Studies on the Chemical Constitution of Agar-agar. XX[1]. *Isolation of a Tetrasaccharide by Enzymatic Hydrolysis of Agar-agar*[2]

By Choji ARAKI and Kiyoshi ARAI

(Received December 1, 1956)

Araki has suggested that agarose, the main polysaccharide of agar, is composed of 1, 3-linked β-D-galactopyranose and 1, 4-linked 3, 6-anhydro-α-L-galactopyranose, the residues being alternately repeated to form a chain[3]. The structure suggested has been based on the chemical composition of agar, the identification of the scission products of methylated agarose[4,5], the isolation of agarobiose (4-*O*-β-D-galactopyranosyl-3, 6-anhydro-L-galactose) and its derivatives from the products of partial acid hydrolysis[6], mercaptolysis[7] and methanolysis[8] of agar, and the isolation of neoagarobiose (3-*O*-3, 6-anhydro-α-L-galactopyranosyl-D-galactose) from the products of enzymatichydrol ysis of agar[9]. In the last-mentioned process, the extract of an agar-digesting bacterium *Pseudomonas kyotoensis* was used as an enzyme solution, and a mixture of four oligosaccharides resulted. The mixture was resolved into its components by chromatography on a charcoal column, and neoagarobiose was eluted from the column with 7.5% ethanol. This paper is concerned with a tetrasaccharide, which has been eluted from the same column with 17.5% ethanol.

The tetrasacccharide, constituting a predominant quantity (40%) among the hydrolysates, forms a crystalline tetrahydrate having the composition $C_{24}H_{38}O_{19}\cdot 4H_2O$, when crystallized from a mixture of methanol and ethanol. The fully acetylated and methylated derivatives have also been obtained as crystals having the respective compositions $C_{24}H_{28}O_{19}(COCH_3)_{10}\cdot 2H_2O$ and $C_{24}H_{28}O_9(OCH_3)_{10}$. The tetrasaccharide has been shown to be *O*-3, 6-anhydro-α-L-galactopyranosyl-(1→3)-*O*-β-D-

1) Part XIX : S. Hirase, This Bulletin, **30**. 68, 70, 75 (1957).

2) The main part of this paper was presented at the 9th Annual Meeting of the Chemical Society of Japan held in Kyoto on April 3, 1956.

3) C. Araki, This Bulletin, **29**, 543 (1956); *Memoirs of the Faculty of Industrial Arts, Kyoto Technical Univ*, **5**, 21 (1956).

4) C. Araki *J Chem. Soc. Japan*, **58**, 1362 (1937).

5) C. Araki, ibid., **61**, 775 (1940).

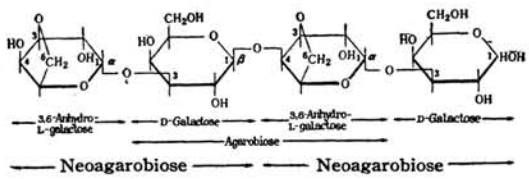
6) C. Araki, ibid., **65**, 533, 627 (1944).

7) S. Hirase and C. Araki, This Bulletin, **27**, 105 (1954).

8) C. Araki and S. Hirase, ibid., **27**, 109 (1954).

9) C. Araki and K. Arai, ibid., **29**, 339 (1956).

galactopyranosyl-(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-galactose(I), or in a simpler expression 4'-*O*-β-neoagarobiosyl neoagarobiose. The linkages involved provide a strong evidence to support the foregoing structure of agarose suggested by one of the authors.



(I) Neoagarotetraose

The structure (I) has ben assigned for the following reasons. Complete methanolysis of the tetrasaccharide afforded 3,6-anhydro-L-galactose dimethylacetal and methyl D-galactoside in an equal molar proportion, indicating that the tetrasaccharide is composed of two moles each of 3,6-anhydro-L-galactose and D-galactose. On reduction of the tetrasaccharide with sodium borohydride, a corresponding glycitol was obtained, which on methanolysis yielded dulcitol together with 3,6-anhydro-L-galactose dimethylacetal and methyl D-galactoside, establishing thereby the reducing end of the parent tetrasaccharide as a D-galactose residue. When the tetrasaccharide was subjected to hydrolysis with the enzyme extract of *P. kyotoensis*, the sole product that could be detected and isolated was neoagarobiose. This evidence suggests that the tetrasaccharide is neoagarobiosyl-neoagarobiose involving two 1→3 linkages. On mild hydrolysis of the tetrasaccharide with 0.04 N-oxalic acid solution, there were obtained equal molar quantities of 3,6-anhydro-L-galactose, D-galactose and agaro- biose, the separation being effected by charcoal chromatography. Identification of the last disaccharide proves the presence of the 1→4 linkage in the parent tetrasaccharide molecule. Thus it is possible to assign the structure(I) to the tetrasaccharide isolated.

