BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 1452—1456 (1967)

Studies on the Chemical Constitution of Agar-agar. XXIV.[1] Isolation of a New Disaccharide as a Reversion Product from Acidic Hydrolysate

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(Received October 20, 1966)

The careful separation of the products of the complete hydrolysis of commercial agar on charcoal-Celite columns has led to the isolation of a new reducing disaccharide, named "isoagarobiose," in a yield of 1.5% of the agar sample. Its crystalline phenylosazone and crystalline hexa-acetate have also been prepared. On methanolysis the disaccharide gave methyl D-galactoside and 3, 6-anhydro-L-galactose dimethyl acetal. Complete methylation, followed by methanolysis, yielded 2, 4, 5-tri-*O*-methyl-3, 6-anhydro-L-galactose dimethyl acetal and methyl 2, 3-di-*O*-methyl-D-galactoside. It follows from these results that the disaccharide has an unusual structure, in which the C_1 of the 3, 6-anhydro-*aldehydo*-L-galactose residue is linked, through cyclic acetal linkages, with the C_4 and C_6 of the D-galactose residue. Evidence is provided that the disaccharide is not involved in the polysaccharide molecule, but has been formed by acid reversion from D-galactose and 3, 6-anhydro-L-galactose during the hydrolysis of the polysaccharide. The formation of the cyclic acetal linkages is accounted for in terms of the peculiar property of the anhydrosugar, which exists in an aldehyde form, with an open-chain structure.

In a previous paper,[1] the products of the complete hydrolysis of commercial agar were carefully separated by chromatography on a charcoal-Celite column which, on elution with water, led to the isolation of several monosaccharides. The present paper is concerned with a disaccharide eluted from the column with 2.5% ethanol in water.

The re-chromatography of the 2.5% ethanol fraction described above has resulted in the isolation of a new reducing disaccharide in a yield of 1.5% of the agar sample. It was obtained as a syrup with a specific rotation of $[\alpha]_D^{30}$ $+42.4°$ in water, and was characterized as its crystalline phenylosazone and crystalline hexa-acetate, the latter compound having the empirical formula $C_{12}H_{14}O_{10}(CH_3CO)_6$.

The disaccharide is highly resistant to acid hydrolysis, as is understandable from manner of isolation. However, violent methanolysis broke down the molecule, thus yielding methyl D-galactoside and 3, 6-anhydro-L-galactose dimethyl acetal. The reduction of the disaccharide with sodium borohydride produced the corresponding glycitol which, on methanolysis, yielded dulcitol and 3, 6-anhydro-L-galactose dimethyl acetal. It is evident therefore that the disaccharide is composed of 3, 6-anhydro-L-galactose and D-galactose, which constitutes a reducing unit of the molecule. We suggest that the disaccharide be named "isoagarobiose."

The structure of isoagarobiose (I) has been elucidated by the methylation study. The complete methylation of the disaccharide afforded a crystalline methyl penta-*O*-methyl-β-isoagarobioside, which, on methanolysis, yielded a mixture of 2, 4, 5-tri-*O*-methyl-3, 6-anhydro-L-galactose dimethyl acetal (II) and methyl 2, 3-di-*O*-methyl-D-galactoside (III). The mixture was then separated into components by distillation under a high vacuum. The former product was hydrolysed to

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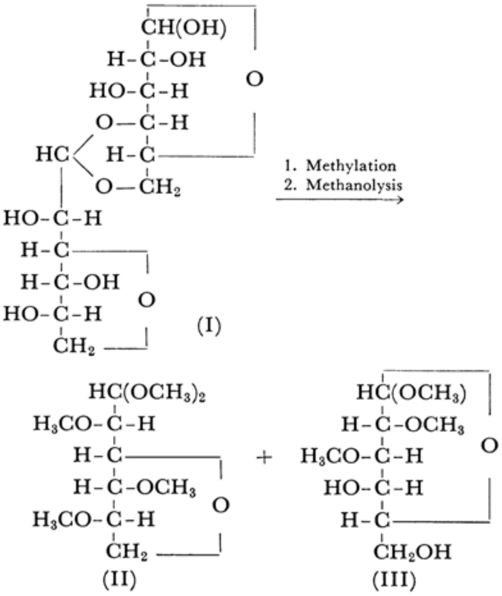
[1] Part XXIII: C. Araki, K. Arai and S. Hirase, This Bulletin, **40**, 959 (1967).



