Studies on the Chemical Constitution of Agar-agar. XVIII[1]. *Isolation of a New Crystalline Disaccharide by Enzymatic Hydrolysis of Agar-agar*

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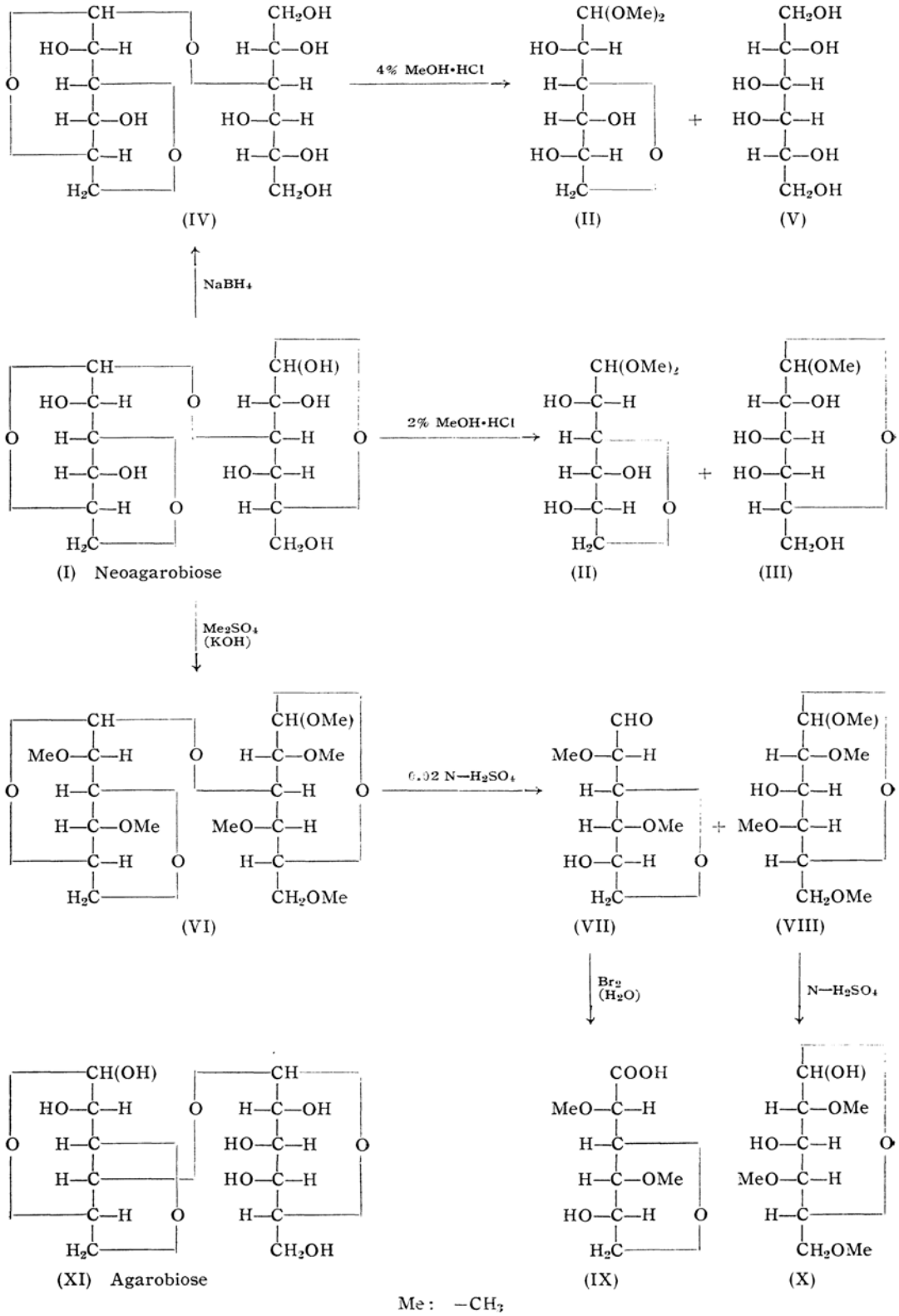
The partial depolymerization of agar-agar by chemical methods, such as acid-hydrolysis[2], methanolysis[1], and mercaptolysis[3], has led to the isolation of agarobiose (4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose) (XI) or its derivatives as the chief oligosaccharide constituent so far identified. The enzymatic hydrolysis would be of great service to examine the structure of the polysaccharide, since the method might bring about oligosaccharide fragments different from those produced by chemical methods. This has been proved to be the case as reported herein.

