# *Studies on the Chemical Constitution of Agar-agar. XIX. Pyruvic Acid as a Constituent of Agar-agar (Part 2)*[1]*. Isolation of a Pyruvic Acid-linking Disaccharide Derivative from the Methanolysis Products of Agar*

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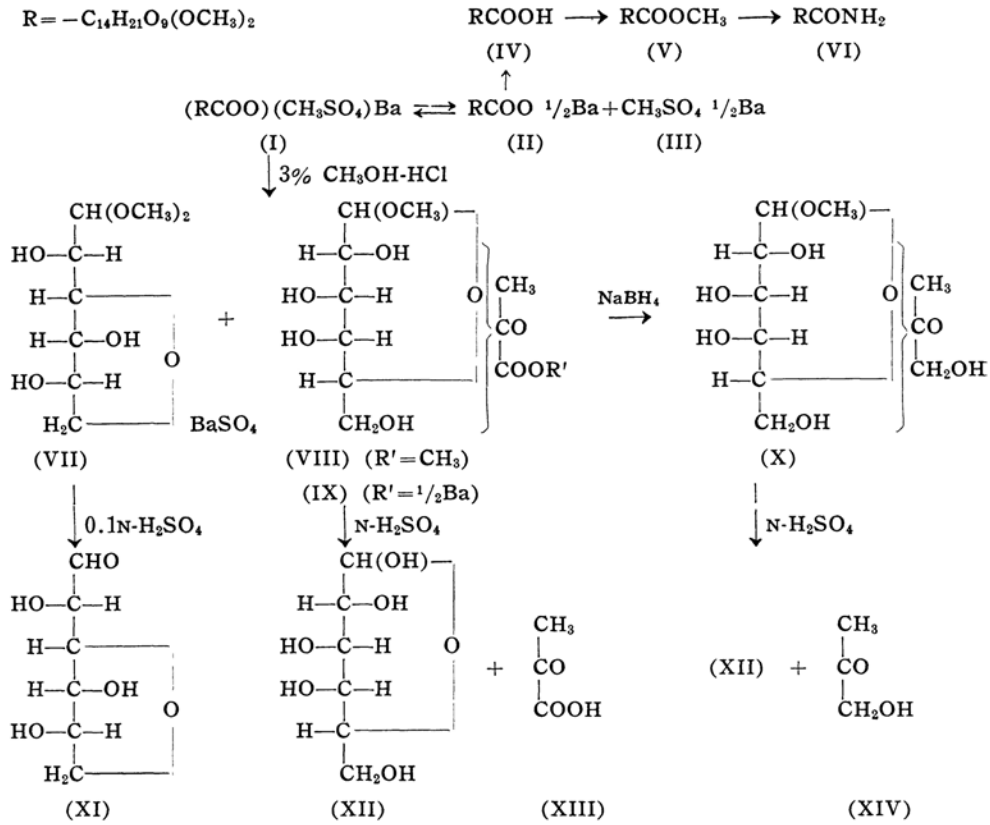
In the previous communication[1], it was suggested that pyruvic acid, produced in 1.06% yield by the hydrolysis of agar, would not be secondary by-product, but an intrinsic constituent. This suggestion has been substantiated herein by the isolation of a crystalline disaccharide derivative carrying a pyruvic acid residue in the molecule from the partial methanolysis products of agar.

The partial methanolysis of agar has been carried out by heating it in 0.5%

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




methanolic hydrogen chloride for two hours according to the method of Araki and the present writer[2]. The product was treated with barium hydroxide solution and passed through columns of cation and anion resins in succession. Acidic compounds, which were retained by the anion resin, were eluted by displacement with excess sulfuric acid. Neutralization with barium hydroxide followed by evaporation afforded a mixture of barium salts, from which a crystalline salt was isolated in 6.3% yield by weight.

