Studies on the Chemical Constitution of Agar-agar. XVII[1] *Isolation of Crystalline Agarobiose Dimethylacetal by Partial Methanolysis of Agar-agar*[2]

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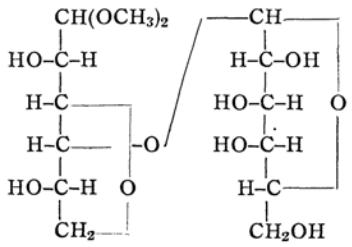
(Received October 14, 1953)

Constitutional studies on agar carried out in this laboratory have revealed that the chief component monosaccharides, so far as known hitherto, are D-galactose and 3,6-anhydro-L-galactose, and that these two are joined by 1-4-linkage to build the agarobiose[3] (4-D-galactopyranosyl 3,6-anhydro-L-galactose) unit in the molecule of agar. Moreover, it has been suggested that agarobiose units would extend over approximately half of the molecule of agar[1,4]. But difficulty was encountered in the quantitative treatment of the material in the preceding works, and consequently it has not been possible to estimate the precise content of agarobiose units. However, the partial methanolysis method, dealt with in the present paper, has proved to make possible not only the isolation of crystalline agarobiose dimethylacetal (I) but also its quantitative separation to some extent to be effected.

The partial methanolysis of agar was accomplished by treatment with boiling methanolic hydrogen chloride for a short period, a portion of agar still remaining undissolved.

The reaction products on solvent fractionation gave rise to a crystalline compound,



(I)

which was identified as agarobiose dimethylacetal (I) for the following reasons. Acetylation gave a crystalline hexaacetate, indicating the presence of six available hydroxyl groups in the molecule. Complete methanolysis produced methyl D-galactoside and 3,6-anhydro-L-galactose dimethylacetal[5] in equimolar proportion. Mild acid hydrolysis regenerated the reducing sugar, the phenylosazone of which was found to be identical with the well-established agarobiose phenylosazone.[3]

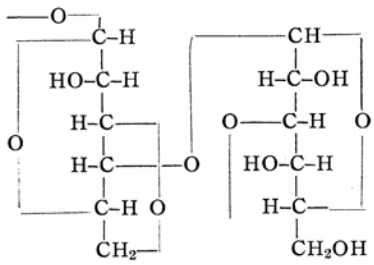
Further constitutional evidence was provided by the fact that, when authentic agarobiose diethylmercaptal[1] was submitted to demercaptalation by the action of mercuric chloride in absolute methanol, there was obtained a crystalline compound having the same characteristics as those of the compound under question. The direct formation[6] of a sugar acetal from the corresponding mercaptal can scarcely occur unless hydroxyl groups in the same molecule are protected by some substituents from the lactol formation. In the case above mentioned, however, the dimethylacetal resulted because the 3,6-anhydro-galactose residue in the agarobiose unit prefers[7] an open-chain structure to a lactol-ring structure on account of the steric hindrance caused by the preexistence of the 3,6-anhydro-ring.

The non-crystalline portion of the methanolysate was separated by means of partition chromatography on a column of starch, which led to the isolation of an additional specimen

1) Part XVI: S. Hirase and C. Araki, This Bulletin. **27**, 105 (1954).
2) Read at 6th Annual Meeting of the Chemical Society of Japan held at Tokyo in 1953.
3) C, Araki, *J. Chem. Soc. Japan*, **65**, 533, 627 (1944).
4) C. Araki, *Memoirs of the Faculty of Industrial Arts, Kyoto Technical Univ., Science and Technology Edition*, **2** (B), 46 (1953).
5) C. Araki, *J. Chem. Soc. Japan*, **65**, 725 (1944).
6) J. W. Green and E. Pacsu, *J. Am. Chem. Soc.*, **60**, 2288 (1938).
7) S. Pea , Advances in Carbohydrate Chemistry, **2**, 37 (1946).

of agarobiose dimethylacetal, together with 3,6-anhydro-L-galactose dimethylacetal and methyl D-galactoside. Thus the total yield of agarobiose units, including not only the yield of agarobiose dimethylacetal itself but also those of advanced methanolytic products, namely, of 3,6-anhydro-L-galactose dimethylacetal and methyl D-galactoside, amounted to 69.5% of the reacted agar, when calculated as the parent free sugar.