The enzymatic hydrolysis of agar was first studied by Gran[4], who assigned the name gelase to the agar-hydrolysing enzymes of a microorganism isolated from sea water. Oshima[5] found gelase in the viscela of *Haliotus giganteus* Gm. (ear-shell), and Mori[6] also found it in the viscela, especially in hepatopancreas, of *Turbocornutus* (top-shell) and of other gastropodes. Kadota[7] prepared the enzyme solution capable of hydrolysing agar from a microorganism isolated from sea water. Fukumoto and Ishimatsu[8] gave the name agarase to the agar-hydrolysing enzymes of a microorganism isolated from putrid *tokoroten* (agar-gel). But little has been studied concerning the isolation and the structure of the hydrolysis product, until Ishimatsu and his co-workers[9] recently reported the characterization of a disaccharide, produced by the action of agarase upon agar, as its phenylosazone (m.p. 197°).

The enzyme extract used in this study has been prepared from *Pseudomonas kyotoensis*, an agar-digesting bacterium, isolated from putrid *tokoroten* by these authors[10]. An aqueous solution of agar was hydrolysed with this enzyme extract under the optimum condition until the reducing power of the solution reached a maximum value. From the resulting solution, there was obtained a mixture of reducing sugars, which were

1) Part XVII: C. Araki and S. Hirase, This Bulletin, **27**, 109 (1954).
2) C. Araki, *J. Chem. Soc. Japan*, **65**, 533, 627 (1944).
3) S. Hirase and C. Araki, This Bulletin, **27**, 105 (1954).
4) H.H. Gran, *Centralbl. Bakt. Abt.*, II, **9**, 562 (1902).
5) K. Oshima, *J. Agr. Chem. Soc. Japan*, **17**, 328 (1941).
6) T. Mori, *J. Agr. Chem. Soc. Japan*, **15**, 1070 (1939); **16**, 886 (1940).
7) H. Kadota, *Mem. Coll. Agr. Kyoto Univ.*, **59**, 54 (1954).
8) J. Fukumoto and K. Ishimatsu, *Bull. of the Osaka Municipal Technical Research Institute*, **13**, 1 (1951); K. Ishimatsu, ibid., **14**, 1 (1953).
9) K. Ishimatsu, Y. Kibesaki and S. Maitani, *Science and Industry (Japanese)*, **28**, 100 (1954).
10) C. Araki and K. Arai, *Memoirs of the Faculty of Industrial Arts, Kyoto Technical Univ.*, **3B**, 7 (1954).


4% MeOH•HCl
NaBH4
2% MeOH•HCl
Me2SO4 (KOH)
0.02 N—H2SO4
Br2 (H2O)
N—H2SO4
(IV)
(II)
(V)
(I) Neoagarobiose
(II)
(III)
(VI)
(VII)
(VIII)
(XI) Agarobiose
(IX)
(X)
Me: —CH3

shown by paper chromatography to be four oligosaccharides. The separation was possible by the charcoal chromatography of Whistler and Durso[11]. An aqueous solution of the mixture was placed on a charcoal column and developed with water and then with successive higher concentrations of ethanol in water. The four oligosaccharides were desorbed from the column on elution with 7.5, 17.5, 30 and 40% aqueous ethanol, respectively.

From the 7.5% ethanol eluate, there has been isolated a new crystalline disaccharide (I) having the composition $C_{12}H_{20}O_{10}$. Its hexaacetate and phenylosazone were also obtained in a crystalline state. The phenylosazone seems to be identical with that obtained by Ishimatsu and his co-workers[9]. The glycoside linkage of the disaccharide is so weak that it was hydrolysed to give D-galactose and 3,6-anhydro-L-galactose, when heated in a 0.02N-oxalic acid solution for two hours. Methanolysis of the disaccharide afforded 3,6-anhydro-L-galactose dimethylacetal (II) and methyl D-galactoside (III). These facts indicate that the disaccharide is composed of 3,6-anhydro-L-galactose and D-galactose residues. When the disaccharide was reduced to the corresponding glycitol (IV) with sodium borohydride and was subsequently methanolysed, 3,6-anhydro-L-galactose dimethylacetal (II) and dulcitol (V) were produced. This proves that the disaccharide is shown to be 3,6-anhydro-L-galactosyl-D-galactose, and hence that it is an isomer of agarobiose (XI) previously obtained. So the disaccharide has been named neoagarobiose.