The crystalline barium salt obtained has been proved to be monohydrates of a double salt (I) consisting of barium methylsulfate (III) and a barium salt of an organic acid (II). This follows from the fact that the double salt was separated into its two component salts II and III by means of an anion exchange resin. When II and III were separately dissolved in methanol and the solutions were mixed, the double salt was immediately regenerated. Methylsulfuric acid constituting a part of the double salt may have arisen from the sulfuric acid residues present in agar.

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

From the barium salt (II), there has been obtained a free acid (IV) having the formula $C_{14}H_{21}O_9(OCH_3)_2COOH$ as its crystalline monohydrates. The acid was converted into the methyl ester (V) by reaction with diazomethane, and the ester was further converted into the amide (VI) by reaction with ammonia. Both derivatives were obtained in a crystalline condition.

In order to examine the components of the acid (IV), the original double salt (I) was subjected to methanolysis. There were produced barium sulfate, 3,6-anhydro-L-galactose dimethylacetal (VII) and a methyl ester methyl glycoside (VIII), the last one being separated from the second through its barium salt (IX). 3,6-Anhydro-L-galactose dimethylacetal (VII) was hydrolysed to give a free sugar (XI), which was identified as its crystalline diphenylhydrazone. The barium salt methyl glycoside (IX) yielded upon hydrolysis D-galactose (XII) and pyruvic acid (XIII). D-Galactose was isolated as crystals, and pyruvic acid was identified as its semicarbazone, phenylhydrazone and *p*-nitrophenylhydrazone. This result indicates that the acid (IV) is a disaccharide derivative composed of 3,6-anhydro-L-

galactose and D-galactose, the latter component carrying a pyruvic acid residue.

On the other hand, the ester group of VIII was reduced to a hydroxyl group with sodium borohydride. Hydrolysis of the resulting product (X) yielded D-galactose (XII) and hydroxyacetone (XIV), the latter product being identified as its semicarbazone. This result demonstrates that the pyruvic acid residue is linked through its carbonyl group with the D-galactose residue.

The isolation of the compound (IV) verifies the view that pyruvic acid is a constituent of agar, because the mild treatment in its isolation completely excludes the possibility that pyruvic acid might be a secondary product derived from some unidentified compound. For instance, oxalacetic acid would be decarboxylated to give pyruvic acid on being heated in an aqueous medium, but it undergoes no decarboxylation on being treated with methanolic hydrogen chloride.

The compound (IV) represents the first example of a carbohydrate with which pyruvic acid is linked through its carbonyl group. The structure will b elater reported.

## Experimental

Unless otherwise stated, concentration and evaporation were carried out under reduced pressure below 40°. Melting points are uncorrected.

**Partial Methanolysis of Agar.**—Commercial agar powder (50 g., dry weight 41.9 g.) was suspended in 0.5% methanolic hydrogen chloride (500 cc.) and heated under reflux for two hours. The undissolved agar (5.5 g.) was removed by filtration, and the filtrate was neutralized with silver carbonate, refiltered and concentrated to a sirup (40.7 g.). It was then saponified by heating in a 0.2N-barium hydroxide solution (500 cc.) at 60° for two hours. Excess barium hydroxide was removed by neutralization with carbon dioxide and filtration, and the filtrate was passed through a column of Amberlite IR-120 (2.8×20 cm.) to remove cations, and then through a column of Amberlite IR-4B (2.8×25 cm.), where anions of acidic compounds were adsorbed. The columns were thoroughly washed with water (5 l.), and the neutral effluent was concentrated to a sirup (32.1 g.); $[\alpha]_D^{26}$-17.7° (water, *c* 1.64). This was a mixture of neutral sugar derivatives, which were not investigated in this communication.

