338. *The Acetylation and Methylation of Agar-agar and the Isolation of 2 : 4 : 6-Trimethyl α-d-Galactose by Hydrolysis.*

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Methods for the acetylation and methylation of agar are described. Hydrolysis of methylated agar with dilute sulphuric acid yields (A) methyl lævulate, (B) a 2 : 4 : 6-trimethyl methylgalactoside, from which the previously unknown 2 : 4 : 6-trimethyl α-galactose has been isolated, and (C) a fraction which gives strong ketose reactions—as does agar itself on mild hydrolysis—and is considered to be a dimethyl methylketoside.

β-*d*-Galactopyranose units linked either directly together by positions 1 and 3 or by means of an intermediary ketose residue appear to be chiefly concerned in the structure of this complex polysaccharide.

Percival and Sim (*Nature*, 1936, **137**, 997) showed that agar could be acetylated to yield a chloroform-soluble agar acetate which on deacetylation regenerated a substance identical with the original polysaccharide in its ability to form a gel. This was regarded as evidence that no substantial degradation had taken place during acetylation. Later Percival, Munro, and Somerville (*ibid.*, 1937, **139**, 512) described some preliminary results of the hydrolysis of the methylated derivative obtained from the acetate. The details of this work and also the progress made in the identification of the acid portion of the hydrolysis product of agar are now described.

Agar acetate was obtained either as a tough colourless glass from chloroform solution or as a white powder by precipitation from solution by light petroleum. Further acetylation had no effect on its properties, and almost ash-free agar was obtained from it on deacetylation. Deacetylation and methylation yielded a product, soluble in chloroform and acetone, unchanged on further methylation. Fractional precipitation from chloroform solution gave four identical fractions, indicating the homogeneous character of the product.

Hydrolysis with 6% sulphuric acid was complete in four hours, the solution acquiring a positive rotation; at the same time methyl alcohol (3—4%) was evolved, and a brown resin (3%) deposited. No furfural derivatives could be detected. After neutralisation with barium carbonate an estimation of barium in the hydrolytic product gave 6·7%. The barium salt, however, obtained by precipitation with alcohol contained no methoxyl residue. Owing to the solubility of this barium salt it was impossible to separate it quantitatively from the sugars (*ca.* 75%) formed on hydrolysis; therefore glucoside formation and esterification were carried out on the entire hydrolytic product. Distillation in a high vacuum yielded: (A) an optically inactive, mobile ester (16%), (B) a crystalline trimethyl methylgalactoside (65%), and (C) a syrup (14%). The yield of (B) thus isolated from methylated agar was *ca.* 50% of the starting material, and by the hydrolysis of methylated agar with methyl-alcoholic hydrogen chloride a yield of *ca.* 60% was obtained. Assuming that methylated agar, as appears likely, is a true derivative of agar, it follows from this that the polysaccharide is composed of galactose residues to the extent of at least 55%, whereas previous authors (*e.g.*, Lüdtke, *Biochem. Z.*, 1929, **212**, 419) were able to detect only 30—40%.

From its physical properties, analytical composition, and comparison of the melting points and mixed melting points of the 2 : 4-dinitrophenylhydrazone and p-*nitrophenylhydrazone* prepared from an authentic specimen, (A) proved to be methyl lævulate. There would seem to be no doubt, therefore, that the acid formerly regarded as a constituent of agar is lævulic acid arising from the decomposition of a fragment of the molecule. Lüdtke (*loc. cit.*) indeed isolated a small amount of lævulic acid from the hydrolysis products of agar itself. It is well known that this acid is more readily produced from ketohexoses than from aldohexoses, and under the experimental conditions employed it was found that galactose gave rise to less than 2% and the trimethyl galactose from (B) to none at all. The hydrolysis of methylated agar with methyl-alcoholic hydrogen chloride yielded scarcely any lævulic ester, fractions (B) and (C) being obtained as before. The application of the Seliwanoff reaction and Bredereck's test on hydrolysed agar, methyl agar, and fraction (C) gave positive indication of the presence of a ketose, and it is considered, therefore, that the lævulic acid is derived from that source. Takahashi and Shirawa (*J. Fac. Agr. Hokkaido Imp. Univer. Japan*, 1934, **35**, 101), in their investigation on agar hydrolysed in an autoclave at 130°, also found a substance giving a ketose reaction. The above ketose tests were negative with fractions (A) and (B).