The structure(I) has been supported also by the identification of the cleavage fragments of the methylated tetrasaccharide. Complete methanolysis of the fully methylated tetrasaccharide afforded a mixture of the following fragments, which were separated on a charcoal column: (a) 2-*O*-methyl-3,6-anhydro-L-galactose derivatives, identified after hydrolysis and bromine oxidation as methyl 2-*O*-methyl-3,6-anhydro-L-galactonate and also as its corresponding amide, (b) 2,4-di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal, identified after hydrolysis and bromine oxidation as 2,4-di-*O*-methyl-3,6-anhydro-L-galactonic acid and its amide, and (c) methyl 2,4,6-tri-*O*-methyl-D-galactoside, identified after hydrolysis as a crystalline sugar and its anilide.

A general nomenclature is suggested here for the oligosaccharides produced by partial break down of agarose and a similar polysaccharide. The prefix "agaro" is added to an oligosaccharide whose non-reducing end is presented by D-galactopyranose. Thus the name agarobiose was assigned to a disaccharide 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose[6]. While, the prefix "neoagaro" is added to an oligosaccharide whose non-reducing end is presented by 3,6-anhydro-L-galactopyranose. Thus the name neoagarobiose was assigned to a disaccharide 3-*O*-3,6-anhydro-α-L-galactopyranosyl-D-galactose[9]. On this basis the tetrasaccharide investigated in this paper is termed neoagarotetraose.

From the fact that neoagarobiose and neoagarotetraose resulted from agar by the action of the enzymes of an agar-digesting bacterium, and also that neoagarotetraose is hydrolysed by the same enzymes to yield neoagarobiose, it may possibly be assumed that agarose is hydrolysed by the enzymes at its occasional 1→4 β-D-galactoside linkages to yield higher homologues of neoagaro-oligosaccharides, which are in turn hydrolysed at their 1→4 linkages to form neoagarobiose as the lowest fragement.

Experimental

General Procedure.—Evaporation and concentration were carried out under reduced pressure below 40°. All the melting points are uncorrected. Unless otherwise stated the specific rotation was measured in the aqueous solution with a 1 dm. tube. The paper chromatograms were irrigated with *n*-butanol-acetic acid-water (4 : 1 : 2 v/v) in the ascending manner and were sprayed with an *o*-aminophenol reagent[10]. For the charcoal chromatography, there was used active carbon Shirasagi[11], which was not mixed with Celite.

Neoagarotetraose.—A part (2.0 g.) of the 17.5% ethanol eluate, $[\alpha]_D$+1.1°, obtained in Part XVIII[9], was dissolved in a few drops of water, and methanol was added while heating to form insoluble precipitates, which were subsequently removed by filtration. Then absolute ethanol (nearly equal in volume to that of the

10) S. Hirase, C. Araki and S. Nakanishi, ibid., **26**, 183 (1953).

11) Product of Takeda Pharmaceutical Industries, Ltd., Osaka.

methanol used) was added to the filtrate while boiling until the solution became slightly turbid, and the resulting solution was kept in a refrigerator. The crystals deposited were filtered and washed successively with ethanol-methanol (2 : 1), ethanol and ether; yield 0.8 g. The filtrate and washings were combined and evaporated, when the second crop of the crystals was obtained; yield 0.6 g.

The two crops of the recrystals were combined and recrystallized by dissolving in hot methanol, adding twice the volume of ethanol while boiling, and then keeping the solution in a refrigerator. Neoagarotetraose was deposited as colorless fine crystals, which were filtered and washed successively with ethanol-methanol (2 : 1), ethanol and ether; yield 0.50 g. It sintered at 190°, melted at 214-218° under decomposition, and had a specific rotation $[\alpha]_D^{21}$ −3.9° (after twenty four hours, *c* 1.0). It gave on a paper chromatogram a spot whose R_{gal} value[9] was 0.71, and showed a reducing power 27.85% of D-galactose. The analysis of the sample dried at 100° in vacuo over phosphorus pentoxide showed that it was a tetrahydrate.

Anal. Found: C, 40.98; H, 6.60. Calcd. for $C_{24}H_{38}O_{19}\cdot 4H_2O$: C, 41.10; H, 6.55%.