**Isolation of the Double Salt (I).**—The above IR-4B resin, by which acidic compounds were retained, was stirred with N-sulfuric acid (300 cc.) under ice cooling for ten minutes, poured into a glass tube (2.8 cm. diameter), and drained. The column was washed with additional 100 cc. of N-sulfuric acid and then with water (2 l.). The effluent and washings were immediately neutralized with a saturated barium hydroxide solution as soon as they percolated, barium sulfate removed by filtration, and the filtrate was concentrated to a sirup. Dissolution in boiling methanol (50 cc.) followed by cooling afforded crystals of the double salt (I), which were filtered, washed with methanol and dried; yield 2.3 g. (6.3% of the agar concerned in methanolysis); $[\alpha]_D^{26}$-29.0° (water, *c* 1.0). The filtrate and washings were concentrated to a sirup (3.0 g.), $[\alpha]_D^{26}$-2.3° (water, *c* 1.75).

The crude crystals above obtained were purified by recrystallization from aqueous methanol until no more change in specific rotation and in barium content was recognized. The pure double salt forms elongated prisms, having $[\alpha]_D^{14}$-30.1° (water, *c* 1.56) and slowly decomposing over 200°C. It is soluble in water and insoluble in organic solvents.

*Anal.* Found: Ba, 19.42; $SO_4$, 13.55; $OCH_3$, 13.09; Ash, 33.01. Calcd. for $(C_{17}H_{27}O_{13})(CH_3SO_4)Ba \cdot H_2O$: Ba, 19.48; $SO_4$, 13.61; $OCH_3$, 13.20; Ash (as $BaSO_4$), 33.09%.

**Separation of the Double Salt (I) into its Component Salts.**—The double salt (I) (6.0 g.) in water (30 cc.) was passed through a column of Amberlite IR-120 (2.8×10 cm.) to remove barium ion, and the column was washed with water (100 cc.). The acidic effluent and washings were combined and immediately passed through a column of Amberlite IR-4B (sulfate form) (2.8×20 cm.), which had been prepared by the reaction of the resin (hydroxide form) with excess N-sulfuric acid and had been washed with large volumes of water (20 l.). The column was then washed with water (400 cc.). In this process, methylsulfuric acid was retained by the resin, while the carboxylic acid (IV) was passed through.

**(a) Barium Salt (II).**—The effluent from the above anion resin was immediately neutralized with a barium hydroxide solution and concentrated to dryness. The residue was dissolved in a small volume of methanol, filtered and poured into dry acetone, giving a white powder of the barium salt (II), which was collected on a glass filter, washed with acetone and dried in vacuo; yield 4.0 g. (93%); $[\alpha]_D^{20}$-40.3° (water, *c* 1.54).

*Anal.* Found: $OCH_3$, 11.95; Ba, 13.34; $SO_4$, nil. Calcd. for $(C_{17}H_{27}O_{13})_2Ba$: $OCH_3$, 12.20; Ba, 13.52%.

**(b) Barium Methyl sulfate (III).**—Methylsulfuric acid, which had been adsorbed by the IR-4B resin, was eluted from the column with N-sulfuric acid (50 cc.) and then with water (300 cc.). Effluents were immediately neutralized with a hot saturated barium hydroxide solution, filtered and evaporated to dryness. The residue was crystallized from aqueous ethanol, giving barium methylsulfate dihydrates; yield 1.4 g. (82%).

*Anal.* Found: Ba, 34.66; $SO_4$, 48.39; $OCH_3$,

15.81. Calcd. for $(CH_3SO_4)_2Ba \cdot 2H_2O$: Ba, 34.73; $SO_4$, 48.58; $OCH_3$, 15.67%.

**Regeneration of I from II and III.**—The barium salt (II) (0.51 g.) and barium methylsulfate dihydrate (III) (0.20 g.) were separately dissolved in methanol. When the solutions were combined, the double salt (I) was immediately crystallized; yield 0.71 g. (100%); $[\alpha]_D^{15}$ -30.6° (water, *c* 1.21).

*Anal.* Found: Ba, 19.33; $SO_4$, 13.40; $OCH_3$, 13.31. Calcd. for $(C_{17}H_{27}O_{13})(CH_3SO_4)Ba \cdot H_2O$: Ba, 19.48; $SO_4$, 13.61; $OCH_3$, 13.20%.