a reducing sugar which, on oxidation with bromine, followed by esterification with methanolic hydrogen chloride, afforded methyl 2, 4, 5-tri-*O*-methyl-3, 6-anhydro-L-galactonate.[2] The latter product was also hydrolysed to 2, 3-di-*O*-methyl-D-galactose, which was then identified as its crystalline aniline derivative.[3-6] The sugar was also identified by its conversion into crystalline 2, 3-di-*O*-methyl-D-galactaric acid bisamide by the method of Luckett and Smith.[7] These results point to the conclusion that, in the molecule of isoagarobiose, the usual glycosidic linkage is not involved, but the C_1 of 3, 6-anhydro-*aldehydo*-L-galactose is connected, through cyclic acetal linkages, with the C_4 and C_6 of D-galactopyranose. This structure agrees with the fact that the disaccharide consumed three moles of periodate.

Isoagarobiose is, then, a disaccharide of a new structural type, which is not covered by the nomenclature rules for carbohydrates. The structure may be tentatively designated as 4, 6-*O*-(3′, 6′-anhydro *aldehydo*-L-galactosylidene)-D-galactose.

There have been so far reported two disaccharides consisting of D-galactose and 3, 6-anhydro-L-galactose; one is agarobiose (4-*O*-β-D-galactopyranosyl-3, 6-anhydro-L-galactose),[8,9] and the other is neoagarobiose (3-*O*-(3′, 6′-anhydro-α-L-galactopyranosyl)-D-galactose),[10] both being involved as repeating units in the molecule of the main polysaccharide of agar.[11] In the case of the isoagarobiose under consideration, re-condensation ("reversion") seems to have occurred between D-galactose and 3, 6-anhydro-L-galactose during the hydrolysis process of agar. This has been demonstrated by the fact that, when agarobiose dimethyl acetal was heated in N sulfuric acid under the same conditions as hold in the hydrolysis of agar, isoagarobiose was obtained in a 1.5% yield.

The formation of the cyclic acetal linkages by reversion can be accounted for in terms of the peculiar property of 3, 6-anhydro-L-galactose, which exists in an aldehyde form with an open-chain structure.[12] The anhydro-sugar in an aldehyde form would condense with D-galactose to form the cyclic acetal structure in a manner similar to that of the condensation of such aldehydes as acetaldehyde and benzaldehyde with various carbohydrate compounds.[13]

Experimental

General Procedure. Solutions were evaporated under reduced pressure at 40°C. Paper chromatograms were run in 1-butanol - acetic acid - water (4 : 1 : 2v/v) and sprayed with *o*-aminophenol in aqueous ethanol acidified with phosphoric acid.[14]

Isolation of Isoagarobiose. As was described in a previous paper,[1] commercial agar (200 g, moisture 19.5%) was subjected to hydrolysis with N sulfuric acid for 20 hr, the mixture of non-fermentable sugars obtained therefrom was then separated on a charcoal-Celite column. Among the four fractions, the fraction eluted from the column with 2.5% ethanol (fraction 2 in Table 1 in the previous paper) will be investigated below.

The fraction was combined with a similar fraction obtained from another run, and the resulting mixture (5.6 g) was re-chromatographed on a charcoal-Celite (1 : 1) column (5×25 cm), which was then eluted with water, 2.5% ethanol, and 5% ethanol in succession. Effluents were examined every 100 m*l* on paper chromatograms, and also for reducing power, and those having the same contents were combined and evaporated to dryness. The five fractions shown in Table 1 were obtained.

Fractions 4 and 5 were obtained as syrups; both of them showed a single spot on a paper chromatogram. The R_f value of 0.21 was exactly the same as that of D-galactose, but the experiments described below have proved that these fractions are isoagarobiose; yield,

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

[3] G. J. Robertson and R. A. Lamb, *J. Chem. Soc.*, **1934**, 1321.

[4] E. Pacsu and S. M. Trister, *J. Am. Chem. Soc.*, **62**, 2301 (1940).

[5] D. J. Bell and G. D. Greville, *J. Chem. Soc.*, **1955**, 1136.

[6] S. Hirase, This Bulletin, **30**, 75 (1957).

[7] S. Luckett and F. Smith, *J. Chem. Soc.*, **1940**, 1106.