Although the chromatographic separation has been unsatisfactory, this result, along with the fact that D-galactose residues are united to other residues at 1,3-positions,[8] and that 3,6-anhydro-L-galactose residues are present as the pyranose form[9] in the molecule of agar, brings us to the conclusion that such constitutional units as illustrated in formula II would extend over approximately two-thirds of the agar molecule. Consequently the constitutional formula for agar, proposed by Jones and Peat[10] in 1942, represents by no means the real feature of the molecule.



(II)

Experimental

Evaporation and concentration were carried out under reduced pressure below 40°. The specific rotation was measured in an aqueous solution unless otherwise stated. All melting points are uncorrected.

Partial Methanolysis of Area. Commercial powdered agar (50 g., dry weight 41.9 g.) was refluxed with 1% (w/v) methanolic hydrogen chloride (500 cc.) for 1.5 hrs. After cooling, the undissolved agar (3.3 g.) was removed by filtration, and the filtrate was submitted to neutralization with silver carbonate followed by filtration and subsequent evaporation. The resulting sirup was treated with 0.2N aqueous barium hydroxide solution (500 cc.) at 60° for 2 hrs. The solution was then neutralized with carbon dioxide, filtered and concentrated to a sirup, which was dried by repeated additions of absolute methanol followed by subsequent evaporation to give rise to an amorphous powder (44.0 g.).

Solvent Fractionation of the Methanolysate. The methanolysate thus obtained was dissolved in absolute methanol (120 cc.), and then *n*-butanol (600 cc.) was added with vigorous shaking. The voluminous precipitate (17.4 g.) formed was filtered, washed first with *n*-butanol and then with ethyl acetate. The combined filtrate and washings were concentrated to dryness, when an amorphous powder (fraction A) was obtained; yield 26.5 g., $[\alpha]_D^{14}$ −13.0°. The precipitate was redissolved in absolute methanol (30 cc.), and then absolute ethanol (150 cc.) was added with shaking. The precipitate formed was again filtered, washed with methanol-ethanol (1:5) and dried, when a hygroscopic white powder (fraction C)was obtained; yield 11.9 g. (30.8% of the reacted agar), $[\alpha]_D^{13}$ −22.8°, OCH_3 found 7.58%. The filtrate and washings were combined and concentrated to dryness, when an amorphous powder (fraction B) was obtained; yield 5.5 g. (14.3% of the reacted agar), $[\alpha]_D^{13}$ −18.1°, OCH_3 found 15.14%.

Isolation of Agarobiose Dimethylacetal. The fraction A was dissolved in absolute ethanol (30 cc.), and acetone (60 cc.) was added, and the transparent solution was left in an ice box for a week. The deposited crystals of agarobiose dimethylacetal were filtered and washed first with ethanol-acetone (1:2) and then with absolute ethanol; yield 13.3 g. (30.2% of the reacted agar, calculated as its parent free sugar), m. p. 160~163°. The combined filtrate and washings upon concentration to dryness gave an amorphous powder (fraction A'); Yield 13.1 g. (33.9% of the reacted agar), $[\alpha]_D^{14}$ +0.65°, OCH_3 found 20.53%. Pure agarobiose dimethylacetal was obtained as prisms on recrystallization of the crude one from absolute ethanol; m. p. 162~164°, $[\alpha]_D^{10}$ − 29.1° (c 1.58) and −37.4° in methanol (c 1.10). It is readily soluble in water and methanol, and scarcely soluble in cold ethanol. *Anal.* Found: C, 45.69; H, 7.23; OCH_3, 16.69. Calcd. for $C_{14}H_{25}O_{11}$: C, 45.46; H, 7.09; OCH_3, 16.76%.