**Free Acid (IV).**—The barium salt (II) (2.0g.) in water (20 cc.) was passed through a column of Amberlite IR-120 (2.8×10 cm.) to remove barium ion, and the column was washed with water (80 cc.). The acidic effluent was partly neutralized with 1 cc. of 0.1N-barium hydroxide solution and concentrated below 30° to a sirup, which was freed from a barium compound by dissolution in dry acetone and filtration. The filtrate was concentrated to a sirup (1.7 g.), which was redissolved in ethyl acetate and kept overnight in a refrigerator. Crystals deposited were filtered, washed with ethyl acetate and dried; yield 1.4 g (78%). Recrystallization twice from ethyl acetate afforded a pure specimen of the acid (IV) in a monohydrate form melting at 118-120° and foaming at 122°; $[\alpha]_D^{20}$ -46.7° (water, *c* 1.05) and -51.6° (methanol, *c* 1.24). It is soluble in water, methanol, ethanol and acetone, and hardly soluble in cold ethyl acetate and ether. Seliwanoff's ketose reaction was strongly positive.

*Anal.* Found: C, 44.39; H, 6.83; $OCH_3$, 13.31%; Neutralization equivalent, 457. Calcd. for $C_{17}H_{28}O_{13} \cdot H_2O$: C, 44.55; H, 6.59; $OCH_3$, 13.53; Neutr. equiv., 458.

**Methyl Ester (V).**—To a solution of the acid (IV) (0.5 g.) in methanol (5 cc.) was added dropwise an ethereal solution of diazomethane, evolution of nitrogen being observed. After being kept for one hour, it was concentrated to a sirup (0.5 g.), which was then dissolved in ethyl acetate, and a small amount of ether was added to form a slight cloudiness. The solution was left in a refrigerator, when the methyl ester (V) was slowly crystallized; yield 0.32 g. An additional crop (0.10 g.) was obtained from the mother liquor. Crude crystals were purified by recrystallization twice from ethyl acetate-ether, giving prisms; m. p. 153-154°; $[\alpha]_D^{15}$ -44.4° (water, *c* 1.04), and -52.9° (methanol, *c* 1.04). It is soluble in water, methanol, ethanol and acetone, hardly soluble in cold ethyl acetate, and insoluble in ether.

*Anal.* H, Found: C, 47.51; 6.75; $OCH_3$, 20.69%; Saponification equivalent, 452. Calcd. for $C_{18}H_{30}O_{13}$: C, 47.57; H, 6.65; $OCH_3$, 20.47%; Sapon. equiv., 454.

**Amide (VI).**—A methanolic solution (5 cc.) of the methyl ester (V) (0.15 g.) was saturated with dry ammonia under ice cooling, and left overnight at room temperature. Evaporation gave a sirup, which solidified on standing. Recrystallization was carried out by dissolving it in acetone and adding ether, the amide (VI) being obtained as monohydrates; yield 0.08 g.; m. p. 102-105°; $[\alpha]_D^{25}$ -43.2° (water, *c* 0.51).

*Anal.* Found: C, 44.66; H, 6.95; N, 3.11. Calcd. for $C_{17}H_{29}O_{12}N \cdot H_2O$: C, 44.64; H, 6.82; N, 3.06%.

**Methanolysis of the Double Salt (I).**—The double salt (I) (4.0g.) in 3% methanolic hydrogen chloride (150 cc.) was heated under reflux for twenty hours. Barium sulfate precipitated was removed by filtration, and the filtrate was neutralized with silver carbonate, filtered and concentrated to a colorless sirup (2.5 g.); $[\alpha]_D^{13}$ +35.1° (water, *c* 0.74). This was proved to be a mixture of VII and VIII, the separation being effected by saponification as described below.

