's reagents to give a methylated methyl ester (VI), a viscous liquid purified by distillation in high vacuum. On hydrolysis it afforded pyruvic acid (X) and 2,3-di-O-methyl-D-galactose (XI). Pyruvic acid was identified as its crystalline phenylhydrazones. 2,3-Di-O-methyl-D-galactose, which had been synthetically known, was identified as follows. (1) Treatment with aniline gave a crystalline anilide, which was in good agreement with a synthetic sample. (2) Treatment with phenylhydrazine and acetic acid resulted in the formation of 3-O-methyl-D-galactosazone, a methoxyl group on the carbon atom C_2 being lost meanwhile. The osazone was identical with a synthetic sample. And, (3) according to the procedure of Luckett and Smith[2], the sugar was oxidized with nitric acid to give 2,3-di-O-methyl-D-galactaric acid, which was then converted to a crystalline bisamide. The identity was again established by comparison with a synthetic sample. The identification of pyruvic acid and 2,3-di-O-methyl-D-galactose as cleavage fragments of VI indicates that the pyruvic acid residue is connected through acetal linkages either with carbon atoms C_4 and C_6 of the D-galactopyranose residue or with C_5 and C_6 of the D-galactofuranose residue. The former structure, namely, methyl 4,6-O-1′-carbomethoxyethylidene-D-galactopyranoside, has been assigned to the methyl ester methyl galactoside (III), because the compound shows a positive value of optical rotation, which is in agreement only with the pyranoside structure.

On the other hand, the ester group of VI was reduced to a hydroxyl group with lithium aluminium hydride and the newly formed hydroxyl group was methylated with Purdie's reagents. The resulting product on hydrolysis yielded methoxyacetone (IX) and again 2,3-di-O-methyl-D-galactose (XI), the former product being identified as its *p*-nitrophenylhydrazone. This fact brings the same conclusion as that already brought as to the linkage between pyruvic acid and D-galactose residues.

Next, in order to study the mode of the linkage between 3,6-anhydro-L-galactose and 4,6-O-1′-carboxyethylidene-D-galactose residues, the methyl ester (II), prepared

1) Part XIX-2: S. Hirase, This Bulletin, **30**, 70 (1957).

2) S. Luckett and F. Smith, *J. Chem. Soc.*, **1940**, 1106.



in the previous work, was methylated with Purdie's reagents. The resulting methylated compound (IV) was obtained as crystals. On methanolysis it afforded a mixture of 2,5-di-O-methyl-3,6-anhydro-L-galactose dimethylacetal (V) and methyl 2,3-di-O-methyl-4,6-O-1′-carbomethoxyethylidene-D-galactoside (VI). The latter, separated from the former through its barium salt (VII), was further hydrolysed to give pyruvic acid (X) and 2,3-di-O-methyl-D-galactose (XI) in the same manner as that already described. 2,5-Di-O-methyl-3,6-anhydro-L-galactose dimethylacetal (V) was hydrolysed to the corresponding reducing sugar, which was then oxidized with bromine water to give crystalline 2,5-di-O-methyl-3,6-anhydro-L-galactonic acid. The amide of the last compound was also prepared in a crystalline condition. These two crystalline derivatives were exactly identical with the corresponding authentic samples. The identification of V and VI as cleavage fragment of IV points to the conclusion that the D-galactopyranose residue is glycosidically joined with the carbon atom C_4 of the 3,6-anhydro-L-galactose residue. Furthermore, on the basis that the compound (I) and its derivatives have negative values of optical rotation, the galactoside linkage has been suggested to be of the β-configuration. Thus, it becomes now possible to assign the structure 4-O-4′, 6′-O-1″-carboxyethylidene-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal (I) to the compound under problem.

Further support for the structure is

derived by the fact that careful hydrolysis of I resulted in the formation of pyruvic acid (X) and agarobiose (4-O-β-D-galactopyranosyl-3, 6-anhydro-L-galactose) (VIII). Moreover, the compound (I) rapidly consumed one mole of periodate in the presence of a sodium bicarbonate buffer, indicating that the presence of a pair of adjacent hydroxyl groups. In conclusion, all the experimental results are compatible with only one structure (I) for the compound isolated from the methanolysis products of agar. However, no information has been made available as to the configuration of the carbon atom C_1 (starred) of the ethylidene group.