The crystalline non-reducing substance (B), $C_{10}H_{20}O_6$, m. p. 64°, $[\alpha]_D^{17°}$ + 107° in water [the rotation, in conjunction with the rotation of the corresponding trimethyl galactose (+ 124°), indicating it in all probability to be a mixture of the α- and the β-form with a preponderance of the former], proved to be a 2 : 4 : 6-*trimethyl methylgalactoside*, this structure being assigned for the following reasons. (1) Complete methylation yielded almost quantitatively 2 : 3 : 4 : 6-tetramethyl galactose, identified by its crystalline anilide. This observation at once identified it as a derivative of galactose and excluded the possibility of substitution at position 5. (2) Hydrolysis yielded a crystalline trimethyl galactose, $C_9H_{18}O_6$, m. p. 104—105°, $[\alpha]_D^{17°}$ + 124° in water, which readily formed a crystalline *dimethyl galactose phenylosazone*, indicating the presence of a methoxyl group in position 2 of the trimethyl galactose. Oxidation with bromine water under conditions favourable to the production of a γ-lactone yielded a trimethyl δ-galactonolactone, $[\alpha]_D^{18°}$ + 150° in water, which fell rapidly on standing to an equilibrium value of + 50° in 16 hours. The probability that position 4 was occupied by a methoxyl residue was confirmed by the observation that at room temperature in contact with 1% methyl-alcoholic hydrogen chloride the crystalline trimethyl galactopyranoside was regenerated, no galactofuranosides being detected. It became clear, therefore, that positions 2 and 4 were occupied by methoxyl residues. The trimethyl galactose cannot therefore be the 2 : 3 : 6-trimethyl galactose of Haworth, Raistrick, and Stacey (*Biochem. J.*, 1935, **29**, 2668) and indeed the physical constants confirm this fact. (3) It remained to decide between 2 : 3 : 4- and 2 : 4 : 6-trimethyl galactose. The former derivative in the form of a syrup has been isolated by Challinor, Haworth, and Hirst (J., 1931, 258) from the aldobionic acid of gum arabic and by Onuki (*Chem. Zentr.*, 1933, II, 367) from stachyose. The properties of the sugar in question, the rotation of the lactone formed by oxidation, and the properties of its derivatives appear to exclude the possibility of identity. Unfortunately we could not obtain a crystalline phenylhydrazide for comparison, but a crystalline *amide*, m. p. 167°, $[\alpha]_D^{20°}$ + 74°, was isolated. This, however, only serves to confirm the absence of identity with the 2 : 3 : 6-trimethyl galactose, since an amide of 2 : 3 : 4-trimethyl galactonolactone has not been described. Repeated attempts to obtain the trimethyl mucic acid which characterised the 2 : 3 : 4-trimethyl galactose of Challinor, Haworth, and Hirst (*loc. cit.*) failed, small yields of compounds of indefinite composition being obtained, of which the reducing properties seemed to indicate them to be derivatives of tartronic or mesoxalic acid. This result is in accordance with the assignment of the structure of 2 : 4 : 6-*trimethyl α-galactose* to the methylated galactose fragment.

It has not yet been found possible to identify the non-reducing fraction (C), which appears to be a dimethyl methylketoside (OMe, 39%). By Freudenberg and Soff's method (*Annalen*, 1932, **494**, 68), it was estimated that one methoxyl group was glycosidic. Treatment with 6% sulphuric acid under the conditions employed for the hydrolysis of methylated agar, followed by esterification, again gave rise to methyl lævulate together with

unchanged material, and this fact together with the strong ketose reactions exhibited seems to point to the presence of a ketose.

The fact that the rotations of acetylated and methylated agar are strongly negative, coupled with the isolation after hydrolysis of *d*-galactose derivatives, makes it clear that there is a preponderance of β-linkages in the molecule. If one assumed it to be composed entirely of β-galactopyranose units linked at positions 1 and 3, the most probable structures would be either a staggered zig-zag chain or a closed loop of six such units. If, however, the former alternative were correct, it would be expected that tetramethyl galactose or some other fully methylated sugar (cf. Haworth, Hirst, and Oliver, J., 1934, 1917) would be observed among the hydrolytic products, and although the possibility exists that some of the methyl lævulate may have been derived from a fully methylated ketose, no definite evidence for this, or for the presence of tetramethyl galactose, has yet been found and it will be necessary to hydrolyse large quantities of methylated agar before the point can be settled. Furthermore the isolation of what appears to be a dimethyl ketose in significant quantity makes it appear likely, unless the methylation of the ketose fragment is hindered in some way, that, if a chain of β-galactopyranose units is present, it may contain at points along its length ketose residues, and that loops (cf. Haworth, Hirst, and Isherwood, this vol., p. 577; Haworth, Hirst, and Oliver, *loc. cit.*) or cross linkages are present at these points. It is hoped that further work will enable progress to be made in this direction.

