Studies on the Chemical Constitution of Agar-agar. XIX[1]*. Pyruvic Acid as a Constituent of Agar-agar (Part 1). Identification and Estimation of Pyruvic Acid in the Hydrolysate of Agar*

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Agar is composed mainly of D-galactose and 3,6-anhydro-L-galactose. Partial depolymerization by means of acid hydrolysis[2], mercaptolysis[3] and methanolysis[4] gave rise to agarobiose(4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose) and its derivatives. Enzymatic hydrolysis[1] led to the isolation of neoagarobiose (3-O-3,6-anhydro-α-L-galactopyranosyl-D-galactose). From this and other evidences, Araki[5] recently suggested that 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose are alternately repeated to build up the chain of agarose, a principal polysaccharide of agar. Through further investigation, however, it has become probable that agar contains a small amount of pyruvic acid as a constituent.

A preliminary experiment involving the use of paper chromatography has indicated that pyruvic acid is produced in the hydrolysate of agar. For confirmation, agar was hydrolysed with very dilute hydrochloric acid, which caused little formation of degradation products of sugar components. Pyruvic acid liberated was easily isolated as its 2,4-dinitrophenylhydrazone though in a slight yield. The fact that pyruvic acid is set free with very dilute acid suggests that it would not

1) Part XVIII : C. Araki, and K. Arai, This Bulletin, **29**, 339 (1956).
2) C. Araki, *J. Chem. Soc. Japan.* **65**, 533, 627 (1944).
3) S. Hirase and C. Araki, This Bulletin, **27**, 105 (1954).
4) C. Araki and S. Hirase, ibid., **27**, 109 (1954).
5) C. Araki, ibid., **29**, 543 (1956).

be a secondary by-product, but an inherent constituent of agar, and also that, in the latter instance, it is engaged in the linkage susceptible to hydrolysis.

In order to examine the content of pyruvic acid, agar was subjected to hydrolysis with N-sulfuric acid. Pyruvic acid produced was completely extracted with ether, and the ether was then removed by distillation. Pyruvic acid obtained was contaminated by a large amount of formic acid, levulinic acid and hydroxymethylfurfural[6], the contaminants arising chiefly from 3,6-anhydro-L-galactose[7], and hence the 2,4-dinitrophenylhydrazine method could not be applied for the estimation of pyruvic acid. The method successfully employed in this study involves the oxidation with sodium metaperiodate at 65° followed by gravimetric determination of carbon dioxide liberated thereby:

$$CH_3COCOOH \xrightarrow{NaIO_4} CH_3COOH + CO_2$$

The content of pyruvic acid found by this method was 1.06% of agar by weight, or one residue for every fifty one hexose residues.

Since it has never been reported that pyruvic acid is present in a polysaccharide, further experiment has been made to examine any distribution of pyruvic acid in several polysaccharides other than agar. But it was detected in none of hydrolysates of mucilages of *Chondrus ocellatus Holmes* and *Gloiopeltis furcata*, gum arabic, apple pectin, konjak mannan and starch. Pyruvic acid seems to be a component peculiar to agar.

It may be of interest from the view point of biochemistry that agar contains pyruvic acid, because agar constitutes the chief cell wall structural material in several red sea weeds and pyruvic acid represents an important intermediate in the metabolism of carbohydrate, fat and protein.

Experimental

Isolation of Pyruvic Acid from Agar.—Commercial agar powder (10.0 g., moisture 17.31%) was hydrolysed with 0.1N-hydrochloric acid (100 cc.) in a boiling water bath for four hours. The solution was decolorized with active carbon, and 2,4-dinitrophenylhydrazine (1 g.) dissolved in warm 4N-hydrochloric acid (150 cc.) was added, when no precipitation occurred. After being kept overnight at room temperature, the solution was extracted four times with 50 cc. portion of ethyl acetate. The extracts were combined, washed once with water, and then treated five times with 10 cc. portion of 5% sodium carbonate solution. The carbonate extracts were combined, and ethyl acetate dissolved in it was removed by evaporation under reduced pressure at 40°. Acidification with concentrated hydrochloric acid under ice cooling afforded fine yellow crystals of pyruvic acid 2,4-dinitrophenylhydrazone, which was then collected on a glass filter, washed with dilute hydrochloric acid and water, and dried at 100° until a constant weight was reached. Yields in duplicate runs were 0.157 g. and 0.162 g., which were equivalent, respectively, to 0.0514 g. and 0.0532 g. of free pyruvic acid. The crystals obtained were nearly pure as indicated by m. p. 214°. Recrystallization from ethanol-ethyl acetate gave a pure specimen melting at 218°. The m. p. was not depressed on admixture with an authentic sample (m. p. 218°).