It is of interest to note that biochemically important pyruvic acid is connected with an agarobiose unit, which is a repeating unit of agarose, a principal polysaccharide of agar[3]. Moreover, it seems probable that the carboxyl group of the pyruvic acid residue may make some contribution to a gel-forming behavior of agar. But further work is needed to solve the problem whether or not pyruvic acid is connected in the same manner of the linkage in the polysaccharide molecule as that in the compound (I) isolated.

Experimental

Unless otherwise stated evaporation and concentration were carried out under reduced pressure below 40°. All the melting points are uncorrected.

Methyl 2,3-Di-O-methyl-4,6-O-1'-carbomethoxyethylidene-D-galactoside (VI).— Methyl 4, 6-O-1'-carbomethoxyethylidene-D-galactoside (III) (2.0 g.), designated as a methyl ester methyl glycoside (VIII) in the previous paper[1], was directly dissolved in methyl iodide (20 g.) and methylated with silver oxide (7 g.) in the usual manner. The methylation was repeated four times, and the final product was purified by high vacuum distillation, VI being obtained as a colorless viscous liquid boiling at 110-115°/0.027 mm Hg; yield 1.7 g.; n_D^{20} 1.4711; $[\alpha]_D^{10}$ +97.1° (water, *c* 1.02) and +98.5° (chloroform, *c* 0.65).

Anal. Found: C, 50.65; H, 7.32; OCH_3, 40.76%; Saponification equivalent, 300. Calcd. for $C_{13}H_{22}O_8$: C, 50.97; H, 7.24; OCH_3, 40.48%; Sapon. equiv., 306.

Hydrolysis of VI.—The methylated compound (VI) (1.25 g.) above obtained was dissolved in N-sulfuric acid (50 cc.) and heated in a boiling water bath until the optical rotation of the solution reached a constant value after five hours ($[\alpha]_D$ +96°→+54°). The solution was treated with ether in a Soxhlet's apparatus for seven hours. The ether extract was used for the identification of pyruvic acid, while the residual solution was used for the isolation of 2,3-di-O-methyl-D-galactose.

(a) Pyruvic Acid (X).—The ether extract was evaporated under atmospheric pressure to remove ether. The residue, which revealed no spot of a sugar derivative on a paper chromatogram, was treated in the same manner as descrived in the previous paper[1]. Phenylhydrazone and *p*-nitrophenylhydrazone of pyruvic acid were prepared, the identity being established by mixed m.p. determination.

(b) 2, 3-Di-O-methyl-D-galactose (XI).— The residual solution, separated from pyruvic acid, was neutralized with barium carbonate, filtered and concentrated to a sirup, which was redissolved in acetone, filtered and again evaporated to dryness. The sugar (XI) was obtained as a colorless sirup; yield 0.70 g.; $[\alpha]_D^{11}$ +61.5° (an initial value)→+84.6° (an equilibrium value, water, *c* 0.78); OCH_3, found: 30.23% (calcd. for $C_8H_{16}O_6$: 29.81%). It migrated on a paper chromatogram to the same position as that of a synthetic sample[4,5], *n*-butanol-ethanol-water (4 : 1 : 2) being used as a mobile phase.

Anilide.—The sugar (0.16 g.) above obtained and aniline (0.07 g.) in ethanol (3 cc.) were heated under reflux for three hours. The anilide was crystallized from acetone-petroleum ether; m.p. 152-154°; $[\alpha]_D^{13}$ −61.9° (an initial value)→+12.9° (an equilibrium value, ethanol, *c* 0.63). Reported values[5] are m.p. 154-155° and $[\alpha]_D$ −56.8°→+12.1° (ethanol). The melting point was not depressed on admixture with an synthetic sample melting at 153-154°.

Anal. Found: C, 59.23; H, 7.46; N, 5.37. Calcd. for $C_{14}H_{21}O_5N$: C, 59.35; H, 7.24; N, 4.95%.

3-O-Methyl-D-galactosazone.—A mixture of 2, 3-di-O-methyl-D-galactose (0.15 g.), phenylhydrazine (0.25 g.) and 50% aqueous acetic acid (0.5 cc.) in water (2 cc.) was heated in a boiling water bath for two hours. Reddish precipitates formed were filtered, washed with water and dried. The precipitates were chromatographed on a column of active alumina, ethyl acetate being used as a developing solvent. The fresh yellow zone at the top of the column was eluted by methanol containing pyridine, and the eluate was concentrated to a small volume. Addition of water precipitated the osazone, which was further purified by dissolution in ethyl acetate and precipitation with petroleum ether; yield 0.05 g.; yellow powder; m. p. 188-191°; $[\alpha]_D^{13}$ −13.3° (ethanol, *c* 0.30). Various values are reported: m. p. 176-179°[4], 178-194°[6] and about 200°[6]. The synthetic sample, prepared and purified exactly in the same manner as above mentioned, melted at188-191°. Mixed m.p. determination showed no depression.