Pirie (*Biochem. J.*, 1936, **30**, 369) on the basis of the isolation of an aldehydo hepta-acetyl *dl*-galactose by the acetolysis of agar suggested that aldehydo galactose was present in the molecule. Freudenberg and Soff (*Ber.*, 1937, **70**, 264) have indicated, however, that aldehydo glucose hepta-acetate can be obtained from glucopyranose derivatives, so the substance isolated by Pirie may be a reversion product. The sulphuric ester grouping apparently present in agar (Neuberg and Ohle, *Biochem. Z.*, 1921, **125**, 311; Lüdtke, *loc. cit.*) is evidently hydrolysed during acetylation and methylation, since no sulphur can be detected in purified agar acetate or methylated agar. The properties of the material obtained on deacetylation do not seem to be affected by this and it may be that, as in the case of the phosphorus residue in starch (Baird, Haworth, and Hirst, J., 1935, 1201; Haworth, Hirst, and Waine, *ibid.*, p. 1299), the presence or absence of sulphuric ester residues has no very obvious effect on the properties of the polysaccharide, contrary to the view of Samec and Ssajevič (*Compt. rend.*, 1921, **173**, 1474).

Experimental.

Prepared Agar.—Powdered agar (B.D.H.) (100 g., ash 3·3%) was washed during 5 days five times with tap water (100 l.). The residue was treated with alcohol, washed with ether, and dried in the air [Found: C, 45·1; H, 6·2; ash, 2·0. Calc. for $(C_6H_{10}O_5)_x$: C, 44·4; H, 6·2%]. Treatment with *N*/20-hydrochloric acid at room temperature or with *N*/50-hydrochloric acid at 48° (1 minute), followed by the addition of alcohol and standing at 30° (½ hour), followed by washing and the further addition of alcohol, failed to reduce the ash content appreciably.

Acetylation of Agar.—Prepared agar (18 g. dry wt.) was shaken with pyridine (100 c.c.) for 1 hour, and a mixture of acetic anhydride (200 c.c.) and pyridine (100 c.c.) added. After heating at 95° for 6 hours and standing for 20 hours, the yellow solution was poured into ice-water (10 l.) with constant stirring. The product was washed free from acid, dried, and extracted with chloroform–acetone (1 : 1), the solution filtered, and the product precipitated by light petroleum and dried in a vacuum (22 g.). The agar acetate softened at 230° and had $[\alpha]_D^{15°}$ — 32·1° in chloroform (*c*, 0·5) (Found: C, 49·1; H, 5·8; CH_3·CO, 39·0. Calc. for $C_{12}H_{16}O_8$: C, 50·0; H, 5·6; CH_3·CO, 44·8%).

Methylated Agar.—Agar acetate (14 g.) in acetone (300 c.c.) was treated with methyl sulphate (70 c.c.) and sodium hydroxide (180 c.c., 30%) in $\frac{1}{10}$th portions every 10 minutes at 56°, followed by heating to 75° during 30 minutes. The white granular product (OMe, 27·7%), after being washed with hot water, was redissolved in acetone and remethylated as before (OMe, 28·3%). This operation was repeated twice more to yield a product, soluble in chloroform, precipitated by light petroleum as a white amorphous powder (10 g.), softening at 220°, $[\alpha]_D$ — 92° in chloroform (*c*, 0·6) (Found: OMe, 30·9%).

The Fractional Precipitation of Methylated Agar.—Methylated agar (5 g.), dissolved in chloro-

form (250 c.c.), was precipitated by successive additions of light petroleum (b. p. 60—80°) to yield four fractions :

(A)	0·9 g., $[\alpha]_D^{15°}$	— 92°	in chloroform	(*c*, 0·4) ;	OMe, 31·0%.
(B)	1·2 g., $[\alpha]_D^{17°}$	— 92°	,, ,,	,,	OMe, 30·8%.
(C)	2·0 g., $[\alpha]_D^{17°}$	— 92°	,, ,,	,,	OMe, 30·9%.
(D)	0·6 g., $[\alpha]_D^{17°}$	— 92·5°	,, ,,	,,	OMe, 30·7%.