[8] C. Araki, *Nippon Kwagaku Kwaisi (J. Chem. Soc. Japan)*, **65**, 533 (1944); **65**, 627 (1944).

[9] C. Araki and S. Hirase, This Bulletin, **27**, 109 (1954).

[10] C. Araki and K. Arai, *ibid.*, **29**, 339 (1956).

[11] C. Araki, *ibid.*, **29**, 543 (1956).

[12] W. N. Haworth, J. Jackson and F. Smith, *J. Chem. Soc.*, **1940**, 620; C. Araki and K. Arai, *Nippon Kwagaku Kwaisi (J. Chem. Soc. Japan)*, **64**, 210 (1943).

[13] A. N. de Belder, *Adv. Carbohydrate Chem.*, **20**, 219 (1965).

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

TABLE 1. RE-CHROMATOGRAPHY

Fraction	Eluant*	Effluent *l*	Yield g	R_f**
1	Water	1.5	0.39	0.10; 0.34
2	Water	2.0	0.50	0.10; 0.21; 0.34
3	2.5% E	1.9	0.23	0.10; 0.21
4	2.5% E	6.8	2.36	0.21
5	5.0% E	4.0	0.13	0.21

* E: Ethanol

** R_f 0.34 corresponds to 6-*O*-methyl-D-galactose, R_f 0.21, to isoagarobiose, and R_f 0.10, to an unidentified compound.

1.5% of the agar sample, $[\alpha]_D^{30}$ +51.7° (4 min)→ +42.4° (48 hr, *c* 0.78, water). It reduces a hot Fehling solution and shows a Seliwanoff ketose reaction.

Isoagarobiose Phenylosazone. To a solution of isoagarobiose (0.25 g) in water (7 m*l*), phenylhydrazine (0.65 g) and 50% aqueous acetic acid (0.7 m*l*) were added. The mixture was then heated at 80°C for 3 hr. Yellow crystalls of the osazone formed were filtered and washed successively with dilute acetic acid, water, ethanol and ether; yield, 0.05 g, mp 215—216°C, $[\alpha]_D^{31}$ −76.5° (17 min)→ −70.1° (24 hr) (*c* 0.70, pyridine-methanol (1 : 1 v/v)).

Found: C, 56.81; H, 5.85; N, 11.10%. Calcd for $C_{24}H_{30}O_8N_4$: C, 57.36; H, 6.02; N, 11.15%.

Hexa-*O*-acetyl-*α*-isoagarobiose. Isoagarobiose (0.22 g) was acetylated with pyridine (3 m*l*) and acetic anhydride (2 m*l*) at room temperature for 48 hr, and then the reaction solution was poured into ice and water. The resulting crystals of the acetate were filtered; mp 185—187°C. The acetate was recrystallized from 94% ethanol; mp 196—197°C, $[\alpha]_D^{15}$ +97.9° (*c* 0.72, chloroform). The highly positive value of the specific rotation indicates that the acetate has an *α*-configuration.

Found: C, 49.95; H, 5.51; CH_3CO, 44.53%. Calcd for $C_{12}H_{14}O_{10}(CH_3CO)_6$: C, 50.00; H, 5.60; CH_3CO, 44.80%.

Periodate Oxidation of Isoagarobiose. Isoagarobiose (0.0510 g) was dissolved in a 0.197 M sodium metaperiodate solution (10.0 m*l*), sodium bicarbonate (0.050 g) being added as a buffer; the solution was then diluted with water to 50.0 m*l* and left at room temperature (17—20°C). Five milliliter portions of the solution were withdrawn at intervals, and there sidual oxidant in it was determined in the usual way.[15] The periodate consumption was 2.83 mol after 7 hr, 2.93 mol after 22 hr, 3.18 mol after 31 hr, and 3.21 mol after 48 hr and 72 hr.