The mother liquor of the recrystallization was diluted with methanol and again evaporated to form hygroscopic crystals, which sintered at 98°, melted at 104-107°, had a specific rotation $[\alpha]_D^{22}$ −2.8° (1.0), and which gave a spot with R_{gal} value 0.71 on a paper chromatogram. The analysis of the sample dried at 65° in vacuo over phosphorus pentoxide indicated that it was a dihydrate.

Anal. Found: C, 43.07; H, 6.42. Calcd. for $C_{24}H_{38}O_{19}\cdot 2H_2O$: C, 43.10; H, 6.31%.

Deca-*O*-acetylneoagarotetraose.—Neoagarotetraose (1.0 g.) in pyridine (20 cc.) was acetylated with acetic anhydride (7.0 g), which was added dropwise under ice cooling. After being left at room temperature (18-19°) for seventy-two hours, the reaction solution was poured with stirring into ice-water (120 cc.), and crystals of the acetate formed were filtered and washed with ice-water; yield 1.0 g.; m. p. 115-116°. Recrystallization was effected first from *n*-butanol and then from methanol-ethanol; m. p. 121°; $[\alpha]_D^8$ −15.8° (chloroform, *c* 1.0) and −11.8° (methanol, *c* 1.0).

Anal. Found: C, 49.15; H, 5.74: CH_3CO, 41.58. Calcd. for $C_{24}H_{28}O_{19}(COCH_3)_{10}\cdot 2H_2O$: C, 49.10; H, 5.72; CH_3CO, 40.00%.

Methyl Nona-*O*-methyl-β-neoagarotetraoside.—A methylation of neoagarotetraose (2.0 g.) was carried out first under ice cooling and then at room temperature (24-25°) over three days in the same manner as in the case of neoagarobiose[9], using dimethylsulfate (63 cc., total) and 32% potassium hydroxide solution (150 cc., total). After completion of the reaction, the reaction mixture was filtered to remove the precipitated potassium sulfate and the filtrate was extracted four times with chloroform (200 cc., total). The extracts were combined, washed once with water, dried over sodium sulfate, and evaporated to a syrup (2.5 g.), which on dissolution in ether and evaporation afforded a semi-solid (2.28 g., OCH_3 36.1%). It was then twice methylated with methyl iodide (25 g.) and silver oxide (15 g.), and the final product was obtained by extraction with ether and subsequent evaporation as a crystalline mass (2.0 g., OCH_3 40.5%), which was triturated with ether. The separated crystals were then filtered; yield 0.48 g.; m.p. 164°. An additional crop was obtained from the filtrate by adding petroleum ether (45-53°) and keeping in a refrigerator; yield 0.72 g.; m. p. 163-164°. The crystals obtained were combined, recrystallized from methanol and washed with ether; m. p. 167°; $[\alpha]_D^{10}$ −87.6° (chloroform, *c* 1.0) and −85.9° (methanol, *c* 1.0). The negative values of the rotation indicate that it is a β-anomer of the methylated neoagarotetraoside.

Anal. Found: C, 52.90; H, 7.64; OCH_3, 40.37; m. w. (Rast), 768. Calcd. for $C_{24}H_{28}O_9(OCH_3)_{10}$: C, 53.12; H, 7.53; OCH_3, 40.27%; m. w., 770.

Methanolysis of Neoagarotetraose.—Neoagarotetraose (1.0 g.) in 4% methanolic hydrogen chloride (50 cc.) was heated under reflux until the optical rotation of the solution reached a constant value: $[\alpha]_D$ −25.0° (initial)→+32.0° (final). After fifteen hours the solution was neutralized with silver carbonate, filtered and evaporated to a syrup (1.15 g.). Paper chromatographic examination showed that the syrup was a mixture of 3,6-anhydro-L-galactose dimethylacetal and methyl D-galactoside. A part (1.0 g.) of the mixture was separated into its components on a powdered filter paper column in the manner previously reported from this laboratory[8,9].

3,6-Anhydro-L-galactose Dimethylacetal.—This substance was eluted from the column with *n*-butanol saturated with water, and was obtained as a colorless syrup on evaporation; 0.50 g.; $[\alpha]_D^{25}$ −31.1° (*c* 0.9); OCH_3, found: 29.62% (calcd. for $C_6H_{10}O_4(OCH_3)_2$: 29.81%). It showed a strong Seliwanoff's ketose reaction and gave on a paper chromatogram a spot corresponding to 3,6-anhydro-L-galactose dimethylacetal. Identification was carried out in the usual manner by hydrolysis to a reducing sugar, which was then converted to 3,6-anhydro-L-galactose diphenylhydrazone[12]; m. p. 153°; $[\alpha]_D^{23}$ −34.6° (initial, methanol, *c* 1.4).

