Studies on the Chemical Constitution of Agar-agar. XXI[1]. *Re-investigation of Methylated Agarose of Gelidium Amansii*

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It has been suggested by Araki[2], one of the present authors, that agar is a mixture of at least two different polysaccharides, one of which is termed agarose having a linear structure composed alternatively of β-D-galactopyranose and 3,6-anhydro-α-L-galactopyranose residues. The structure assignment to the agarose has been partly based on the fact that complete methanolysis of methylated agarose led to the isolation of methyl 2,4,6-tri-*O*-methyl-D-galactoside[3,4] and 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal[5]. But the separation of the cleavage fragments was incomplete and the latter fragment was isolated only in a yield insufficient to interpret the whole molecule of agarose. This paper is concerned with the examination of the agarose structure by carefully re-investigating the methanolysis products of methylated agarose prepared from the *Gelidium amansii* agar.

Following the complete methanolysis of the methylated polysaccharide with 3% methanolic hydrogen chloride, the products were separated into neutral and acidic components by means of ion-exchange resins. The acidic component was obtained in too small a yield to be of structural significance. The mixture of neutral sugar derivatives was solved into its components by fractionation with petroleum ether followed by chromatography on charcoal-Celite columns. The mixture was found to contain methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside in addition to the previously isolated 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal and methyl 2,4,6-tri-*O*-methyl-D-galactoside. It was of significance to observe that, well in agreement with the fore-going Araki's formula of agarose, the 2-*O*-methyl-3,6-anhydro-L-galactose derivatives could be obtained in molar quantities equivalent to the 2,4,6-tri-*O*-methyl-D-galactose derivative.

The methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside, a new compound, was obtained as crystals, melting at 67~69°C and showing a specific rotation $[\alpha]_D^{12}$ +66° in water. Its structure was assigned by the rotation, by the hydrolysis to give 2-*O*-methyl-3,6-anhydro-L-galactose, which was identified as crystalline 2-*O*-methyl-3,6-anhydro-L-galactonic acid and its methyl ester and amide, and by further methylation to give crystalline methyl 2,4-di-*O*-methyl-3,6-anhydro-β-L-galactoside. The finding of the methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside among the methanolysis products of the methylated agarose accounts for the direct origin of methyl 2,4-di-*O*-methyl-3,6-anhydro-β-L-galactoside, which had been isolated[6] after methanolysis and subsequent re-methylation of the methylated polysaccharide.

If the agarose molecule were branched, it would be expected that either methyl di-*O*-methyl-D-galactoside or 3,6-anhydro-L-galactose dimethylacetal or both would be derived from units of branch points when the methylated polysaccharide was subjected to methanolysis. In spite of careful examination, however, neither of them could be detected among the methanolysis products under investigation. It is therefore convincing that the molecule is a chain as previously suggested by Araki[2]. As for the non-reducing end of the chain, repeated experiments failed to detect any of the fully methylated component sugars among the methanolysis products of the methylated polysaccharide with the exception of only one experiment, in which 2,3,4,6-tetra-*O*-methyl-D-galactose was isolated as its crystalline aniline derivative in a yield of twelve milligrams from fifteen grams of the methylated polysaccharide. This exception, however, suffers from the

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[1] Part XX: C. Araki and K. Arai, This Bulletin, **30**, 287 (1957).

[2] C. Araki, This Bulletin, **29**, 543 (1956); *Memoirs Fac. Ind. Arts, Kyoto Tech. Univ.*, **5**, 21 (1956).

[3] C. Araki, *J. Chem. Soc. Japan* (*Nippon Kwagaku Kwaishi*), **58**, 1362 (1937).

[4] E. G. V. Percival and J. C. Somerville, *J. Chem. Soc.*, **1937**, 1615.

[5] C. Araki, *J. Chem. Soc. Japan* (*Nippon Kwagaku Kwaishi*), **61**, 775 (1940).