In order to elucidate further the structure of neoagarobiose (I), it was methylated with dimethylsulfate and a potassium hydroxide solution and subsequently with Purdie's reagents. The resulting methyl penta-O-methyl-β-neoagarobioside (VI) was obtained in a crystalline state. On hydrolysis with .002 N-sulfuric acid the methylated neoagarobioside yielded a mixture of methyl 2,4,6-tri-O-methyl-β-D-galactoside (VIII) and 2,4-di-O-methyl-3,6-anhydro-L-galactose (VII). The former was separated from the latter by solvent extraction, and was obtained as crystals. On hydrolysis it gave crystalline 2,4,6-tri-O-methyl-D-galactose (X), the anilide of which was also obtained as crystals. This trimethyl-galactose and its anilide coincided in all respects with the corresponding authentic specimens. 2,4-di-O-methyl-3,6-anhydro-L-galactose (VII) was oxidised with bromine water to give crystalline 2,4-di-O-methyl-3,6-anhydro-L-galactonic acid (IX), which was further converted into the crystalline amide. These crystals were also in good agreement with the corresponding authentic specimens. The identification of VIII and VII as cleavage fragments establishes the structure of methyl penta-O-methyl-β-neoagarobioside (VI) as methyl 3-O-2′,4′-di-O-methyl-3′,6′-anhydro-L-galactopyranosyl-2,4,6-tri-O-methyl-β-D-galactopyranoside, and hence the structure of neoagarobiose (I) as 3-O-3′,6′-anhydro-L-galactopyranosyl-D-galactose.

The glycoside linkage has been suggested to be of the α'-type on the following basis. The specific rotations of α- and β-forms of methyl 3,6-anhydro-L-galactopyranoside would be in the neighborhood of $-80°$ and $+115°$, respectively, in water, the inference being drawn from the values recorded for their enantiomorphs[12]. If the 3,6-anhydro-L-galactopyranosyl group is combined through a β-type likage with D-galactose, which proper has a positive optical rotation, the resulting disaccharide would have a highly positive optical rotation. Meanwhile, if they are combined with each other through an α-type linkage, the resulting disaccharide would have a relatively low value of optical rotation, as a result of the compensation between positive and negative rotatory values of the components. The specific rotation of neoagarobiose ($[\alpha]_D +34.4° \rightarrow +20.3°$) seems to fall in with the latter instance. The specific rotation of neoagarobitol (IV) ($[\alpha]_D^{aq.} -20.0°$) also supports the α'-configuration on a similar consideration. In addition, the downward mutarotation observed when neoagarobiose is dissolved in water indicates that it crystallizes with the α-configuration.

Experimental

Evaporation and concentration were carried out under reduced pressure below 40°. All the melting points are uncorrected. The optical rotations are determined in aqueous solutions unless otherwise stated.

Preparation of Enzyme Extract.—A small amount of *P. kyotoensis* was inoculated to sterized media (50 cc.) having the following composition: sodium chloride 0.1%, dipotassium phosphate 0.1%, potassium nitrate 0.1%, magnesium sulfate heptahydrate 0.05%, and agar 0.5%; pH 7, adjusted with a 0.1 N-sodium hydroxide solution. The inoculated media were incubated at 30° for three days, and were diluted with the same media (400 cc.), the pH being again adjusted to 7 with a 0.1 N-sodium hydroxide solution. After incubation at 30° for a week, toluene (3 cc.) was added, and the mixture was digested at 30° for

11) R.L. Whistler and D.F. Durso, *J. Am. Chem. Soc.*, 72, 667 (1950).

12) W.N. Haworth, J. Jackson and F. Smith, *J. Chem. Soc.*, 1940, 620.