Anal. Found: N, 15.31; OCH_3, 8.52. Calcd. for $C_{19}H_{24}O_4N_4$: N, 15.05; OCH_3, 8.38%.

2,3-Di-O-methyl-D-galactaric Acid Bisamide.—

3) C. Araki, This Bulletin, **29**, 543 (1956).

4) G. J. Robertson and R. A. Lamb, *J. Chem. Soc.*, **1934**, 1321.

5) D. J. Bell and G. D. Greville, ibid., **1955**, 1136.

6) F. Rebber and T. Reichstein, *Helv. Chim. Acta*, **28**, 1164 (1945).

2,3-Di-*O*-methyl-D-galactose (0.15 g.) obtained was treated by the procedure of Luckett and Smith[2], 2,3-di-*O*-methyl-D-galactaric acid bisamide was obtained as prisms; m.p. 226–228°, not depressed on admixture with a synthetic sample (m.p. 226–228°).

Anal. Found: C, 40.50; H, 6.90. Calcd. for $C_8H_{16}O_6N_2$: C, 40.67; H, 6.83%.

Reduction of VI.—A solution of VI (1.3 g.) in dry ether (20 cc.) was added dropwise to a solution of lithium aluminium hydride (0.5 g.) in dry ether (30 cc.) with stirring. After thirty minutes, an excess of water was added to the reaction mixture, which was then filtered, and the residue was washed thoroughly. Evaporation of the filtrate and washings gave a sirup, which was purified by dissolution in ether and filtration. Evaporation gave methyl 2,3-di-O-methyl-4,6-O-hydroxyisopropylidene-D-galactoside, a colorless sirup; yield 1.2 g.; n_D^{20} 1.4780; $[\alpha]_D^{23}$+101.3° (water, *c* 0.78); OCH_3, found: 32.50% (calcd. for $C_{12}H_{22}O_7$: 33.42%).

Methyl 2,3-Di-O-methyl-4,6-O-methoxyisopropylidene-D-galactoside and its Hydrolysis.—The reduction product (1.1 g.) above obtained was twice methylated with methyl iodide and silver oxide in the usual manner. Methyl 2,3-di-O-methyl-4,6-O-methoxyisopropylidene-D-galactoside was obtained as a colorless sirup; yield 1.0 g.; n_D^{20} 1.4681; $[\alpha]_D^{24}$+108.1° (water, *c* 0.74); OCH_3, found: 41.77% (calcd. for $C_{13}H_{24}O_7$: 42.42%).

The sirup (0.9 g.) above obtained was dissolved in N-sulfuric acid (20 cc.) and heated in a boiling water bath for four and a half hours, at which time the optical rotation of the solution reached a constant value ($[\alpha]_D$+107°→+66°). Methoxyacetone liberated in the hydrolysate was distilled in steam under atmospheric pressure, 60 cc. of the distillate being collected. The residual solution was used for the isolation of 2,3-di-O-methyl-D-galactose.

(a) Methoxyacetone (IX).—To the above distillate was added a methanolic solution of *p*-nitrophenylhydrazine, which caused an immediate precipitation of methoxyacetone *p*-nitrophenylhydrazone; golden needles; yield 0.35 g.; m.p. 112–113°, not depressed on admixture with an authentic sample (m.p. 112–113°).

(b) 2,3-Di-O-methyl-D-galactose (XI).—The distillation residue was neutralized, filtered and evaporated to dryness, giving 2,3-di-O-methyl-D-galactose, a colorless sirup; yield 0.55 g.; $[\alpha]_D^{24}$ +55.6° (an initial value)→+82.8° (an equilibrium value, water, *c* 0.70); OCH_3, found: 29.62% (calcd. for $C_8H_{16}O_6$: 29.81%). It migrated to the same position on a paper chromatogram as that of a synthetic sample. Identification was carried out by its conversion into the anilide, 3-O-methyl-D-galactosazone and 2,3-di-O-methyl-D-galactaric acid bisamide exactly in the same manner as that already described.