**(a) Barium Salt Methyl Glycoside (IX).**—The above sirup was heated in 0.3N-barium hydroxide solution (150 cc.) at 60° for two hours. The solution was neutralized with carbon dioxide, filtered and concentrated to a sirup (3.1 g.). It was then dissolved in a small volume of methanol and poured into dry acetone with stirring, the barium salt methyl glycoside (IX) being precipitated as a white powder, which was collected on a glass filter, washed with acetone and dried in vacuo; yield 2.3 g.; $[\alpha]_D^{14}$ +56.2° (water, *c* 0.89).

*Anal.* Found: $OCH_3$, 9.05; Ba, 20.66. Calcd. for $(C_{10}H_{15}O_8)_2Ba$: $OCH_3$, 9.34; Ba, 20.70%.

**(b) 3,6-Anhydro-L-galactose Dimethylacetal (VII).**—The filtrate and washings, separated from the above salt (IX), were combined and evaporated to dryness, when VII was obtained as a colorless sirup[3]; yield 0.7 g.; $[\alpha]_D^{13}$ -27.0° (water, *c* 0.74); $OCH_3$, found: 26.32% (calcd. for $C_8H_{16}O_6$: 29.81%). It migrated on a paper chromatogram at the same rate as an authentic sample ($R_f$ 0.67[2]), *n*-butanol-ethanol-water (4:1:2) being used as a solvent and *o*-aminophenol reagent[4] being sprayed.

**3,6-Anhydro-L-galactose Diphenylhydrazone.**—The dimethylacetal (0.15 g.) above obtained was hydrolysed with 0.1N-sulfuric acid at 95° for two hours in the usual manner, giving 3,6-anhydro-L-galactose (XI) (0.12 g.). It was then converted into its diphenylhydrazone in the usual manner[3]; m. p. 153-155°, not depressed on admixture with an authentic sample; $[\alpha]_D^{13}$ −34.4° → −21.5° (methanol, *c* 0.93).

**Methyl Ester Methyl Glycoside (VIII).**—The barium salt methyl glycoside (IX) (2.6 g.) above obtained was esterified by heating in 4% methanolic hydrogen chloride (100 cc.) for four hours. The solution was neutralized with silver carbonate, filtered and concentrated to a sirup, which was purified by extraction with hot acetone and evaporation. The methyl ester methyl glycoside

3) C. Araki, *J. Chem. Soc. Japan*, **65**, 725, (1944).
4) S. Hirase, C. Araki and S. Nakanishi, This Bulletin, **26**, 183 (1953).

(VIII) was obtained as a pale yellow viscous sirup; yield 1.7 g.; $[\alpha]_D^{13}$ +74.8° (water, *c* 1.27) and +69.0° (methanol, *c* 1.26).

*Anal.* Found: $OCH_3$, 21.80%; Saponification equivalent, 278. Calcd. for $C_{11}H_{18}O_8$: $OCH_3$, 22.29 %; Sapon. equiv., 278.

**Hydrolysis of IX.**—The barium salt methyl glycoside (IX) (0.8 g.) was dissolved in a small amount of water and N-sulfuric acid (15 cc.) was added with stirring. Barium sulfate precipitated was removed by filtration, and the filtrate was heated in a boiling water bath until the optical rotation of the solution reached a constant value after five hours ($[\alpha]_D$ +52° → +28°). The solution was placed in a Soxhlet's apparatus and treated with ether for seven hours to extract pyruvic acid. The residual solution was used for isolation of D-galactose.

(a) **D-Galactose (XII).**—The residual solution just mentioned was neutralized with barium carbonate, filtered and concentrated to a sirup, which gave D-galactose on dissolution in hot methanol and cooling; yield 0.30 g.; m. p. 166-168°, not depressed on admixture with an authentic sample; $[\alpha]_D^{14}$ +80.6° (after twentyfour hours, water, *c* 1.08).