TABLE I

CHROMATOGRAPHIC SEPARATION OF ENZYMATIC HYDROLYSATES OF AGAR-AGAR

Ethanol	%	0	5	7.5	10	15	17.5	22	30	40	50
	l.	2.5	1.2	3.3	1.8	1.1	4.2	2.8	3.0	1.5	2.7
Yield	g.	0.67	0.1	3.7	0.15	0.15	5.3	0.6	1.63	0.65	0.40
$[\alpha]_D^{\circ}$ (H_2O)		—	+2.8	+17.7	+6.6	+6.8	+1.1	−9.1	−7.9	−16.9	−15.5

sixteen hours with occasional shaking. It was then filtered through an asbestos-bed, concentrated to 50 cc. below 35°, and was dialysed in a fish skin sack against running water until chlorine ions and reducing sugars were diffused (thirteen hours). The dialysed contents (110~130 cc.) were used as the enzyme extract to hydrolyse agar.

Enzymatic Hydrolysis of Agar.—Agar powder (5.5 g., or 5.27 g. as dry material), which had been defatted with acetone, was dissolved in hot water (1400 cc.) and sterized in steam, the pH of the solution being 5.2. After cooling, the enzyme extract (110–130 cc.) and three drops of toluene were added, and the mixture was kept at 40° in an incubator for about seventy two hours, when the reducing power reached a maximum value. After being heated in steam for fifteen minutes, it was filtered through double filter papers and concentrated to a sirup, to which methanol was added so that the concentration became 85 %. Insoluble precipitates were removed by filtration and washing with 85 % methanol, and the filtrate and washings were concentrated to a sirup (about 5 g.). The sirup was combined with three similar batches and extracted with hot 95 % methanol. Evaporation of the extract gave a mixture of reducing sugars as a pale brown sirup; yield 18–19 g. (moisture 10.6 %), or 71–81 % of agar in weight; $[\alpha]_D$+5.3° (*c* 1); reducing power, 26.1 % of galactose; aldose value, 43.0 % of hexose.

Paper Chromatography of Hydrolysates.—An aliquote of the above sirup was analysed on paper chromatograms (Tōyō Filter Paper No. 52) by the usual ascending method. *n*-Butanol-acetic acid-water (4:1:2 v/v) was used as an irrigating solvent and an ethanolic solution of O-aminophenol phosphate was sprayed[13]. Four yellowish brown spots were revealed: I (R_{gal} 1.30), II (R_{gal} 0.62), III (R_{gal} 0.34) and IV (R_{gal} 0.15), where R_{gal} is the rate of movement relative to that of galactose. The color of the spots presumably indicates the presence of 3,6-anhydro-galactose component.

Column Chromatography of Hydrolysates. A 10 % aqueous solution of the hydrolysates (13.4 g) obtained above was placed on a column (6.0×19.0 cm.) of active carbon Shirasagi[14] (Celite was not mixed with it), and the column was eluted with water, 5, 7.5, 10, 15, 17.5, 22, 30, 40 and 50 % aqueous ethanol in succession. Aliquotes of every 100 cc. portion of the eluates were withdrawn for examination of reducing power, and the next higher developer was added, after several continuous portions were recognized to be non-reducing. Each eluate was separately evaporated to dryness to recover the material. The result is quoted in Table I.

The water eluate left inorganic substances on evaporation. The 7.5, 17.5 and 30% ethanol eluates were chromatographically pure fractions, showing R_{gal} values 1.30, 0.62 and 0.34, respectively. Both 40 and 50% ethanol eluates were shown by paper chromatography to be mixtures of two components, whose R_{gal} values were 0.34 and 0.15. Solely the 7.5% ethanol fraction is treated in this communication.

Isolation of Neoagarobiose(I).—The 7.5% ethanol fraction was a colorless amorphous powder, which amounted to 28% of the hydrolysates. One gram of it was dissolved in a few drops of hot water, methanol was added while heating, and insoluble precipitates were removed by filtration. Crystallization of neoagarobiose was facilitated by addition of further methanol to the filtrate while heating. After standing overnight in a refrigerator, crystals were filtered, washed with methanol and ether, and dried; yield 0.85 g.; m.p. 197–198°. Recrystallization was repeated several times by dissolving it in a small amount of water and adding ethanol, until no more change of the physical constants was recognized; m.p. 207–208°; $[\alpha]_D^9$+34.4° (initial)→+20.3° (after twenty fuor hours, *c* 2.0); reducing power, 40.6% of galactose.