The Hydrolysis of Methylated Agar with Sulphuric Acid.—Methylated agar (8 g.) was heated at 95° with sulphuric acid (170 c.c., 6%). The substance gradually dissolved and a brown resin was deposited, which was filtered off (0·25 g.; OMe, nil). A constant rotation ($[\alpha]_D^{20°}$ + 45°) was reached in 4 hours and the hydrolysis was continued during a further hour. The hydrolysate was neutralised with barium carbonate, and the filtered solution evaporated almost to dryness at 50°/10 mm.; alcohol then precipitated a barium salt contaminated by reducing sugars. Solution in water and reprecipitation with alcohol gave a white powder (0·7 g.; OMe, nil). The aqueous-alcoholic residues on evaporation to dryness gave a pale yellow, reducing syrup (6 g.; OMe, 44%) which still contained some of the barium salt. By a modification of the method of Freudenberg and Soff (*loc. cit.*) it was estimated that methylated agar lost 3·5% of methyl alcohol during the hydrolysis.

Simultaneous Esterification and Glucoside Formation.—The neutralised hydrolytic product (7 g.) from another hydrolysis, which still contained the barium salt, was boiled with 5% methyl-alcoholic hydrogen chloride for 6 hours; the solution was neutralised with silver carbonate, filtered, and evaporated at 40°/15 mm. to a non-reducing syrup (5·9 g.), which was fractionally distilled to yield the following fractions : (A) 0·98 g., bath temp. 90—100°/0·01 mm.; (B) 3·8 g., bath temp. 150—160°/0·01 mm.; (C) 0·85 g., bath temp. 180—190°/0·01 mm.; residue, 0·4 g.

Identification of (A) *as methyl lævulate.* The colourless mobile liquid, which gave the iodoform reaction, had $n_D^{15°}$ 1·4250, $[\alpha]_D^{15°}$ ± 0° (Found : C, 54·8; H, 7·9; CO_2Me, 48·0. Calc. for $C_6H_{10}O_3$: C, 55·3; H, 7·8; CO_2Me, 45·3%). The 2 : 4-dinitrophenylhydrazone had m. p. 136°, alone or mixed with the 2 : 4-dinitrophenylhydrazone of methyl lævulate (m. p. 137°) (Found : N, 15·9. Calc. for $C_{12}H_{14}O_6N_4$: N, 16·2%). The *p*-nitrophenylhydrazone had m. p. 136°, alone or mixed with *methyl lævulate* p-*nitrophenylhydrazone* (m. p. 136°) (Found : C, 54·25; H, 5·65; N, 16·2. $C_{12}H_{15}O_4N_3$ requires C, 54·3; H, 5·7; N, 15·85%).

Identification of (B) *as* 2 : 4 : 6-*trimethyl methylgalactoside.* This fraction, which solidified completely during distillation, was recrystallised from light petroleum and had m. p. 62—64°, $[\alpha]_D^{15°}$ + 107° in water (*c*, 0·4) (Found : C, 50·0; H, 8·6; OMe, 51·1. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%). 2 : 4 : 6-*Trimethyl methylgalactoside* may also be isolated as a crystalline *hydrate*, m. p. 37°, $[\alpha]_D^{15°}$ + 101° in water (*c*, 0·4) (Found : C, 47·0; H, 8·6; OMe, 47·1. $C_{10}H_{20}O_6,H_2O$ requires C, 47·2; H, 8·7; OMe, 48·8%).

Complete Methylation and the Isolation of 2 : 3 : 4 : 6-*Tetramethyl Galactose Anilide.*—The crystalline galactoside (1 g.) was methylated in the usual way once with methyl sulphate and sodium hydroxide and once with silver oxide and methyl iodide. After extraction and distillation in a high vacuum the glucosidic residue was removed by heating for 2 hours with hydrochloric acid (7%), the fully methylated sugar being isolated and treated with aniline. Tetramethyl galactopyranose anilide was obtained (0·6 g.), m. p. 192°, unchanged on admixture with an authentic specimen.