Anal. Found: N, 8.61. Calcd. for $C_{18}H_{20}O_4N_2$: N, 8.53%.

Methyl D-Galactoside.—After removal of the 3,6-anhydro-L-galactose dimethylacetal from the column, methyl D-galactoside was eluted with 80% aqueous methanol, and was obtained as a syrup (0.48 g.) on evaporation. Crystallization from methanol-acetone-(1 : 2) afforded methyl α-D-galactoside monohydrate: m. p. and mixed m. p. 108-109°; $[\alpha]_D^{23}$+180.5° (*c* 1.0); OCH_3, found: 14.73% (calcd. for $C_7H_{14}O_6\cdot H_2O$: 14.62%).

Reduction and Methanolysis of Neoagarote-

12) C. Araki, *J. Chem. Soc. Japan*, **65**, 725 (1944).

traose.—A solution of neoagarotetraose (1.50 g.) in water (30 cc.) was combined with a solution of sodium borohydride (0.15 g.) in water (10 cc.), and the resulting solution was left at room temperature (7°) withoccasional shaking for two and a half hours, at which time the solution showed no longer reducing power. It was then carefully neutralized with 0.25 N-aqueous acetic acid under ice cooling, and evaporated to dryness. Crude neoagarotetra-itol, obtained as an amorphous material (1.65 g.), was crystallized from methanol-ethanol (1 : 1); m. p. 60–63°; $[\alpha]_D^{11}$ −32.0° (*c* 1.3, *l* 0.5).

The above glycitol (1.60 g.) in 4% methanolic hydrogen chloride (100 cc.) was heated under reflux until the optical rotation of the solution reached a constant value ($[\alpha]_D$ −25.0° → +3.1°) After eighteen hours the solution was neutralized with silver carbonate, filtered and evaporated to give a syrupy mixture (1.45 g.) of the following three products, which were separated by chromatography.

3,6-Anhydro-L-galactose Dimethylacetal.—The above mixture was placed on a starch column (4.5×25 cm.) and eluted with *n*-butanol saturated with water. The eluate showing the optical rotation in the negative direction was evaporated to dryness, 3,6-anhydro-L-galactose dimethylacetal being obtained as a colorless syrup; yield 0.60 g; $[\alpha]_D^{22}$ −29.8° (*c* 0.9); OCH_3, found: 28.8% (calcd. for $C_6H_{10}O_4(OCH_3)_2$: 29.81%). It was identified in exactly the same manner as that above-mentioned.

Methyl D-Galactoside.—The chormatographic column was then eluted with *n*-butanol-ethanol-water (4 : 1 : 2), and the eluate showing the optical rotation in the positive direction was evaporated to dryness. Methyl D-galactoside was obtained as a syrup (0.40 g.) and was identified in exactly the same manner as that above-mentioned.

Dulcitol.—The elution with the same solvent was continued and the eluate (200 cc.) showing no optical rotation was concentrated to a syrup (0.20 g.). Then the eluant was changed to 80% aqueous methanol and the eluate (200 cc.) was concentrated to a syrup (0.20 g.). The syrups obtained were combined and subjected to crystallization from methanol, affording dulcitol; yield 0.20 g.; m. p. 186–187°, not depressed on admixture with an authenic sample; $[\alpha]_D^9 \pm 0°$ (*c* 1.0).

Anal. Found: C, 39.55; H, 7.42. Calcd. for $C_6H_{14}O_6$: C, 39.56; H,7.75%.

Hydrolysis of Neoagarotetraose with 0.04 N-Oxalic Acid.—Neoagarotetraose (1.50 g.) in 0.04 N-oxalic acid solution (100 cc.) was heated in a boiling water bath, the progress of the hydrolysis being followed polarimetrically: $[\alpha]_D$ −1.7° (initial), −4.0° (15 min.), −3.3° (30 min.), −2.7° (1 hr.), +0.7° (1.5 hr.), +5.5° (2 hr.), +6.0 (2.5 and 3 hr., constant). After three hours the acid was removed by neutralization with calcium carbonate and filtration, the filtrate was concentrated to a syrup, which was then extracted with methanol, and the extract was again concentrated to give a light yellow syrup (1.50 g.). When chromatographed on a paper, it showed a brown spot corresponding to galactose, and two yellow with tails spots, the centres of which had the respective R_{gal} values 2.07 and 3.10, corresponding to agarobiose and 3,6-anhydro-galactose, respectively. The separation of the mixture was effected by the charcoal chromatography of Whistler and Durso[13].