Anal. Found: C, 44.46; H, 6.20%; aldose equiv., 325., Calcd. for $C_{12}H_{20}O_{10}$: C, 44.44; H, 6.22%; aldose equiv., 324.

Neoagarobiose Phenylosazone.—A mixture of neoagarobiose (0.5 g.), phenylhydrazine (0.75 g.) and 50% acetic acid (0.75 cc.) in water (25 cc.) was heated at 90° for two hours. After cooling, crystals of the osazone were filtered, washed successively with water, ethanol, and ether, and dried; yield 0.35 g. The pure osazone was obtained as yellow needles on recrystallization from ethanol; m.p. 199–200°; $[\alpha]_D^9$+59.8° (initial)→+56.1° (after twenty four hours, *c* 0.82) in pyridine-methanol (2:3).

Anal. Found: C, 57.25; H, 6.12; N, 11.37. Calcd. for $C_{24}H_{30}O_8N_4$: C, 57.36; H, 6.02; N, 11.15%.

Hydrolysis of Neoagarobiose with 0.02N-Oxalic Acid Solution.—Neoagarobiose (0.60 g.) in a 0.02 N-oxalic acid solution (30 cc.) was heated in a boiling water bath. The progress of the reaction was followed by determination of the optical rotation of the solution; $[\alpha]_D$+17.5° (initial), +15.0° (15 min.), +14.5° (30 min.), +12.5° (1 hr.), +8.0° (2 hr.), +6.0° (2.5 and 3 hr., constant). After three hours, the solution was neutralized with calcium carbonate, filtered, and

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

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

concentrated to a sirup (0.55 g.). Paper chromatographic examination revealed a distinct spot of galactose together with a diffused tailing presumably of 3,6-anhydro-galactose. This indicative evidence was made conclusive by their isolations. A 10% aqueous solution of the sirup was placed on a charcoal column and developed with water. Every 30 cc. of the eluate was determined for the optical rotation, and fractions with positive and negative rotations were separately combined and concentrated to dryness.

a) D-Galactose.—From the first eight fractions with positive rotations, there was obtained a sirup (0.35 g.), from which D-galactose was crystallized; yield 0.25 g.; m.p. and mixed m.p. with an authentic specimen 166–167°; $[\alpha]_D^{26}$+81.5° (after twenty fourhours *c* 1.5). Nitric acid oxidation afforded mucic acid (m.p. 213°).

b) 3,6-Anhydro-L-galactose.—Next, eleven fractions with negative rotations gave on evaporation a sirupy 3,6-anhydro-L-galactose (0.18 g.). It reduced Fehling's solution at room temperature, and showed a strong Seliwanoff's reaction. Treatment with phenylhydrazine and acetic acid in the usual manner afforded 3,6-anhydro-L-galactose phenylosazone; m.p. and mixed m.p. with an authentic specimen[15] 217–218°.

Anal. Found: N, 16.23. Calcd. for $C_{18}H_{20}O_3N_4$: N, 16.46%.

Methanolysis of Neoagarobiose.—Neoagarobiose (1.0 g.) in 2% methanolic hydrogen chloride (50 cc) was boiled until the optical rotation became constant: $[\alpha]_D$+16.0° (initial), −38.0° (15 min.), −41.0° (30 min.), −35.0° (1 hr.), −30.0° (1.5 and 2 hr.). The solution was neutralized with silver carbonate, filtered, and concentrated to a sirup (1.1 g.). Paper chromatographic examination indicated that it was a mixture of 3,6-anhydrogalactose dimethylacetal and methyl galactoside. The mixture was chromatographed on a starch column exactly in the same way as described by Araki and Hirase[1]. 3,6-Anhydro-galactose dimethylacetal was eluted with *n*-butanol saturated with water, and then methyl galactoside was eluted with 80% aqueous methanol.

a) 3,6-Anhydro-L-galactose Dimethylacetal (II).—Evaporation of butanolic eluate gave 3,6-anhydro-L-galactose dimethylacetal as a colorless sirup; yield 0.50 g.; $[\alpha]_D^{12}$−24.6° (*c* 1.05); OCH_3, found 26.2 (calculated for $C_6H_{10}O_4(OCH_3)_2$ 29.8%). Identification was carried out by hydrolysing it to the free sugar, which was then converted into the diphenylhydrazone in the usual manner[15], m.p. and mixed m.p. with and authentic specimen 153°; $[\alpha]_D^{22}$−34.6° (initial, methanol, *c* 1.21).