Anal. Found: C, 40.43; H, 3.34; N, 20.68. Calcd. for $C_9H_8O_6N_4$: C, 40.30; H, 3.01; N, 20.89 %.

Examination of Polysaccharides other than Agar.—Each 10 g. of mucilages of *Chondrus ocellatus Holmes* and *Gloiopeltis furcata*, gum arabic and apple pectin was hydrolysed with 0.1N-hydrochloric acid (100 cc.) in a boiling water bath for four hours. Konjak mannan and starch (each 10 g.) was hydrolysed with N-hydrochloric acid (100 cc.) for five hours. Each hydrolysate was treated with 2,4-dinitrophenylhydrazine exactly in the same manner as that described for agar. None of them afforded 2,4-dinitrophenylhydrazone of pyruvic acid.

Quantitative Determination of Pyruvic Acid.—*Apparatus and Procedure.*—The quantitative determination of carbon dioxide evolved from pyruvic acid during the periodate oxidation was carried out by passing a slow current of carbon dioxide-free air through the reaction flask to sweep the evolved gas into an adsorption vessel through a train assembled as follows: (1) a washing bottle containing a 30% potassium hydroxide solution, and an U-tube filled with soda-lime, (2) the reaction flask (an 100 cc. Erlenmeyer flask) with two-holed rubber stopper carrying the inlet and outlet tubes, (3) a trap kept at −10° for the removal of most of moisture, (4) an adsorption vessel charged with concentrated sulfuric acid for the prevention of any moisture and volatile organic material from getting into the next adsorption vessel, and (5) a weighed adsorption vessel charged with a 30% potassium hydroxide solution for the quantitative determination of carbon dioxide.

A sample containing 0.05-0.3 g. of pyruvic acid was dissolved in water (20 cc.) in the reaction flask. Crystals of sodium metaperiodate (1.0g.) were added, and the flask was immediately connected to the remainder of the apparatus. The mixture was then heated at 65°, while a slow stream of air was bubbled through the apparatus. The increase in weight of the carbon dioxide adsorption vessel was measured after two

6) C. Araki, *J. Chem. Soc. Japan*, **58**, 1214 (1937).
7) C. Araki and K. Arai, ibid., **63**, 1720 (1942).

hours, and the amount of pyruvic acid was calculated from the weight of carbon dioxide found.

Control Analysis. — Pyruvic acid alone and contaminated by arbitrary amounts of a mixture of formic acid, levulinic acid and hydroxymethylfurfural was analysed by the above method. These contaminants exhibited no harmful influence upon the results. Some typical results are given in Table I.

TABLE I

QUANTITATIVE DETERMINATION OF PYRUVIC ACID IN THE PRESENCE OF CONTAMINANTS

PA, g. taken	FA, LA and HMF, g. added	PA, g. found	Error, g.
0.1119	—	0.1123	+0.0004
0.1116	—	0.1105	−0.0011
0.3387	—	0.3377	−0.0010
0.1211	each 0.5	0.1215	+0.0004
0.1100	each 1.5	0.1145	+0.0045
0.1048	each 3.0	0.1065	+0.0017

PA: Pyruvic acid. FA: Formic acid.
LA: Levulinic acid. HMF: Hydroxymethylfurfural.

Quantitative Determination of Pyruvic Acid in the Hydrolysate of Agar.—Each 10 g. of agar powder (moisture 17.31%) was hydrolysed with N-sulfuric acid (100 cc.) in a boiling water bath for two, four and six hours, respectively. Humus substances formed were removed by filtration through a charcoal bed, and the filtrate was extracted with ether for thirty hours in a Soxhlet's apparatus. A control experiment indicated that the extraction was complete. Water (10 cc.) was added to the extract, and ether was then removed by careful distillation. The residual solution was quantitatively transferred to the reaction flask for analysis, and the pyruvic acid present in the solution (20 cc.) was determined by the method above described. The results obtained were 0.0777 g. in a two hours hydrolysate, 0.0853 g. and 0.0877 g. in a four hours hydrolysate, and 0.0885 g. and 0.0873 g. in a six hours hydrolysate, respectively. The average value excluding the result of two hours hydrolysis is 0.0872 g. or 1.06% of moisture-free agar by weight. This figure indicates the presence of one residue of pyruvic acid for every fifty one residues of hexose.

Summary

1. Pyruvic acid has been isolated as its 2,4-dinitrophenylhydrazone from the hydrolysate of agar for the first time.

2. Quantitative determination has indicated that the content of pyruvic acid is 1.06% of agar or one residue for every fifty one hexose residues.

3. It has been suggested that pyruvic acid would be an intrinsic constituent of agar.

4. Several polysaccharides other than agar have been examined, but none of them produced pyruvic acid on hydrolysis.

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