A 10% aqueous solution of the syrup (1.50 g.) was placed on a charcoal column (3.3×7.0 cm.) and eluted with water and with successive higher concentrations of ethanol in water. The eluate (30 cc. per fraction) was examined both polarimetrically and paper chromatographically, and the fractions containing the same material were combined and evaporated to recover the material. The result is given in Table I, and the yields cited indicate the presence of D-galactose, 3,6-anhydro-L-galactose and agarobiose in the approximate mcle ratio 1 : 1 : 1. The identification of each product was carried out in the manner described below.

D-Galactose.—Fraction I, which completely solidified, was recrystallized from methanol, affording D-galactose; yield 0.25 g.; m. p. 166–167°; $[\alpha]_D^6$ +96.5° (initial) → +79.5° (after twenty four hours, *c* 1.0).

Anal. Found: C, 40.15; H, 6.89. Calcd. for $C_6H_{12}O_6$: C, 40.00; H, 6.67%.

The filtrate of the recrystallization was concentrated to a syrup, which afforded D-galactose phenylosazone on treatment with phenylhydrazine in the presence of acetic acid; m. p. and mixed

TABLE I

CHROMATOGRAPHIC SEPARATION OF THE HYDROLYSATES OF NEOAGAROTETRAOSE WITH 0.04 N-OXALIC ACID SOLUTION

Fraction	I	II	III	IV	V	VI	VII
Eluted by	W	W	2.5%E	5.0%E	7.5%E	10%E	17.5%E
Volume (cc.)	330	420	360	570	450	420	450
$[\alpha]_D$(°)	+53.7	−18.8	−15.6	−7.8	−17.5	−2.8	−3.0
Yield (g.)	0.35	0.20	0.13	0.41	0.17	0.05	0.08
Component identified	D-Galactose	3,6-Anhydro-L-galactose		Agarobiose		Neoagarotetraose unchanged	

W: Water, E: Ethanol

13) R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc.*, **72**, 677 (1950).

m. p. 187–188°; N, found: 15.63% (calcd. for $C_{18}H_{22}O_4N_4$: 15.70%).

3,6-Anhydro-L-galactose.—Fractions II and III showed a distinct Seliwanoff's ketose reaction and reduced a Fehling's solution at room temperature. The phenylosazone prepared therefrom agreed with 3,6-anhydro-L-galactose phenylosazone[12]; m. p. and mixed m. p. 216–217°; $[\alpha]_D^6$ −65.3° (initial) → −37.6° (after twenty four hours, pyridine-ethanol (2 : 3), *c* 0.5, *l* 0.5).

Anal. Found: N, 16.62. Calcd. for $C_{18}H_{20}O_3N_4$: N, 16.46%.

Agarobiose.—Fractions IV and V showed a Seliwanoff's reaction and reduced a Fehling's solution at room temperature. The phenylosazone prepared therefrom agreed with agarobiose phenylosazone[6]; m. p. 218–219°; $[\alpha]_D^6$ −155.7° (initial) → −137.6° (after forty eight hours, pyridine-ethanol (2 : 3), *c* 0.6, *l* 0.5).

Anal. Found: N, 11.23. Calcd. for $C_{24}H_{30}O_8N_4$: N, 11.15%.

Neoagarotetraose Recovered.—Fractions VI and VII did not reduce a Fehling's solution at room temperature and gave on a paper chromatogram a single spot corresponding to neoagarotetraose (R_{gal} 0.71), proving thereby to be an unchanged material.

Hydrolysis of Neoagarotetraose with Enzymes.—The enzyme solution used for the following experiments was prepared on a 100 cc. scale from *P. kyotoensis* exactly in the same manner as that reported in Part XVIII[9]. Twenty five cc. of the solution was buffered with N/15-potassium dihydrogen phosphate (10 cc.) and was diluted with water to 40 cc., and a drop of toluene was added, the pH value of the resulting solution being 5.6. Three similar batches were prepared: (A) with addition of neoagarotetraose (0.5 g.), (B) with addition of neogarotetraose (0.5 g.) after being heated to kill the enzymes, and (C) without addition of neoagarotraose. These were kept at 40° in an incubator, and aliquotes (2.0 cc.) were periodically withdrawn to determind the reducing power by Bertrand's method (table II). Only the batch A showed an increase in the reducing power, corresponding to 21.8% hydrolysis.