[6] (a) C. Araki, ibid., **59**, 304 (1938). (b) C. Araki and K. Arai, ibid., **61**, 503 (1940). (c) S. Hands and S. Peat, *Nature*, **142**, 797 (1938). (d) E. G. V. Percival, J. C. Somerville and I. A. Forbes, ibid., **142**, 797 (1938).

possibility that the polysaccharide molecule might have been degraded before or during the methylation process. Percival and his coworkers reported, that no tetra-*O*-methyl-D-galactose could be detected among the products of hydrolysis of the methylated polysaccharide⁷⁾. Consequently, it may be still difficult at this time to decide whether D-galactose and 3,6-anhydro-L-galactose constitutes the non-reducing end of the molecule. In any case, since the end group, if present, would exist only in a trace quantity, it seems likely that agarose is a chain with a very high degree of polymerization, although the possibility is not completely excluded yet that the polysaccharide may have an endless, closed loop-structure.

In connection with the methylation data mentioned above, the information concerning the paper chromatography of the methylated derivatives of 3,6-anhydro-L-galactose is also reported here in. Cyclohexanol saturated with water was found to serve as an effective solvent for separating the anhydrosugar derivatives, the spots of which could be easily revealed when chromatograms were sprayed with an *o*-aminophenol reagent previously reported from this laboratory⁸⁾. Table I shows the R_f values and the colors of spots observed by this method for some available samples of the anhydrosugar derivatives.

TABLE I. PAPER CHROMATOGRAPHY OF SOME 3,6-ANHYDRO-L-GALACTOSE DERIVATIVES

Compound	R_f	Color
Methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside	0.77	brown
2-*O*-Methyl-3,6-anhydro-L-galactose dimethylacetal	0.67	brown
Methyl 2,4-di-*O*-methyl-3,6-anhydro-β-L-galoctoside	0.84	brown
2,4-Di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal	0.84	yellow
2,5-Di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal	0.84	brown

Filter paper used: Tōyō Filter Paper No. 52.
Developing solvent: cyclohexanol saturated with water.
Spraying reagent: *o*-aminophenol in aqueous ethanol acidified with phosphoric acid⁸⁾.

Experimental

General Procedure.—Evaporation and concentration of solutions were carried out below 40°C under reduced pressure. Specific rotations were measured in aqueous solutions unless otherwise stated. Melting points are uncorrected.

Paper Chromatography.—Paper chromatograms were run in cyclohexanol saturated with water and sprayed with *o*-aminophenol in aqueous ethanol acidified with phosphoric acid⁸⁾ for the purpose of identifying 3,6-anhydro-L-galactose derivatives; while, they were run in *n*-butanol-ethanol-water (4 : 1 : 2 V./V.) and sprayed with aniline hydrogen phthalate in moist *n*-butanol⁹⁾ for the purpose of identifying reducing D-galactose derivatives.

Methylated Agarose.—Methylated agarose was prepared by acetylating the *Gelidium amansii* agar with acetic anhydride and pyridine, extracting the agarose acetate from the resulting acetylation products with chloroform and then treating the agarose acetate repeatedly with dimethylsulfate and sodium hydroxide as previously described by Araki¹⁰⁾, one of the present writers. The methoxyl value found was 33.8% in reasonable agreement with the value 34.3% calculated for $[C_{12}H_{14}O_5(OCH_3)_4]_n$.

Methanolysis of Methylated Agarose and Separation of Neutral and Acidic Components.—Methylated agarose (15.0 g.) was suspended in 3% methanolic hydrogen chloride (100 ml.) and heated under reflux for 35 hr. The resulting brown solution was neutralized with silver carbonate, filtered and evaporated to a sirup. It was then dissolved in 0.2 N barium hydroxide solution (100 ml.) and heated for 2 hr. at 60°C to saponify the methyl ester of the acidic component. After removal of the excess of barium hydroxide by neutralization with carbon dioxide and subsequent filtration, the solution was allowed to pass through columns of Amberlite IR-120 (80 ml.) and IR-4B (100 ml.) in succession, and the columns were thoroughly washed with water. The effluent and washings were combined and evaporated to dryness, when the mixture of neutral sugar derivatives was obtained as a colorless sirup; yield, 15.2 g., $[\alpha]^{20}_{D}+60.0°$ (c 1.20).