4-O-2′,3′-Di-O-methyl-4′,6′-O-1″-carbomethoxyethylidene-β-D-galactopyranosyl-2,5-di-O-methyl-3,6-anhydro-L-galactose Dimethylacetal (IV).—The methyl ester (II) (1.0 g.), prepared in the previous paper[1], was directly dissolved in methyl iodide (35 g.), and was methylated by occasional additions of silver oxide (10 g.) in the usual manner. The methylation was repeated five times. The final product, isolated by extraction with ether and evaporation, was a colorless sirup (1.0 g.), which solidified on standing for a few months. It was recrystallized by dissolving in a small amount of absolute ether, adding petroleum ether (b.p.34–45°) and keeping the solution in a refrigerator for a few weeks. Pure IV was obtained as brilliant prisms; yield 0.78 g., m. p. 78–80°; $[\alpha]_D^{20}$−31.6° (water, *c* 1.33) and −20.9° (chloroform, *c* 1.15).

Anal. Found: C, 51.96; H, 7.39; OCH_3, 42.50%; Saponification equivalent, 508; m.w. (Rast), 510. Calcd. for $C_{22}H_{38}O_{13}$: C, 51.75; H, 7.50; OCH_3, 42.55%; Sapon. equiv. and m.w., 511.

Methanolysis of IV.—The methylated compound (IV) (2.7 g.) in 3% methanolic hydrogen chloride (30 cc.) was heated under reflux for twentyfive hours. It was neutralized with silver carbonate, filtered and concentrated to a sirup (2.7 g.), which was proved to be a mixture of V and VI. The separation was effected by saponification as described below.

(a) Barium Salt of Methyl 2,3-Di-O-methyl-4,6-O-1′-carboxyethylidene-D-galactoside (VII).—The above mixture (2.7 g.) was heated in a 0.3 N-barium hydroxide solution (50 cc.) at 60° for two hours. The solution was neutralized with carbon dioxide, filtered and concentrated to a sirup (3.0 g.). It was then dissolved in methanol (10 cc.) and ether (100 cc.) was added with shaking, the barium salt (VII) being precipitated as a fine powder; yield 2.1 g.; $[\alpha]_D^{19}$+48.8° (water, *c* 1.33). The filtrate was kept aside for the isolation of V.

Anal. Found: OCH_3, 25.32; Ba, 19.11. Calcd. for $(C_{12}H_{19}O_8)_2$ Ba: OCH_3, 25.84; Ba, 19.08%.

Methyl Ester (VI).—The above barium salt (2.2 g.) was converted to the methyl ester by heating in 3%methanolic hydrogen chloride (20 cc.) for five hours. The product, isolated in the usual manner, was purified by distillation in high vacuum, yielding VI as a colorless viscous liquid; yield 1.2 g.; b. p. 110–115°/0.030 mmHg; $[\alpha]_D^{21}$+67.7° (water, *c* 0.93) and +72.4° (chloroform, *c* 1.16); n_D^{20} 1.4685.

Anal. Found: C, 50.78; H, 7.26; OCH_3, 40.42%; Saponification equivalent, 304. Calcd. for $C_{13}H_{22}O_8$: C, 50.97; H, 7.24; OCH_3, 40.48%; Sapon. equiv.; 306.

Hydrolysis.—The methyl ester (VI) above obtained was hydrolysed with N-sulfuric acid (30 cc.) for five hours. Pyruvic acid (X) and 2,3-di-*O*-methyl-D-galactose (XI) produced were identified in the same manner as that already described.

(b) 2,5-Di-O-methyl-3,6-anhydro-L-galactose Dimethylacetal (V).—The filtrate, separated from the barium salt (VII), was evaporated to dryness, V being obtained as a colorless sirup; yield 0.50 g.; $[\alpha]_D^{19}$−6.67° (water, *c* 0.90); n_D^{20} 1.4563; OCH_3, found: 52.15% (calcd. for $C_{10}H_{20}O_6$: 52.48%).

2,5-Di-O-methyl-3,6-anhydro-L-galactonic Acid.

—The above dimethylacetal (0.35 g.) was hydrolysed with 0.1N-sulfuric acid (5 cc.) in a boiling water bath for two hours, neutralized with barium carbonate, filtered and concentrated to dryness. 2,5-Di-O-methyl-3,6-anhydro-L-galactose was obtained as a slightly colored sirup; yield 0.25 g.; $[\alpha]_D^{21}$ −13.3° (water, *c* 0.75); OCH_3, found: 32.02% (calcd. for $C_8H_{14}O_5$: 32.60%).