After forty eight hours, batches A and C were respectively heated for five minutes in a boiling water bath to stop the reaction. All the batches were separately concentrated, the residues were repeatedly extracted with hot methanol, and the extracts were again evaporated to dryness. The resulting syrups weighed (A) 0.40 g., (B) 0.40 g. and (C) 0.10g., respectively. Paper chromatographic examination indicated that syrup A was a mixture of neoagarobiose and neoagarotetraose, syrup B contained neoagarotetraose only, and the syrup C contained neither of them, proving thereby that the neoagarobiose in the syrup A was derived by the hydrolysis of neoagarotetraose with enzymes.

For the isolation of the neoagarobiose, the experiment A was repeated five times, and the combined syrups (2.4 g.) were chromatographed on a charcoal column (4.5×10 cm.) in a manner similar to the separation of the acid-hydrolysates of neoagarotetraose, the result being shown in Table III.

Fraction I was chiefly inorganic substances. Fraction II gave on a paper chromatogram a spot (R_{gal} 1.15) corresponding to neoagarobiose, fraction III two spots (R_{gal} 1.15 and 0.71) corresponding to neoagarobiose and neoagarotetraose, and fractions IV, V and VI a spot (R_{gal} 0.71) corresponding to neoagarotetraose, respectively. The identification was carried out as follows.

Neoagarobiose.—Fraction II was amorphous powders, a part (0.25 g.) of which was dissolved in a few drops of water, twice its volume of methanol was added while heating, the solution was filtered, and additional methanol was added to the filtrate while heating. When the resulting solution was kept in a refrigerator, neoagarobiose

TABLE II

HYDROLYSIS OF NEOAGAROTETRAOSE WITH ENZYMES*

	Initial	8 hr.	24 hr.	32 hr.	48 hr.	±**
A	170.4	184.0	195.0	198.5	207.6	+37.2
B	176.4	179.0	182.9	179.0	167.6	−8.8
C	6.9	8.1	10.9	9.2	10.2	+3.3

A: A buffered enzyme solution (40 cc.) + neoagarotetraose (0.5 g.).
B: A buffered enzyme solution (40 cc.) killed + neoagarotetraose (0.5 g.).
C: A buffered enzyme solution (40 cc.) only.
* The figures are indicated by Bertrand's copper value (mg.), calculated for the whole solution (40 cc.).
** An increase or decrease in copper value after 48 hr.

TABLE III

CHROMATOGRAPHIC SEPARATION OF ENZYMATIC HYDROLYSATES OF NEOAGAROTETRAOSE

Fraction	I	II	III	IV	V	VI
Eluted by	W	5%E	7.5%E	10%E	15%E	17.5%E
Volume (cc.)	640	750	1230	480	1050	720
Yield (g.)	0.1	0.30	0.25	0.25	1.20	0.25
$[\alpha]_D$ (°)	±0	+16.5	−0.9	−1.8	−2.9	−3.0

W: Water, E: Ethanol.

was deposited; yield 0.15 g.; m. p. 202–203°, not depressed on admixture with an authentic sample[9]; $[\alpha]_D^{15}$ +25.0° → +22.5° (*c* 1.1).

Anal. Found: C, 44.25; H, 6.33. Calcd. for $C_{12}H_{20}O_{10}$: C, 44.44; H, 6.22%.

The filtrate from the above crystals was concentrated to a syrup, which was then treated with phenylhydrazine in the presence of acetic acid. The phenylosazone obtained had m. p. 198–199°, not depressed on admixture with neoagarobiose phenylosazone[9].

Anal. Found: N, 11.37. Calcd. for $C_{24}H_{30}O_8N_4$: N, 11.15%.

Neoagarotetraose Recovered.—All the fractions IV, V and VI were amorphous powders, which on acetylation with acetic anhydride and pyridine gave neoagarotetraose deca-acetate, m. p. 120–121°, not depressed on admixture with the sample described earlier.

Methanolysis of Methyl Nona-*O*-methyl-β-neoagarotetraoside.—The methylated tetraoside (1.70 g.), described earlier, was dissolved in 4% methanolic hydrogen chloride (100 cc.) and the solution was heated under reflux until the optical rotation of the solution reached a constant value ($[\alpha]_D$ −39.9° → +32.4°). After seventeen hours the solution was neutralized with silver carbonate, filtered and evaporated to a syrup, which was then dried by dissolution in ether and re-evaporation; yield 1.60 g.; OCH_3, found: 44.5%.