The anion-exchange resin used above was transferred to a beaker, stirred with 2 N sulfuric acid (100 ml.) for a few minutes under ice-cooling to displace the acidic component, and re-transferred to a glass tube to form a column. After the excess of the liquid was allowed to drain from the column, the resin was washed with additional 40 ml. of 2 N sulfuric acid followed by water (600 ml.). All the effluents and washings were combined and immediately neutralized with a saturated barium hydroxide solution, the precipitates of barium sulfate were removed by filtration, and the filtrate was evaporated to dryness. The acidic component in a barium salt form was obtained as a thick sirup; yield, 0.42 g., $[\alpha]^{20}_{D}$ $+2.7°$ (c 0.75). It showed positive color reactions both for ketose and uronic acid. But the yield was so small that no further investigation was carried out.

Fractionation of the Neutral Components with Petroleum Ether.—The mixture of the neutral

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7) E. G. V. Percival and T. G. H. Thomson, *J. Chem. Soc.*, **1942**, 750.

8) S. Hirase, C. Araki and S. Nakanishi, This Bulletin, **26**, 183 (1953).

9) S. M. Partridge, *Nature*, **164**, 443 (1949).

10) C. Araki, *J. Chem. Soc. Japan* (*Nippon Kwagaku Kwaishi*), **58**, 1338, 1351 (1937).

sugar derivatives (15.2 g.) obtained above was dissolved in water (80 ml.). The solution was continuously extracted with petroleum ether (b. p. 30~37°C) for 30 hr. in a Soxhlet's apparatus. The extract was evaporated to a thin sirup, which on standing crystallized partly; yield, 2.05 g., $[\alpha]^{22}_{D}$ +76.1° (*c* 0.71). The residual aqueous solution was also evaporated to a sirup; yield, 13.0 g., $[\alpha]^{22}_{D}$ +56.0° (*c* 1.0).

Chromatographic Separation of the Components Insoluble in Petroleum Ether.—One third quantity of the petroleum ether-insoluble components obtained above was chromatographed at 20~22°C on a column (5.2×22 cm.) packed with a mixture of charcoal (50 g.) and Celite (40 g.). The solvents used for elution were water and ethanol of successively increasing concentrations in water. The effluents were collected of every one liter in separate receivers and concentrated to give 100 ml. fractions, which were then examined both polarimetrically and paper chromatographically. The fractions showing the same contents were combined and evaporated to recover components, which were then purified by dissolution in acetone, filtration and re-evaporation to a constant weight. The total recovery amounted to 4.12 g. or 93% of the applied sample. The recovered components were determined for methoxyl content and specific rotation. The results are shown in Table II. None of the fractions showed on a paper chromatogram the presence of non-methylated or fully methylated 3,6-anhydro-L-galactose derivatives, which might be, derived from units of branch points or the non-reducing end of the methylated polysaccharide molecule, respectively.

Identification of the Components Insoluble in Petroleum Ether.—*(a) 2-O-Methyl-3,6-anhydro-L-galactose Dimethylacetal.*—Fraction II in Table II was a colorless sirup showing a refractive index n^{25}_{D} 1.4620. When examined on a paper chromatogram, it showed a single spot having R_f 0.67. The methoxyl value found was in good agreement with the value 41.90% calculated for $C_6H_9O_3 \cdot (OCH_3)_3$. The fraction was therefore considered to be pure 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal. One of the present writers has reported $[\alpha]^{8}_{D}$ −13.79° and n^{25}_{D} 1.4647 about the previously isolated sample of 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal[5].

Fraction III in Table II was a colorless sirup, which on standing crystallized partly. When examined on a paper chromatogram, it showed the presence of two spots having R_f 0.67 and 0.77, respectively, one agreed with the dimethylacetal obtained above, the other with methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside. Since the optical rotations of these pure compounds are −30.7° and +66.0°, respectively, as reported herein, the fraction was considered to be a mixture of 28% of the dimethylacetal (0.14 g.)