Anal. Found: N, 8.67. Calcd for a $C_{18}H_{20}O_4N_2$: N, 8.53%.

b) Methyl D-Galactoside.—Evaporation of the 80% methanal eluate gave a sirup (0.54 g.), which on crystallization from acetone-methanol (2:1) afforded crystals of methyl α-D-galactoside monohydrate; yield 0.35 g.; m.p. 109°, not depressed on admixture with an authentic specimen; $[\alpha]_D^{28}$+174.1° (*c* 1.1).

Anal. Found: C, 39.25; H, 7.59; OCH_3, 14.60. Calcd. for $C_7H_{14}O_6 \cdot H_2O$: C, 39.60; H, 7.60; OCH_3, 14.62%.

Hydrolysis with N-sulfuric acid gave D-galactose, m.p. and mixed m.p. 168°; $[\alpha]_D^{27}$+81.5° (after twenty four hours, *c* 1.5).

Reduction and Methanolysis of Neoagarobiose.—Neoagarobiose (1.0 g.) in water (20 cc.) was mixed with an aqueous solution (10 cc.) of sodium borohydride (0.15 g.). The mixture was kept at room temperature (10–13°) with occasional shaking for two hours, when it showed no longer the reducing power. The solution was then ice-cooled, carefully neutralized with an 1.5% aqueous acetic acid, and concentrated to dryness. Crude neoagarobitol (IV) was obtained as an amorphous solid; yield 1.2 g.; $[\alpha]_D^{16}$−20.0° (*c* 1.25).

The glycitol (1.15 g.) was heated in boiling 4% methanolic hydrogen chloride (50 cc.) until a constant optical rotation was observed: $[\alpha]_D$−28.3° (initial), −22.6° (15 min.), −21.7° (30 min.), −20.9° (45 min.), −20.4° (1 hr.), −21.3° (2 and 3 hr.). The solution was neutralized with silver carbonate, filtered, and concentrated to give a semi-crystalline residue.

a) Dulcitol (V).—The above residue was triturated with methanol. Crystals of dulcitol (0.35 g.), separated by filtration, were recrystallized from ethanol-water (1:1); m.p. and mixed m.p. with an authentic specimen 187–188°; $[\alpha]_D^{19}$±0° (*c* 1.37).

Anal. Found: C, 39.66; H, 8.06. Calcd. for $C_6H_{14}O_6$: C, 39.56; H, 7.75%.

b) 3,6-Anhydro-L-galactose Dimethylacetal (II).—The filtrate separated from crystals of dulcitol was concentrated, and chromatographed on a starch column in the same manner as described before. 3,6-Anhydro-L-galactose dimethylacetal was obtained as a colorless sirup; yield 0.48 g.; $[\alpha]_D^{17}$−25.3° (*c* 1.2); OCH_3, found 28.4% (calcd. for $C_8H_{14}O_6$ 29.8%).

An additional specimen of dulcitol (0.12 g.) was also recovered from the column.

Hexa-O-acetyl-neoagarobiose. — Neoagarobiose (1.0 g.) was acetylated with pyridine (25 cc.) and acetic anhydride (7.0 g.) for seventy two hours in the usual manner. The reaction mixture was poured into ice-water (100 cc.) with stirring, neutralized with sodium bicarbonate, and extracted with chloroform. The extract was washed twice with water, dried with sodium sulfate, and evaporated. The hexaacetate (0.70 g.) obtained was recrystallized from ethanol; yield 0.46 g.; m.p. 112°; $[\alpha]_D^{27}$+1.57° (chloroform, *c* 1.27); $[\alpha]_D^{29}$+0.82° (methanol, *c* 1.22).