Separation of the Methanolysates by Charcoal Chromatography.—A 10%aqueous solution of the above methanolysates (1.60 g.) was placed on a charcoal column (3.5×10 cm.) and eluted with water and with successive higher concentrations of ethanol in water. The eluates were collected at 30 cc. intervals, and the fractions showing the same content were combined and concentrated to dryness (Table IV). The eluates with water (450 cc.) and with 2.5% ethanol (first 750 cc.) were discarded, since they left nothing on evaporation. The compounds recovered by evaporation were identified in the manner described below.

a) 2-*O*-Methyl-3, 6-anhydro-L-galactose Part.—Fractions I and II, showing a distinct Seliwanoff's reaction, were proved to be derivatives of 2-*O*-methyl-3, 6-anhydro-L-galactose by the conversion into the following crystatalline compounds.

Methyl 2-O-Methyl-3, 6-anhydro-L-galactonate.—A part (0.27 g.) of the fraction I was hydrolysed with 3% hydrobromic acid (6 cc.) in the boilng water bath for ten minutes, and was then oxidized with bromine (0.4 cc.), which was dropped into the hydrolytic solution kept at 40° during four hours. After being left overnight room temperature, the reaction solution was aerated to remove excess bromine, neutralized with silver carbonate, filtered before and after treatment with hydrgen sulfide, and evaporated to a syrup, which solidified on dissolution in methanol and evaporation; yield 0.23 g.

The oxidation product obtained above was esterified by heating in 4% methanolic hydrogen chloride for five hours. The methyl ester, isolated in the usual manner, was separated from a syrupy contaminant by tiling; yield 0.25 g.; m. p. 85–86°. Recrystallization from benzene afforded pure methyl 2-*O*-methyl-3, 6-anhydro-L-galactonate[5]; yield 0.17 g.; m. p. 91°, not depressed on admixture with an authentic specimen; $[\alpha]_D^{24}$ −61.6° (*c* 0.8, *l* 0.5).

Anal. Found: C, 46.79; H, 6.68; OCH_3, 29.1. Calcd. for $C_6H_9O_4(OCH_3)_2$: C, 46.58; H, 6.85; OCH_3, 30.12%.

2-O-Methyl-3, 6-anhydro-L-galactonamide.—The methyl ester obtained above was converted in the usual manner into an amide[5]; m. p. 170–171°; $[\alpha]_D^{23}$ −74.3° (*c* 2.3, *l* 0.5).

Anal. Found: N, 7.15; OCH_3, 16.22. Calcd. for $C_7H_{13}O_5N$: N, 7.33; OCH_3, 16.23%.

b) 2, 4, 6-Tri-*O*-methyl-D-galactose Part.—Solid fractions III and IV were methyl 2, 4, 6-tri-*O*-methyl-D-garactoside, contaminated by a small amount of methyl 2-*O*-methyl-3, 6-anhydro-L-galactoside, which was removed as follows. The two fractions were combined and treated with N/50-sulfuric acid in a boilng water bath for two hours to hydrolyse the contaminant, and then the solution was neutralized with barium carbonate, filtered and evaporated to dryness. The resulting residue (0.70 g.), which solidified on dissolution in methanol and re-evaporation, was extrated with boiling petroleum ether (b. p. 45–55°). On cooling the extract afforded crystals of methyl 2, 4, 6-tri-*O*-methyl-D-galactoside; yield 0.40 g.; m. p. 70–72°; $[\alpha]_D^{23}$ +54.2° (*c* 1.8); OCH_3, found: 51.37% (calcd. for $C_6H_9O_2(OCH_3)_4$: 52.54%). Evaporation of the mother liquor afforded an additional crop (0.25 g.).