TABLE II. CHROMATOGRAPHIC SEPARATION OF THE COMPONENTS INSOLUBLE IN PETROLEUM ETHER

	Receiver number	Solvent*	Yield g.	$[\alpha]^{22}_{D}$ ° (H_2O)	OCH_3 %	Identified component**
I	1—3	W	nil	—	—	—
II	4—8	2.5% E	1.10	−30.7	42.37	A
III	9—10	2.5% E	0.50	+40.3	38.15	A (28%) + B (72%)
IV	11—12	5 % E	0.19	+66.7	32.83	B
V	13—15	5 % E	0.22	+92.8	44.65	B (40%) + C (60%)
VI	16—21	7.5% E	0.77	+110.2	52.25	C
VII	22—28	10 % E	0.90	+110.9	52.32	C
VIII	29—33	15 % E	0.06	+111.0	52.35	C
IX	34—38	25 % E	0.25	−29.0	43.11	?
X	39—41	50 % E	0.13	+35.3	39.37	?

* W: Water; E: Ethanol.

** A: 2-*O*-Methyl-3,6-anhydro-L-galactose dimethylacetal;
B: Methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside;
C: Methyl 2,4,6-tri-*O*-methyl-α, β-D-galactoside.

TABLE III. CHROMATOGRAPHIC SEPARATION OF THE COMPONENTS SOLUBLE IN PETROLEUM ETHER

	Receiver number	Solvent*	Yield g.	$[\alpha]^{22}_{D}$ ° (H_2O)	OCH_3 %	Identified components**
I	1—3	2.5% E	—	—	—	—
II	4—7	5 % E	0.60	+71.7	48.40	B (70%) + C (30%)
III	8—9 10—14 15—19	5 % E 7.5% E 10 % E	1.22	+88.9	52.22	C
IV	20—24 25—28	15 % E 30 % E	0.04	+96.0	—	C + D (?)

* E: Ethanol.

** B: Methyl 2-*O*-methyl-3, 6-anhydro-β-L-galactopyranoside;
C: Methyl 2,4,6-tri-*O*-methyl-α, β-D-galactoside;
D: Methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside.

and 72% of the methyl glycoside (0.36 g.). This proportion is in agreement with that calculated on the basis of methoxyl values. For the actual separation, the fraction was re-chromatographed on a column (2.5×20 cm.) of charcoal-Celite at 20∼22°C. The dimethylacetal was eluted from the column chiefly with 4% ethanol in water; yield, 0.10 g., $[\alpha]_D^{17}$ −30.3° (*c* 0.54), n_D^{25} 1.4615, OCH_3 41.27%; while, the methyl glycoside was removed from the column with 6% ethanol in water and was obtained as crystals; yield, 0.31 g., m. p. 65∼67°C, $[\alpha]_D^{17}$ +66.0° (*c* 0.50). Admixture with the sample obtained from fraction IV described below showed no depression of the melting point.

For the identification of 2-*O*-methyl-3, 6-anhydro-L-galactose dimethylacetal, the following experiments were carried out:

i) 2-O-Methyl-3,6-anhydro-L-galactose.—2-*O*-Methyl-3,6-anhydro-L-galactose dimethylacetal (0.40 g.) in 0.02 N sulfuric acid (20 ml.) was heated in a boiling water bath for 2 hr., at which time the rotation of the solution reached a constant value ($[\alpha]_D$ −31°→−10°). The solution was neutralized with barium carbonate, filtered and evaporated to a sirup which was then dissolved in acetone. Filtration and evaporation of the acetone solution afforded 2-*O*-methyl-3, 6-anhydro-L-galactose as a colorless sirup; yield, 0.35 g., $[\alpha]_D^{12}$ −14.3° (*c* 0.63), n_D^{25} 1.4870, OCH_3; found: 17.45% (calcd. for $C_6H_9O_4(OCH_3)$: 17.59%). It reduced a Fehling solution at room temperature and restored the color to a Schiff's reagent. One of the present writers has reported $[\alpha]_D^{34}$ +33.9° of the sugar[5]. The previously reported value in a positive direction seems to be ascribed to some contaminants.