Methanolysis of Isoagarobiose. A solution of isoagarobiose (1.0 g) in 2.5% methanolic hydrogen chloride (75 m*l*) was heated under reflux for 50 hr, neutralized with silver carbonate, and filtered. The filtrate was evaporated to a syrup which, on dissolution in methanol-acetone (1 : 1), afforded crystals of methyl *α*-D-galactoside monohydrate; yield, 0.14 g; mp 99—100°C. The filtrate was again evaporated to a syrup (0.95 g), which was then subjected to chromatography on a cellulose column (3×34 cm). The column was eluted first with 1-butanol-water (6 : 1 v/v) until the 3, 6-anhydro-L-galactose dimethyl acetal had been removed from the column. It was then eluted with 80% methanol (300 m*l*) to remove any methyl D-galactoside.

a) 3, 6-*Anhydro-L-galactose Dimethyl Acetal.* The butanolic effluent obtained above was evaporated to a syrup; yield, 0.44 g; $[\alpha]_D^{26}$ −28.6° (*c* 0.57, water), R_f 0.67. These properties are in agreement with those of 3, 6-anhydro-L-galactose dimethyl acetal.[9,16]

Found: OCH_3, 28.85%.

For conclusive identification, the dimethyl acetal (0.20 g) was hydrolysed with 0.05 N oxalic acid (7 m*l*) for 2 hr; the resulting reducing sugar (0.15 g, $[\alpha]_D^{26}$ −11.5° (*c* 1.07, water)) was then converted into its osazone[16] in the usual way; yellow crystals, mp and mmp 217—218°C, $[\alpha]_D^{26}$ −55.5° (*c* 1.0, pyridine-methanol (2 : 3)).

b) Methyl D-*Galactoside.* The methanolic effluent from the column described above was evaporated to a syrup (0.30 g) which, on dissolution in methanol-acetone, afforded crystals of methyl *α*-D-galactopyranoside monohydrate; yield, 0.13 g; mp 104—105°C. This was combined with the crystals obtained before chromatography, and the mixture was recrystallized from the same mixed solvent; mp and mmp 107—108°C, $[\alpha]_D^{26}$ +176.8° (*c* 0.78, water).

Found: OCH_3, 14.30%.

Reduction and Subsequent Methanolysis of Isoagarobiose. A solution of sodium borohydride (0.20 g) in water (10 m*l*) was mixed with a solution of isoagarobiose (1.25 g) in water (20 m*l*). The resulting solution showed no reducing power after about 2 hr, at which time it was neutralized with dilute acetic acid and then deionized by passing it through columns of Amberlite IR-120 (20 m*l*) and Amberlite IR-4B (20 m*l*). The evaporation of the deionized solution afforded isoagarobi-itol as a syrup; yield, 0.77 g, $[\alpha]_D^{30}$ −11.7° (*c* 1.02, water).

When the glycitol obtained above was subjected to methanolysis with 3% methanolic hydrogen chloride (60 m*l*) for 30 hr in the usual way, the product was obtained as a syrup (0.50 g). This was chromatographed on a cellulose column (2×33 cm) in the manner described for the direct methanolysis products of isoagarobiose. The evaporation of the butanolic effluent afforded 3, 6-anhydro-L-galactose dimethyl acetal; yield, 0.25 g, $[\alpha]_D^{25}$ −25.4° (*c* 1.2, water), R_f 0.67.

Found: OCH_3, 28.56%.

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15) E. L. Jackson, "Organic Reactions," Vol. 2, p. 341 (1944).

16) C. Araki, *Nippon Kwagaku Kwaisi (J. Chem. Soc. Japan)*, **65**, 725 (1944).

TABLE 2. DISTILLATION OF THE METHANOLYSIS PRODUCT OF METHYL PENTA-*O*-METHYL-β-ISOAGAROBIOSIDE

Fraction	Bath temp. °C	Yield* g	Bp** °C	n_D^{25}	CH_3O %	$[\alpha]_D(H_2O)$ °
1	140—160	0.88	83—85	1.4440	59.31	−15.5
2	161—181	0.18	86—120	1.4508	55.71	−5.1
3	181—200	0.56	136—143	1.4643	43.21	+15.4
4	208—215	0.68	145—153	1.4751	46.20	+37.5

* The distillation residue weighed 0.30 g.

** Under 0.025—0.030 mmHg.

On the other hand, the evaporation of the methanolic effluent afforded a syrup (0.23 g) from which dulcitol was crystallized; yield, 0.15 g, mp 180—181°C. It was recrystallized from methanol; mp and mmp 184°C, $[\alpha]_D^{31}$ ±0° (*c* 0.65, water).

Found: C, 39.72; H, 7.86%.