Hexaacetyl Agarobiose Dimethylacetal. Agarobiose dimethylacetal (1.0 g.) was acetylated with pyridine and acetic anhydride in the usual way. Crude crystals were deposited when the reaction mixture was poured into ice-water; yield 1.6 g., m. p. 84~86°. Recrystallization twice from methanol-water (1:2) gave flakes of the pure substance; m.p. 87~88°, $[\alpha]_D^{13}$ −5.76° in chloroform (c 1.22) and −12.5° in benzene (*c* 1.20). *Anal.* Found: C, 50.09; H, 6.00; CH_3CO, 41.30. Calcd. for $C_{12}H_{14}O_9(CH_3CO)_5(OCH_3)_2$: C, 50.16; H, 6.15; CH_3CO, 41.49%.

Methanolysis of Agarobiose Dimethylacetal. Agarobiose dimethylacetal (2.0 g.) was refluxed with 2% methanolic hydrogen chloride (100 cc.), the reaction being followed polarimetrically: $[\alpha]_D$ −38.5° (initial); −6.0° (1 hr.); +10.0° (3 hrs.); +18.0° (10 hrs.); +21.5° (15 hrs.); +22.5° (20 hrs.); +23.0° (25, 30 and 35 hrs.). After 35 hrs. the reaction solution was neutralized with silver

8) C. Araki, *J. Chem. Soc. Japan*, **58**, 1362 (1937); E. G. V. Percival and J. C. Somerville, *J. Chem. Soc.*, **1937**, 1615.
9) C. Araki, *J. Chem. Soc. Japan*, **61**, 775 (1940).
10) W. G. M. Jones and S. Peat, *J. Chem. Soc.*, **1942**, 225.

carbonate, filtered and concentrated to a sirup (2.0 g.), which was shown by paper chromatography to be a mixture of 3, 6-anhydrogalactose dimethylacetal and methyl galactoside. This indicative evidence was confirmed by the isolation of each component by means of the chromatographic separation on a starch column, at first *n*-butanol saturated with water and then 80% methanol being used as a mobile phase. 3,6-Anhydro-L-galactose dimethylacetal was obtained on solvent removal of the butanol effluent as a colorless sirup: yield 46.7% of the sample used, $[\alpha]_D^{10}$ −28.7° (*c* 0.76), OCH_3 found 25.8%. Its structure was confirmed by hydrolysing it to free sugar and then converting the latter to the crystalline diphenylhydrazone in the usual way[5]; m.p. and mixed m.p. 154°.

Methyl D-galactoside, obtained on evaporation of the 80% methanol effluent in a yield of 53.6% of the sample used, was hydrolysed with N-sulfuric acid in the usual way to give free D-galactose, m.p. and mixed m.p. 165°, $[\alpha]_D^{16}$ +81.9° (equilibrium, *c* 0.56).

Hydrolysis of Agarobiose Dimethylacetal. Agarobiose dimethylacetal (5.0 g.) was hydrolysed with 0.01 N-aqueous oxalic acid solution (50 cc.) in a boiling water bath, the reaction being followed polarimetrically; $[\alpha]_D$ −29.0° (initial); −21.0° (0.5 hr.); −18.5° (1.0 hr.); −17.5° (1.5 and 2.0 hrs.). After 2 hrs. the solution was neutralized by means of an anion exchange resin Amberlite IR-4B, and then evaporated to a sirup, which was dried over phosphorus pentoxide in vacuum to form a hygroscopic powder of agarobiose; yield 4.4 g., $[\alpha]_D^{23}$ −17.4° (initial, c2.18)→−14.2° (equilibrium). Crystallization from methanol, ethanol, acetic acid, or from combined solvent systems of them was unsuccessful.

Phenylosazone: m.p. 220~221° (decomposition), not depressed on admixture with an authentic specimen[1,3], $[\alpha]_D^2$ −136.8 (initial)→−108.8° (after 24 hrs.) in pyridine-ethanol (2:3) (*c* 0.432).