ii) 2-O-Methyl-3,6-anhydro-L-galactonic acid.—The reducing sugar (0.30 g.) obtained above was dissolved in water (5 ml.) and bromine (0.4 ml.) was added dropwise during 4 hr. to the solution kept at 40°C. The treatment of the solution in the usual manner afforded 2-*O*-methyl-3, 6-anhydro-L-galactonic acid; yield, 0.35 g., m. p. 137∼140°C. Recrystallization twice from ethyl acetate furnished the pure compound; m. p. and mixed m. p. 141∼142°C, $[\alpha]_D^{12}$ −70.3° (*c* 0.84). One of the present writers has reported m. p. 142°C and $[\alpha]_D^{12}$ −70.0° for 2-*O*-methyl-3, 6-anhydro-L-galactonic acid[5].

Found: C, 43.63; H, 6.32; OCH_3, 16.34. Calcd. for $C_6H_9O_5(OCH_3)$: C, 43.75; H, 6.27; OCH_3, 16.15%.

iii) Methyl 2-O-methyl-3,6-anhydro-L-galactonate.—The acid obtained above was converted to the methyl ester by the reaction with methanolic hydrogen chloride in the usual manner. The resulting methyl ester was recrystallized from benzene, forming stout needles; m. p. 90∼91°C, $[\alpha]_D^{12}$ −70.8° (*c* 1.06). One of the present writers has reported about m. p. 91°C[1,5] $[\alpha]_D^{10}$ −55.3°[5] and $[\alpha]_D^{24}$ −61.6°[1].

Found: C, 46.54; H, 7.02; OCH_3, 30.23. Calcd. for $C_8H_{14}O_6$: C, 46.60; H, 6.84; OCH_3, 30.12%.

iv) 2-O-Methyl-3,6-anhydro-L-galactonamide.—The amide was prepared from the methyl ester obtained above by the reaction with methanolic ammonia and purified by recrystallization from ethanol; m. p. 171∼172°C, $[\alpha]_D^{12}$ −75.4° (*c* 0.53). Araki and Arai have reported m. p. 170∼171°C and $[\alpha]_D^{23}$ −74.3° for the amide[1].

Found: C, 43.88; H, 6.95; OCH_3, 16.01. Calcd. for $C_7H_{13}O_5N$: C, 43.97; H, 6.85; OCH_3, 16.23%.

(b) Methyl 2-O-Methyl-3,6-anhydro-β-L-galactopyranoside.—Fraction IV in Table II crystallized completely on standing and melted at 64°C. It showed on a paper chromatogram as a single spot having R_f 0.77. This was combined with the crystals obtained by re-chromatography of fraction III described above and was recrystallized twice from petroleum ether, affording pure methyl 2-*O*-methyl-3, 6-anhydro-β-L-galactopyranoside as elongated prisms; m. p. 67∼69°C, $[\alpha]_D^{12}$ +66.0° (*c* 1.00).

Found: C, 50.49; H, 7.46; OCH_3, 32.55; mol. wt. (Rast), 190. Calcd. for $C_6H_8O_3(OCH_3)_2$: C, 50.52; H, 7.42; OCH_3, 32.65%; mol. wt., 190.2.