2, 4, 6-Tri-O-methyl-D-galactose.—The above

TABLE IV

CHROMATOGRAPHIC SEPARATION OF THE METHANOLYSATES OF METHYLATED NEOAGAROTETRAOSIDE

Fraction	I	II	III	IV	V	VI	VII
Eluted by	2.5%E	5%E	5%E	7.5%E	7.5%E	10%E	10%E
Volume (cc.)	600	600	750	660	600	600	600
Yield (g.)	0.30	0.06	0.48	0.20	0.15	0.22	0.05
n_D^{25}	1.4647	1.4655	solid	solid	1.4539	1.4526	1.4500
OCH_3 (%)	32.6	33.5	47.8	49.0	49.0	48.3	49.3
$[\alpha]_D^{25}$ (°)	−29.6	+34.2	+73.9	+63.2	+50.3	+26.1	+54.0
	2-*O*-Methyl-3, 6-anhydro-L-galactose part		2, 4, 6-Tri-*O*-methyl-D-galactose part		2, 4-Di-*O*-mehyl-3, 6-anhydro-L-galactose part		

E: Ethanol

methyl glycoside (0.40 g.) was hydrolysed with N-sulfuric acid (12 cc.) in the usual manner. The resulting 2,4,6-tri-*O*-methyl-D-galactose was crystallized from ether; yield 0.25 g.; m. p. 97–98°. Recrystallization from the same solvent afforded a pure specimen; yield 0.20 g.; m. p. 113–114°, not depressed on admixture with an authentic sample[4]; $[\alpha]_D^{30}$ +111.7° (initial) → +91.3° (after twenty four hours, *c* 1.7).

Anal. Found: C, 48.65; H, 8.27; OCH_3, 41.20. Calcd. for $C_6H_9O_3(OCH_3)_3$: C, 48.65; H, 8.11; OCH_3, 41.89%.

2,4,6-Tri-O-*methyl*-L-*phenyl*-D-*galactosylamine.*—The above sugar was treated with aniline in the usual manner to give an anilide[4]; m. p. 172–173°; $[\alpha]_D^{31}$ −81.5° (initial) → +38.6° (after forty eight hours; acetone, *c* 1.4, *l* 0.5).

Anal. Found: N, 4.78; OCH_3, 31.54. Calcd. for $C_{15}H_{23}O_5N$: N, 4.71; OCH_3, 31.31%.

c) **2,4-Di-*O*-methyl-3,6-anhydro-L-galactose Part.**—Fractions V, VI and VII were fairly positive to Seliwanoff's test and gave on a paper chromatogram a yellow spot corresponding to 2,4-di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal.

2,4-Di-O-*methyl-3,6-anhydro*-L-*galactonic Acid.*—The fractions VI and VII were combined and a part (0.25 g.) of the resulting mixture was hydrolysed and then oxidized in exactly the same manner as that described for 2-*O*-methyl-3,6-anhydro-L-galactose derivatives. The oxidation product (0.18 g.) was crystallized from ethyl acetate; m. p. 150°, not depressed on admixture with 2,4-di-*O*-methyl-3,6-anhydro-L-galactonic acid[14]; $[\alpha]_D^{17}$ −61.4° (*c* 1.2, *l* 0.5).

Anal. Found: C, 46.34; H, 6.86; OCH_3, 29.66. Calcd. for $C_6H_5O_4(OCH_3)_2$: C, 46.58; H, 6.85; OCH_3, 30.15%.

2,4-Di-O-*methyl-3,6-anhydro*-L-*galactonamide.* The above acide (0.1 g.) was esterified with boiling 4% methanolic hydrogen chloride (3 cc.) and the methyl ester obtained was further converted to the amide[14] in the usual manner; m. p. 150°; $[\alpha]_D^{24}$ −73.4° (*c* 1.3, *l* 0.5).

Anal. Found: N, 6.95; OCH_3, 29.65. Calcd. for $C_6H_{15}O_5N$: N, 6.83; OCH_3, 30.25%.

14) C. Araki, *J. Chem. Soc. Japan*, **61**, 503 (1940).

Summary

1. A tetrasaccharide, forming a crystalline tetrahydrate, has been isolated from the products of enzymatic hydrolysis of agar. Its fully acetylated and methylated derivatives have also been obtained as crystals.

2. Identification of both monosaccharide and disaccharide constituents has led to the assignment of the structure, *O*-3,6-anhydro-α-L-galactopyranosly-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-garactose, to the tetrasaccharide isolated. The structure has been supported also by methylation data.

3. The tetrasaccharide has been termed neoagarotetraose, based on the general nomenclature suggested for oligosaccharides derived from agarose and a similar polysaccharide.

4. A probable course has been assumed for the enzymatic degradation of agarose.

The authors are indebted to Mr. N. Nakata for assistance in this study and to Mr. S. Hirase for help in the preparation of the manuscript. This study has been aided by the Grant for Fundamental Scientific Research from the Ministry of Education, for which our thanks are also expressed.

Institute of Chemistry, Faculty of Industrial Arts, Kyoto Technical University, Matsugasaki, Kyoto