Anal. Found: C, 49.75; H, 5.80; CH_3CO, 45.01. Calcd. for $C_{12}H_{14}O_{10}(CH_3CO)_6$: C, 50.00; H, 5.60; CH_3CO, 44.80%.

Methyl Penta-O-methyl-β-neoagarobioside

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

(VI).—A 32% potassium hydroxide solution (38 cc.) and dimethylsulfate (16 cc.) were alternatively dropped during eight hours into an ice-cooled and vigorously stirred solution of neoagarobiose (2.0 g.) in water (7 cc.), care being taken to keep the medium alkaline. More potassium hydroxide solution (5 cc.) was added, and the solution was cooled and stirred for two hours. After standing overnight, the potassium hydroxide solution (106 cc.) and dimethylsulfate (47 cc.) were run in during twenty hours at room temperature (23–25°), the same care being taken. After the addition, the solution was stirred for ten additional hours, and then extracted with four 50 cc. portions of chloroform. The extracts were dried with sodium sulfate, evaporated, redissolved in ether, and again evaporated, when a crystalline mass was obtained; yield 2.16 g.; OCH_3 42.8%. This was twice methylated with methyl iodide (25 g.) and silver oxide (15 g.) in the usual manner. The final product was isolated as a crystalline mass by extraction with ether and evaporation; yield 2.20 g., OCH_3 46.0%. Recrystallization from petroleum ether (b.p. 38–43°) gave a pure sample of methyl penta-O-methyl-β-neoagarobioside; m.p. 127–128°; $[\alpha]_D^{30}$ −19.6° (methanol, *c* 1.28); $[\alpha]_D^{30}$ −22.0° (chloroform, *c* 1.28).

Anal. Found: C, 52.89; H, 8.12; OCH_3, 45.45%; M.W. (Rast's), 408. Calcd. for $C_{12}H_{14}O_4(OCH_3)_6$: C, 52.93; H, 7.90; OCH_3, 45.62%; M.W., 408.

Hydrolysis of Methyl Penta-O-methyl-β-neoagarobioside.—The sample (2.0 g.) in 0.02N-sulfuric acid (100 cc.) was heated in a boiling water bath until the optical rotation reached a constant value: $[\alpha]_D$ −18.0° (initial), −16.0° (15 min.), −15.0° (30 min.), −13.5° (45 min.), −12.5° (1 hr.), −3.5° (1.5 hr.), −1.5° (2 hr.), +1.0° (2.5 hr.), +2.0° (3 and 4 hr., constant). It was neutralized with barium carbonate, filtered, concentrated, redissolved in ether, and again evaporated, when a mixture of methyl 2,4,6-tri-O-methyl-β-D-galactoside and 2,4-di-O-methyl-3,6,-anhydro-L-galactose was obtained as a semi-crystalline residue (1.68 g.).

Methyl 2,4,6-Tri-O-methyl-β-D-galactoside (VIII).—The above mixture was extracted several times with boiling petroleum ether, an insoluble sirup (0.5 g.) being set aside. Evaporation of the extracts afforded methyl 2,4,6-tri-*O*-methyl-β-D-galactoside as a crystalline mass (1.10 g., OCH_3 47.1%). Recrystallization twice from petroleum ether gave pure silky needles (0.37 g.); m.p. 110–112°; $[\alpha]_D^{14}$ +2.90° (*c* 1.71). Araki[15] recorded m.p. 110–112° and $[\alpha]_D^{22}$ +6.38°.

Anal. Found: C, 50.61; H, 8.56; OCH_3, 51.97. Calcd. for $C_6H_8O_2(OCH_3)_4$: C, 50.85: H, 8.47; OCH_3, 52.54%.

An additional crop (0.50 g., OCH_3 50.2%) was recovered by evaporating the mother liquor of the recrystallization and extracting again with boiling petroleum ether. A petroleum ether-insoluble sirup (0.22 g.) was also obtained.