The structure assignment to this compound has been based on the following experiments:

i) 2-O-Methyl-3,6-anhydro-L-galactose.—The methyl glycoside (0.20 g.) obtained above was hydrolyzed with 0.02 N sulfuric acid (20 ml.) for 2 hr. during which time the specific rotation of the solution changed from +66° to −11°, the final value being in good agreement with that observed when 2-*O*-methyl-3, 6-anhydro-L-galactose dimethyl-acetal was hydrolyzed. The resulting 2-*O*-methyl-3, 6-anhydro-L-galactose was isolated in the same manner as described already; yield, 0.15 g., $[\alpha]_D^{12}$ −15.0° (*c* 0.50), n_D^{25} 1.4867; OCH_3 found 17.50%. The sugar was identified as 2-*O*-methyl-3, 6-anhydro-L-galactonic acid and its methyl ester and amide in exactly the same manner as described already.

ii) Methyl 2,4-di-O-methyl-3,6-anhydro-β-L-galactoside.—Methyl 2-*O*-methyl-3, 6-anhydro-β-L-galactopyranoside (0.08 g.) was dissolved in methyl iodide (4 ml.), silver oxide (3 g.) added, and the mixture was refluxed for 6 hr. The reaction mixture was diluted with dry acetone and filtered. Evaporation of the filtrate afforded methyl 2, 4-di-*O*-methyl-3, 6-anhydro-β-L-galactoside melting at 81∼82°C, which was then recrystallized from petroleum ether; yield, 0.07 g., m. p. and mixed m. p. 83°C, $[\alpha]_D^{10}$ +76.7° (*c* 0.60). Methyl 2, 4-di-*O*-methyl-3, 6-anhydro-β-L-galactoside has been recorded as: m. p. 83°C and $[\alpha]_D^{14}$ +78.1°[6a]; m. p. 82∼83°C and $[\alpha]_D$ +73.0°[6c] and m. p. 81°C and $[\alpha]_D$ +75.0°[6d].

Found: C, 53.02; H, 7.88; OCH_3, 45.45. Calcd. for $C_6H_7O_2(OCH_3)_3$: C, 52.93; H, 7.90; OCH_3, 45.59%.

(c) Methyl 2,4,6-Tri-O-methyl-α, β-D-galactoside.—All fractions VI—VIII in Table II were obtained as crystals with ill-defined melting points. Examination on paper chromatograms using the *o*-aminophenol reagent revealed no spot, indicating thereby the absence of any 3, 6-anhydro-L-galactose derivative. The methoxyl values found were in good agreement with the value 52.52% calculated for $C_6H_8O_2(OCH_3)_4$. These fractions

were therefore composed entirely of methyl 2,4,6-tri-*O*-methyl-α,β-D-galactoside.

Fraction V in Table II showed a spot corresponding to methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside on a paper chromatogram. When the fraction was hydrolyzed with 1 N sulfuric acid for 5 hr. at 98°C, the product showed a single spot corresponding to 2,4,6-tri-*O*-methyl-D-galactose on a paper chromatogram, the anhydrosugar derivative being completely destroyed during the hydrolysis. Fraction V was therefore a mixture of 40% of methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside (0.09 g.) and 60% of methyl 2,4,6-tri-*O*-methyl-α,β-D-galactoside, the proportion being calculated on the basis of the methoxyl values.

i) 2,4,6-Tri-O-methyl-α-D-galactose.—Fractions VI—VIII were combined and hydrolyzed with 1 N sulfuric acid (20 ml.) for 4.5 hr. at 98°C. The reaction solution was then treated in the usual manner, when 2,4,6-tri-*O*-methyl-D-galactose was obtained; yield, 1.40 g., m. p. 104∼105°C. Paper chromatographic examination indicated the presence of 2,4,6-tri-*O*-methyl-D-galactose only. Recrystallization, twice from ether, furnished the pure compound; m. p. and mixed m. p. 110∼111°C, $[\alpha]_D^{15}$ +115.9° (initial)→+90.7° (after 24 hr., constant) (*c* 1.07). The physical constants are in good agreement with literature values[3,4].

Found: C, 48.56; H, 8.30; OCH_3, 41.78. Calcd. for $C_6H_9O_3(OCH_3)_3$: C, 48.64; H, 8.16; OCH_3, 41.90%.

ii) 2,4,6-Tri-O-methyl-N-phenyl-D-galactosylamine.—The tri-*O*-methyl-D-galactose recovered from the mother liquor of recrystallization mentioned above was converted into its aniline derivative in the usual manner[3,4]; m. p. and mixed m. p. 174°C, $[\alpha]_D^{14}$ −120.0° (initial)→−82.0° (constant) in acetone (*c* 0.50).