Methyl Penta-*O*-methyl-β-isoagarobioside. To a solution of isoagarobiose (2.5 g) in water (8 m*l*), 32% potassium hydroxide solution (61 m*l*) and dimethyl sulfate (24 m*l*) were added alternately by drop over an 8-hr period with vigorous stirring under ice-cooling. After stirring and cooling had been continued for two more hours, the solution was left overnight. An additional 159 m*l* of the alkali solution and 71 m*l* of dimethyl sulfate were added at 24—25°C with vigorous stirring over a 13-hr period, after which the reaction mixture was stirred for 10 more hr. The product was then extracted four times with 75-m*l* portions of chloroform, and the combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The resulting syrup was further methylated twice, with methyl iodide (25 g) and silver oxide (15 g). The product was obtained as a syrup which, on standing, gradually crystallized; yield, 2.3 g, $[\alpha]_D^{9}$ +7.7° (*c* 1.16, water), OCH_3, 45.73%. Recrystallization was effected by dissolving it in ether and adding petroleum ether; yield, 0.8 g, mp 90—91°C, $[\alpha]_D^{7}$ −18.5° (*c* 1.08, water), $[\alpha]_D^{7}$ −29.0° (*c* 1.0, methanol), $[\alpha]_D^{7}$ −25.2° (*c* 1.03, chloroform). Further recrystallization did not raise the melting point. The negative value of its specific rotation indicates that the glycosidic methoxyl group has a β-configuration.

Found: C, 53.11; H, 7.99; OCH_3, 45.73%; mol wt (Rast), 409. Calcd for $C_{12}H_{14}O_4(OCH_3)_6$: C, 52.91; H, 7.90; OCH_3, 45.66%; mol wt, 408.

Methanolysis of Methyl Penta-*O*-methyl-β-isoagarobioside. A solution of methyl penta-*O*-methyl-β-isoagarobioside (2.9 g) in 2.5% methanolic hydrogen chloride (100 m*l*) was refluxed for 20 hr, after which 5% methanolic hydrogen chloride (20 m*l*) was added; the mixture was then heated for 15 more hr. The resulting dark solution was neutralized with silver carbonate, filtered, and evaporated to a syrup which was then extracted with chloroform. The extract was dried with anhydrous magnesium sulfate and again evaporated to a syrup; yield, 3.2 g, $[\alpha]_D^{6}$ +29.1° (*c* 0.93, water), n_D^{25} 1.4653, OCH_3 48.44%. When a 3.0-g portion of the syrup was distilled under high vacuum, the four fractions shown in Table 2 were obtained.

From the data in Table 2, it may be presumed that fraction 1 is tri-*O*-methyl-3, 6-anhydro-L-galactose dimethyl acetal and that fraction 3 is methyl di-*O*-methyl-D-galactoside. This will be proved below. Fraction 2 must be a mixture of the above two compounds, while fraction 4 must be the unchanged disaccharide derivative.

2, 4, 5-Tri-*O*-methyl-3, 6-anhydro-L-galactose Dimethyl Acetal. Fraction 1 in Table 2 was further purified by re-distillation; bp 85—86°C/0.024 mmHg (bath temperature 140—142°C), n_D^{25} 1.4438, $[\alpha]_D^{17}$ −24.3° (*c* 1.0, water). It showed a strong Seliwanoff ketose reaction and gave a spot with a R_f value of 0.92 on a paper chromatogram. These properties are in agreement with those of 2, 4, 5-tri-*O*-methyl-3, 6-anhydro-L-galactose dimethyl acetal.2)

Found: C, 52.57; H, 8.99; OCH_3, 60.6%.

Methyl 2, 4, 5-Tri-O-methyl-3, 6-anhydro-L-galactonate. When the dimethyl acetal (0.50 g) obtained above was hydrolysed with 0.02 N sulfuric acid (20 m*l*) at 98°C for 2 hr in the usual way, 2, 4, 5-tri-*O*-methyl-3, 6-anhydro-L-galactose was obtained as a syrup; n_D^{25} 1.4621, $[\alpha]_D^{20}$ −19.4° (*c* 1.03, water), OCH_3, 45.3%. This sugar (0.30 g) was oxidized with bromine water in the usual way to the corresponding aldonic acid, which was then esterified by heating it with 4% methanolic hydrogen chloride (10 m*l*) under reflux for 10 hr. Methyl 2, 4, 5-tri-*O*-methyl-3, 6-anhydro-L-galactonate was obtained as a syrup; yield, 0.25 g, n_D^{25} 1.4481, $[\alpha]_D^{8}$ −52.2° (*c* 0.83, water). The literature values2) are n_D^{25} 1.4467 and $[\alpha]_D^{13}$ −53.1° (water).