2,4,6-Tri-O-methyl-D-galactose (X).—The recovered crystals of methyl 2,4,6-tri-O-methyl-β-D-galactoside (0.50 g.) were hydrolysed with N-sulfuric acid for three hours in the usual manner. 2,4,6-Tri-O-methyl-α-D-galactose (0.45g.) was obtained as crystals, and recrystallized from ether; m.p. 113–114°, not depressed on admixture with an authentic specimen[16]; $[\alpha]_D^{24}$ +112.6° (initial)→+90.9° (after twenty four hours, *c* 1.2).

Anal. Found: C, 48.90; H, 8.04 OCH_3, 42.00. Calc for $C_6H_9O_3(OCH_3)_3$: C, 48.65; H, 8.11; OCH_3, 41.89%.

Anilide: m.p. 172–173°, not depressed on admixture with an authentic specimen[13]; $[\alpha]_D^{24}$ −84.0° (initial)→−44.8° (after twenty four hours)→+2.8° (after fourty eight hours, *c* 0.45) in acetone.

Anal. Found: N, 4.80; OCH_3, 30.20. Calcd. for $C_{15}H_{23}O_5N$: N, 4.71; OCH_3, 31.31%.

2,4-Di-O-methyl-3,6-anhydro-L-galactose (VII).—Petroleum ether-insoluble residue, separated from methyl 2,4,6-tri-O-methyl-β-D-galactoside, was proved to be 2,4-di-O-methyl-3,6-anhydro-L-galoctose[17]; a pale yellow sirup; yield 0.72 g., n_D^{25} 1.4871; $[\alpha]_D^{23}$ −6.9° (*c* 0.88); OCH_3 31.1% (Calcd. for $C_6H_8O_3(OCH_3)_2$ 32.64%). It reduced a Fehling's solution at room temperature, decolorized a neutral permanganate solution, showed a strong Seliwanoff's reaction, and restored the color to a Schiff's reagent.

2,4-Di-O-methyl-3,6-anhydro-L-galactonic Acid (IX).—Bromine (0.75 cc.) was dropped during five hours into an ice-cooled and vigorously stirred aqueous solution (30 cc.) of 2,4-di-O-methyl-3,6-anhydro-L-galactose (0.50 g.), in the presence of barium carbonate (6 g.). The stirring and cooling were continued for three more hours. After standing overnight, the non-reducing reaction mixture was aerated to remove excess bromine, filtered, and concentrated to a sirup, which was extracted with boiling chloroform. The residual barium salt was dissolved in water, and passed through a column of a cation exchange resin Amberlite IR-120, the column being then washed thoroughly with water. The acidic percolate and washings were combined, neutralized with silver carbonate, filtered before and after treatment with hydrogen sulfide, and concentrated to a sirup. Extraction with ethanol-benzene (1: 1) followed by evaporation afforded a semi-crystalline residue (0.30 g.), from which crystals of 2,4-di-O-methyl-3,6-anhydro-L-galactonic acid were separated by use of a porous plate; yield 0.22 g. The pure acid was obtained by recrystallization from ethanol-benzene (1: 1); m.p. 150°, not depressed on admixture with an authentic specimen[12,17]; $[\alpha]_D^{24}$ −60.8° (*c* 1.94).

Anal. Found: C, 46.47; H, 7.02; OCH_3, 29.8. Calcd. for $C_6H_8O_4(OCH_3)_2$: C, 46.58; H, 6.85; OCH_3, 30.12%.

Amide: The above acid was esterified with methanolic hydrogen chloride in the usual manner. The methyl ester obtained was converted into the amide in the usual manner; m.p. 150°, not

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

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

18) I.A. Forbes and E.G.V. Percival, *J. Chem. Soc.*, **1939**, 1844.

depressed on admixture with an authentic specimen[17,18]; $[\alpha]_D^{24}$ −74.0° (*c* 0.94).

Anal. Found: N, 7.00; OCH_3, 30.12. Calcd. for $C_8H_{15}O_5N$: N, 6.83; OCH_3, 30.25%.

Summary

1. A new crystalline disaccharide has been isolated from enzymatic hydrolysates of agar. Its hexaacetate and phenylosazone have been prepared in crystalline states.

2. The name neoagarobiose has been assigned to the disaccharide isolated.

3. The structure of neoagarobiose has been shown beyond doubt to be 3-O-3′,6′-anhydro-L-galactopyranosyl-D-galactose. It has been suggested that the glycoside linkage is of α'-configuration.

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