(d) Unidentified Components.—Fractions IX and X in Table II seemed to be methylated dissaccharide derivatives having molecular weight, respectively, 423 and 398. The structures will be studied in the subsequent paper.

Separation and Identification of the Components Soluble in Petroleum Ether.—*(a) Separation.*—The sirup (2.05 g.), which had been obtained by extracting the neutral components of the methanolysis products with petroleum ether, was chromatographed at 22∼24°C on a column (5.0× 15 cm.) packed with charcoal (40 g.) and Celite (28 g.) in exactly the same manner as in the separation of the components insoluble in petroleum ether. The results are shown in Table III.

(b) Identification.—Fraction II was a colorless sirup, showing a spot corresponding to methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside on a paper chromatogram. When it was hydrolyzed with 1 N sulfuric acid for 5 hr. at 98°C, the resulting product showed a spot corresponding to 2,4,6-tri-*O*-methyl-D-galactose on a paper chromatogram, the anhydrosugar derivative being completely destroyed during the hydrolysis. From these facts, fraction II was considered to be a mixture of 70% of methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside (0.42 g.) and 30% of methyl 2,4,6-tri-*O*-methyl-D-galactoside (0.18 g.), the proportion being calculated on the basis of the methoxyl values.

Fraction III was proved to be entirely methyl 2,4,6-tri-*O*-methyl-α,β-D-galactoside in exactly the same manner as in the case of fractions VI—VIII in Table II.

Fraction IV in Table III gave no spot on a paper chromatogram sprayed with an *o*-aminophenol reagent, indicating thereby the absence of any 3,6-anhydro-L-galactose derivative. On hydrolysis it produced 2,4,6-tri-*O*-methyl-D-galactose which was identified on a paper chromatogram. Fraction IV contained therefore methyl 2,4,6-tri-*O*-methyl-α,β-D-galactoside only. However, a similar fraction obtained from the other batch was found to contain methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside in addition to the tri-*O*-methyl-D-galactose derivative as described below.

Confirmation of Methyl 2,3,4,6-Tetra-*O*-methyl-D-galactoside.—From the other batch of methylated agarose (15 g.), there was obtained a fraction similar to fraction IV of Table III; yield, 0.07 g. Paper chromatographic examination indicated the absence of any 3,6-anhydro-L-galactose derivative. The sirup was hydrolyzed with 1 N sulfuric acid (1 ml.) for 4.5 hr. at 98°C and the product was isolated in the usual manner as a colorless sirup; yield, 62 mg. Examination on a paper chromatogram indicated the presence of both 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose. The mixture was resolved into the components on a column (2.5×32 cm.) packed with filter paper powder, a 2-butanone-water azeotrope being used as a mobile phase. A faster moving fraction containing the tetra-*O*-methyl-D-galactose was obtained as a sirup; yield, 20 mg., $[\alpha]_D^{10}$ +44.0° (*c* 0.50). It was dissolved in ethanol (1 ml.), aniline (20 mg.) added, and the solution was refluxed for 3 hr., when 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosyl amine was obtained; yield, 12 mg., m. p. 186∼189°C. Recrystallization from ethanol afforded the pure compound; m. p. and mixed m. p. 192°C, $[\alpha]_D^{13}$ −75.8° (initial) in acetone (*c* 0.132).

Found: OCH_3, 40.18. Calcd. for $C_{16}H_{25}O_5N$: OCH_3, 40.51%.

Yields of the Components.—As shown in Tables II and III, the yields of 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal, methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside and methyl 2,4,6-tri-*O*-methyl-α,β-D-galactoside are 3.72, 2.34 and 7.02 g., respectively, when calculated on the basis of the whole neutral components (15.2 g.). Consequently, the molar proportion of the sum of the former two to the last one is 1 : 1.03.

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