Agarobiose Dimethylacetal from the Corresponding Diethylmercaptal. Agarobiose diethylmercaptal[1] (3.0 g.) in absolute methanol (60 cc.) was treated with mercuric chloride (5.6 g.) and yellow mercuric oxide (6 g.), according to the procedure of Green and Pacsu[11] for the preparation of thioglycosides. The resulting product was a colorless sirup, which gave crystals of agarobiose dimethylacetal on crystallization from ethanol-acetone (1:2); yield 1.1 g., m.p. 161~163°. Recrystallization from ethanol afforded a pure substance, m.p. 162~164°, not depressed on admixture with the specimen isolated from the agar-methanolysate described above; $[\alpha]_D^{17}$ −28.9° (*c* 1.21), OCH_3 found 16.62 (calcd. for $C_{14}H_{26}O_{11}$ 16.76%),

The mother liquor separated from the crude crystals was concentrated to a sirup (1.2 g.); $[\alpha]_D^{17}$ −23.2° (*c* 1.11), OCH_3 found 16.25%. Paper chromatographic examination revealed the spot of agarobiose dimethylacetal only.

Examination of the Fraction A', B and C on Paper Chromatograms. A small portion of each of the fraction A', B and C was examined by paper chromatography, using *n*-butanol-ethanol-water (4:1:2) as a mobile phase and *o*-aminophenol as a color-developer[12]. All of the revealed spots showed a yellow coloration caused by 3,6-anhydrogalactose. Thus, the fraction A' gave the spots of 3,6-anhydrogalactose dimethylacetal (*Rf* 0.66), an unidentified compound (a trace, *Rf* 0.43) and of agarobiose dimethylacetal (*Rf* 0.34). The fraction B showed the spots of agarobiose dimethylacetal and unidentifiable compounds (*Rf* 0.43 and 0.19). The fraction C showed the spots of agarobiose dimethylacetal (a trace) and unidentified, probably higher oligosaccharide derivatives (*Rf* 0.19 and those less than 0.19). Methyl galactoside was detected nowhere, although expected to be present, as it moves to the position (*Rf* 0.31) near the agarobiose derivative in the above condition. The same results were obtained when paper strips were sprayed with a lead tetraacetate reagent[13].

Separation of the Fraction A', B and C on Starch Columns. General Procedure:—The sample to be separated was dissolved in *n*-butanol nearly saturated with water (if necessary, a small amount of methanol was added), and placed on the top of the chromatographic column, which had been prepared by pouring a thick slurry of potato starch in *n*-butanol saturated with water into a glass tube and allowing the excess solvent to percolate down. The column was then washed with *n*-butanol saturated with water. Small portions of the effluent were examined on the paper chromatograms in the same way as described in the preceding section, and the effluent was divided into several sub-fractions in such a manner as to lead to the highest possible recovery of each component. After agarobiose dimethylacetal had been completely washed out, the column was then washed with 80% aqueous methanol until nothing came into the effluent. The sub-fractions thus obtained were separately evaporated to dryness.

The Fraction A':—The sample (799 mg.) was separated on a column (4×18 cm.) into four sub-fractions listed in Table I. The sub-fraction A'

Table I. The chromatographic separation of the fraction A'

Sub-fraction	Yield %a	Yield %b	$[\alpha]_D^{15}$	*Rf* value
A'—I	18.5	4.9	−25.0°	0.67
A'—II	7.2	—	−12.0°	0.67, 0.43, 0.34
A'—III	50.1	14.9	−15.2°	0.34
A'—IV	20.0	6.3	+45.3°	(0.31)

a. of the sample used, by weight.
b. of the reacted agar when calculated as the parent free sugar.

11) J. W. Green and E. Pacsu, *J. Am. Chem. Soc.*, **59**, 1205 (1937).

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

13) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, **1950**, 3162.