(b) **Pyruvic Acid (XIII).**—The ether extract above described was distilled under atmospheric pressure to remove ether, and the residue was dissolved in water (9 cc.). The solution was used to prepare the following derivatives of pyruvic acid in the usual manner.

*Phenylhydrazone.*—Needles; m. p. and mixed m. p. 192°.

*p-Nitrophenylhydrazone.*—Yellow fine crystals; m. p. and mixed m. p. 226°.

*Semicarbazone.*—Prisms; m. p. and mixed m. p. 212°.

**Reduction of VIII and Hydrolysis.**—Sodium borohydride (0.3 g.) was added to a solution of VIII (0.9 g.) in water (10 cc.). The reaction solution was left aside with occasional shaking for five days at room temperature. Then it was neutralized with dilute sulfuric acid, deionized by passing through columns of Amberlite IR-120 and IR-4B in succession, and concentrated to a sirup, which was purified by dissolution in absolute methanol and filtration. Evaporation afforded a viscous sirup of the reduction product (X); yield 0.8 g.; $[\alpha]_D^{24}$ +74.5° (water, *c* 0.50); $OCH_3$, found: 12.15% (calcd. for $C_{10}H_{18}O_7$: 12.40%).

The product (X) (0.40 g.) obtained was heated in N-sulfuric acid (10 cc.) until the optical rotation of the solution reached a constant value after four and a half hours ($[\alpha]_D$ +74°→+40°). The solution was neutralized with barium carbonate and filtered. The filtrate, diluted with water up to 40 cc., was distilled under atmospheric pressure, the same volume of water as that of the distillate being supplied as the distillation progressed. Forty cc. of the distillate was collected to identify hydroxyacetone therein, while the residual solution was used for the isolation of D-galactose.

(a) **D-Galactose (XII).**—The residual solution of the distillation was concentrated to dryness, giving D-galactose; m. p. and mixed m. p. 166-167°; $[\alpha]_D^{25}$ +80.5° (after twentyfour hours, water, *c* 1.15).

(b) **Hydroxyacetone (XIV).**—To the distillate above obtained, semicarbazide hydrochloride (0.5 g.) and sodium acetate trihydrate (0.8 g.) were added. After being left overnight at room temperature, the solution was concentrated to a small volume, when hydroxyacetone semicarbazone was crystallized; yield 0.06 g.; m. p. 195-196°, not depressed on admixture with an authentic sample.

**Decarboxylation of Oxalacetic Acid.**—Oxalacetic acid (0.3064 g.) in water (25 cc.) was heated in a boiling water bath, the evolved gas being swept by a slow current of carbon dioxide-free air into an adsorption vessel charged with a 30% potassium hydroxide solution. Carbon dioxide found was 0.1013 g. (99.3% of theory). From the reaction solution, pyruvic acid was obtained as its phenylhydrazone melting at 192°. When oxalacetic acid (0.3106 g.) was heated under reflux in 0.5% methanolic hydrogen chloride (25 cc.), which was used for the isolation of I from agar, carbon dioxide evolved was only 0.0042 g. (4.06% of theory). From the reaction solution, methyl oxalacetate was obtained as its phenylhydrazone, melting at 116-118°.

## Summary

1. Partial methanolysis of agar has led to isolation of a crystalline double barium salt, which is separable into its components, barium methylsulfate and a barium salt of an organic acid.

2. From the last salt above mentioned, there has been obtained a free acid having the formula $C_{14}H_{21}O_9(OCH_3)_2COOH$ as its crystalline monohydrates. Its methyl ester and amide have also been prepared in a crystalline condition.

3. The acid obtained, a new compound, has been proved to be a disaccharide derivative composed of 3,6-anhydro-L-galactose, D-galactose and pyruvic acid.

4. It has also been proved that the pyruvic acid residue is linked through its carbonyl group with the D-galactose residue.

5. The isolation of the pyruvic acid-linking disaccharide derivative substantiates the view that pyruvic acid is an intrinsic constituent of agar.

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