The sugar (0.20 g.) obtained was oxidized by bromine water in the usual manner[7]. The product was crystallized from ethyl acetate. 2,5-Di-O-methyl-3,6-anhydro-L-galactonic acid was obtained as colorless prisms; yield 0.12 g.; m.p. 159–161°, not depressed on admixture with an authentic sample[7]; $[\alpha]_D^{21}$ −61.9° (water, *c* 0.42).

The mother liquor from the above crystals was concentrated to a sirup (0.08 g.), which was esterified by diazomethane in ether. The methyl ester obtained was converted to the amide by reaction with ammonia in the usual manner[7]; m.p. 171–172°, not depressed on admixture with an authentic sample[7]; $[\alpha]_D^{22}$ −71.7° (water, *c* 0.46).

Partial Hydrolysis of I.—The crystalline acid (I) (0.50 g.) in 0.05N-hydrochloric acid (10 cc.) was heated in a boiling water bath until the optical rotation of the solution reached a constant value: $[\alpha]_D$ −46.7° (initial), −30.0° (0.5 hour), −22.0° (1 hour), −18.6° (1.5 hours), −14.6° (2.0 and 2.5 hours). The solution was neutralized with silver carbonate, filtered and concentrated to a sirup. Extraction with absolute methanol separated agarobiose from insoluble silver pyruvate.

(a) Pyruvic Acid.—The silver pyruvate (0.20 g.) obtained was dissolved in water (2 cc.), N-hydrochloric acid (2 cc.) was added, and precipitates of silver chloride were removed by filtration. When phenylhydrazine (0.10 g.) was added to the filtrate, pyruvic acid phenylhydrazone was crystallized; yield 0.12 g.; m.p. and mixed m.p. 190–191°.

(b) Agarobiose (VIII).—The methanol extract, separated from silver pyruvate, gave on evaporation agarobiose as a colorless sirup, which reduced Fehling's solution at room temperature; yield 0.33 g.; $[\alpha]_D^{14}$ −13.8° (water, *c* 0.80). It was identified by conversion into its dimethylacetal.

Agarobiose Dimethylacetal.—The above sirup (0.30 g.) was dissolved in 0.5% methanolic hydrogen chloride (3 cc.) and heated under reflux for two hours. The solution was neutralized with silver carbonate, filtered and concentrated to a sirup (0.30 g.), which was then chromatographed on a cellulose powder column, *n*-butanol-water (6 : 1) being used as a mobile phase. Agarobiose dimethylacetal was obtained as crystals, which were further purified by recrystallization from ethanol-acetone (1 : 2); yield 0.20 g.; m.p. 163–164°, not depressed on admixture with an authentic sample[8]; $[\alpha]_D^{14}$ −29.5° (water, *c* 0.98); OCH_3, found; 16.39% (calcd. for $C_{14}H_{26}O_{11}$: 16.76%).

Periodate Oxidation of I.—The compound (I) (0.1615 g.) was dissolved in water (10 cc.), and 0.215M-solution of sodium metaperiodate (10.0 cc.) was added. The solution was immediately neutralized with sodium bicarbonate until it became slightly alkaline to a methyl redi ndicator, and then was diluted exactly to 50.0 cc. Aliquotes were withdrawn periodically for the determination of the residual oxidant in the usual manner. The sample consumed 0.63, 1.02 and 1.05 mole of the oxidant, respectively, after six, twenty-four and forty-eight hours. On standing for a longer period, up to two moles of the oxidant were consumed on account of the gradual hydrolysis of the pyruvic acid residue.

Summary

1. The structure of the acidic compound, isolated from the partial methanolysis products of agar in the previous work, has been investigated.

2. Methylation data have indicated that the structure of the compound is 4-O-4′, 6′-O-1″-carboxyethylidene-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal, the β-linkage being suggested on the basis of the optical rotatory behavior.

3. The structure assigned is also supported by the result of partial hydrolysis and periodate oxidation of the compound.

4. No information has been obtained as to the configuration of the carbon atom C_1 of the ethylidene group in the molecule.

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7) C. Araki, *J. Chem. Soc. Japan*, **65**, 627 (1944).