—I was confirmed to be 3,6-anhydro-L-galactose dimethylacetal by its *Rf* value and its hydrolysis to the free sugar followed by subsequent conversion to the crystalline diphenylhydrazone,[5] m.p. and mixed m.p. 154°. A′—II was a mixed fraction. A′—III was chromatographically pure agarobiose dimethylacetal, and when crystallized from ethanol-acetone, it gave a crystalline specimen, m.p. and mixed m.p. 161~163°, $[\alpha]_D^{17}$ −28.8° (*c* 0.67). A′—IV, a methanol effluent, gave only a faint spot on a paper chromatogram when sprayed with an *o*-aminophenol reagent, but it revealed an apparent zone moving to the same position (*Rf* 0.31) as α-methyl D-galactoside when sprayed with a lead tetraacetate reagent. When crystallized from ethanol, it gave crystalline α-methyl D-galactoside hydrate, m.p. and mixed m.p. 108~110°, $[\alpha]_D^{16}$ +175.8° (*c* 0.41). Thus A′—IV was considered to be mainly a mixture of α- and β-methyl D-galactoside.

The Fraction B:—The sample (799 mg.) was separated on a column (4×14 cm.) into three sub-fractions shown in Table II. The sub-fraction B—I was a mixed fraction. B—II was pure agarobiose dimethylacetal which crystallized completely; m.p. and mixed m.p. 161~163°, $[\alpha]_D^{16}$ −28.8°(c 0.66). B—III, a methanol effluent, was shown to contain an unidentified substance and a trace of methyl galactoside on paper chromatograms.

TABLE II THE CHROMATOGRAPHIC SEPARATION OF THE FRACTION B

Sub-fraction	Yield %a	Yield %b	$[\alpha]_D^{15}$	*Rf* value
B—I	3.9	—	−13.8°	0.43, 0.34
B—II	69.3	8.7	−24.2°	0.34
B—III	20.7	—	−3.4°	0.19, (0.31)

a. of the used sample, by weight.
b. of the reacted agar when calculated as the parent free sugar.

The Fraction C:— This was considered from its methoxyl content and its paper chromatographic examination to be chiefly composed of higher oligosaccharide derivatives. Hence, it was submitted again to mild methanolysis, and agarobiose dimethylacetal produced thereby was analysed: a portion of the fraction C (2.38 g.) was boiled with 1% methanolic hydrogen chloride (20 cc.) for 1.5 hrs., and the reaction solution was treated in the similar manner to that described for the methanolysis of agar. The product (2.40 g.) was divided into two parts, the ethanol-methanol (5 : 1)-soluble part (1.55 g.); $[\alpha]_D^{16}$ −10.3°, and the insoluble part (0.78 g.); $[\alpha]_D^{16}$ −22.9°. Paper chromatographic examination indicated that the former contained agarobiose derivative, but the latter did not. Separation of the former on a starch column exactly in the same way as described for the fraction A′ afforded the chromatographically pure sub-fraction of agarobiose dimethylacetal in a yield of 25.5% of the used sample (5.1% of the reacted agar, when calculated as its parent free sugar), from which a crystalline specimen could be isolated; m.p. and mixed m.p. 161—163° $[\alpha]_D^{17}$—28.5° (c 0.42). Other sub-fractions were shown paper-chromatographically to contain unidentified compounds, which will be studied later.

Thus, the total yield of agarobiose units was calculated by summing up the yield of agarobiose dimethylacetal (the crystalline specimen isolated from A, the sub-fraction A′—III and B—II, and a sub-fraction from C), 3,6—anhydro-L-galactose dimethylacetal (the sub-fraction A′—I) and of methyl D-galactoside (the sub-fraction A′—IV), namely, 69.5% of the reacted agar (38.6 g.) when each is calculated as its parent free sugar.

The authors wish to express their thanks to Mr. K. Arai for carrying out the microanalysis and to Mr. K. Yamanouchi for his assistance in this study. This research has been aided by the Grant for Fundamental Scientific Research from the Ministry of Education to whom thanks are also expressed.

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