Found: C, 51.06; H, 7.81; OCH_3, 52.13; $COOCH_3$, 24.85%. Calcd for $C_6H_{10}O_2(OCH_3)_4$: C, 51.25; H, 7.75; OCH_3, 53.00; $COOCH_3$, 25.21%.

2, 3-Di-*O*-methyl-D-galactose. Fraction 3 (0.50 g) in Table 2 was hydrolysed with N sulfuric acid (10 m*l*) in the usual way; 2, 3-di-*O*-methyl-D-galactose was thus obtained as a syrup; yield, 0.35 g, n_D^{25} 1.4844, R_f 0.44, $[\alpha]_D^{11}$ +61.5° (9 min) → +82.2° (24 hr) (*c* 1.0, water). These physical constants are in agreement with those of 2, 3-di-*O*-methyl-D-galactose.3−6)

Found: C, 46.05; H, 7.58; OCH_3, 31.00%. Calcd for $C_6H_{10}O_4(OCH_3)_2$: C, 46.12; H, 7.75; OCH_3, 29.81%.

2, 3-Di-O-methyl-N-phenyl-D-galactosylamine. The sugar (0.11 g) obtained above was converted into its anilide in the usual way, and the product (0.06 g) was purified by recrystallization from acetone-petroleum ether; mp 129°C, $[\alpha]_D^{15}$ −30.2° (13 min) → +12.8° (96 hr) (*c* 1.0, ethanol). Two forms have been reported for the anilide; one has a lower mp 129°C4) or 130—131°C and $[\alpha]_D^{16}$ +119.4° (ethanol),3) while the other has a higher mp 154—155°C and $[\alpha]_D$ −56.8°→ +12.1°5) or mp 152—154°C and $[\alpha]_D^{13}$ −61.9°→ +12.9° (ethanol).6) The anilide prepared above agreed with the former form in melting point and with the latter form in specific rotation.

Found: C, 59.25; H, 7.28; N, 4.93; OCH_3, 31.32%. Calcd for $C_{14}H_{21}O_5N$: C, 59.35; H, 7.24; N, 4.95; OCH_3, 21.91%.

2, 3-Di-O-methyl-D-galactaric Acid Bisamide. The di-*O*-methyl-D-galactose (0.20 g) was converted into 2, 3-di-*O*-methyl-D-galactaric acid bisamide by the method of Luckett and Smith.7) The product was purified by recrystallization from water; mp 226—227°C. The melting point is in agreement with the reported value.7)

Found: C, 40.48; H, 6.99; N, 11.99; OCH_3, 25.71%. Calcd for $C_8H_{16}O_6N_2$: C, 40.67; H, 6.83; N, 11.86; OCH_3, 26.28%.

Formation of Isoagarobiose by Reversion. Agarobiose dimethyl acetal9) mp (165—166°C) (10 g) was heated with N sulfuric acid (100 m*l*) in a boiling water bath for 20 hr, after which the resinous substances formed were removed by filtration. The filtrate was neutralized with barium carbonate, re-filtered, deionized with Amberlite IR-120 (150 m*l*) and Amberlite IR-45 (150 m*l*), and evaporated to a syrup (4.9 g). D-Galactose was then removed therefrom both by crystallization and fermentation, and a mixture of non-fermentable sugars was obtained as a syrup (0.65 g). The syrup showed three spots, with R_f values of 0.10, 0.21 and 0.34, on a paper chromatogram, the second one corresponding to that of isoagarobiose.

The mixture was chromatographed on a charcoal-Celite (1 : 1) column (3×16 cm), which was then eluted with water, 2.5% ethanol and 5% ethanol in succession, isoagarobiose being removed from the column with the last eluant. The evaporation of the effluent afforded the disaccharide in a syrup; yield, 0.15 g, R_f 0.21 (R_{gal} 1.00), $[\alpha]_D^{20}$ +39.5° (*c* 1.0, water). Acetylation with acetic anhydride and pyridine gave hexa-*O*-acetyl-*α*-isoagarobiose; mp 196—197°C. Admixture with the sample described earlier showed no depress ions of the melting point.