The Isolation of 2 : 4 : 6-*Trimethyl* α-*Galactose.*—Hydrolysis of the trimethyl methylgalactoside (1 g.) during 2 hours with 7% hydrochloric acid, followed by neutralisation with silver carbonate and evaporation, gave a syrup, which crystallised on treatment with ether (0·7 g.). Recrystallisation from ether–light petroleum gave colourless needles, m. p. 104—105°, $[\alpha]_D^{15°}$ + 124° in water (*c*, 0·9), falling to + 93° (equilibrium value). This substance is shown below to be 2 : 4 : 6-*trimethyl* α-*galactose* (Found : C, 48·2; H, 7·9; OMe, 40·0. $C_9H_{18}O_6$ requires C, 48·7; H, 8·1; OMe, 41·9%).

Osazone Formation and the Isolation of 4 : 6-*Dimethyl Galactosazone.*—The above sugar (0·5 g.) on treatment with phenylhydrazine (1 c.c.) and acetic acid (1 c.c.) and heating at 90° for 2 hours gave rise to yellow needles (0·4 g.), which were recrystallised from aqueous alcohol. They had m. p. 158°, $[\alpha]_D^{20°}$ — 25° in alcohol (*c*, 0·3) (Found : C, 62·3; H, 6·5; OMe, 14·9; N, 15·6. $C_{20}H_{26}O_4N_4$ requires C, 62·1; H, 6·8; OMe, 16·1; N, 14·5%).

Isolation of 2 : 4 : 6-*Trimethyl* δ-*Galactonolactone and its Crystalline Amide.*—The trimethyl galactose (1 g.) in water (14 c.c.) was treated with bromine (2 c.c.) for 26 hours at 35° and for 22 hours at 18°. Bromine was removed by aeration, the solution neutralised with silver carbonate, and the silver precipitated by hydrogen sulphide. Water was removed at 50°/15 mm. and

the yellow syrup (0·7 g.) was finally heated at 100°/0·01 mm. for 2 hours. It had $[\alpha]_D^{15°}$ + 152° in water (*c*, 0·2) (initial value); + 122° (45 minutes); + 112° (2 hours); + 90° (4 hours); + 50° (16 hours, constant value) (Found: OMe, 40·4; 0·165 g. required 14·9 c.c. of *N*/20-NaOH. Calc. for $C_9H_{16}O_6$: OMe, 42·4%; *N*/20-NaOH, 15·0 c.c.). A crystalline *amide* (0·3 g.) was formed by treating the lactone (0·3 g.) overnight with methyl-alcoholic ammonia, removing the solvent, and recrystallising the product from acetone; it formed lustrous plates, m. p. 167°, $[\alpha]_D^{12°}$ + 74° in water (*c*, 0·3) (Found: C, 46·0; H, 7·8; OMe, 37·6; N, 6·2. $C_9H_{19}O_6N$ requires C, 45·6; H, 8·1; OMe, 39·2; N, 5·9%).

Oxidation of the Lactone with Nitric Acid.—The lactone (2 g.) was oxidised with nitric acid under the conditions laid down by Challinor, Haworth, and Hirst (*loc. cit.*). No crystalline trimethyl mucic ester was obtained, but an ester (0·2 g.) resulted, which was distilled in a high vacuum and converted into an amide (0·05 g.) of zero rotation; this (Found: OMe, 1%) reduced ammoniacal silver nitrate, melted to a red liquid at 165—170°, and was probably impure tartronamide.

Regeneration of 2 : 4 : 6-*Trimethyl Methylgalactoside from* 2 : 4 : 6-*Trimethyl Galactose.*—The sugar in the form of a syrup (0·1 g.) was dissolved in 0·7% methyl-alcoholic hydrogen chloride (10 c.c.), and the change in rotation observed: $[\alpha]_D^{15°}$ + 52° (initial value); + 55° (13 hours); + 82° (26 hours); + 100° (70 hours, constant value). Neutralisation with silver carbonate and removal of solvent gave crystalline 2 : 4 : 6-trimethyl methylgalactoside in quantitative yield.

Examination of Fraction (C).—Redistillation at 190°/0·01 mm. gave a colourless non-reducing syrup, $[\alpha]_D^{15°}$ + 29° in chloroform (*c*, 0·4) [Found: OMe, 39·9; glucosidic OMe, 14·0 (Freudenberg and Soff, *loc. cit.*). $C_9H_{18}O_6$ requires OMe, 41·9%]. The syrup gave a strong Seliwanoff reaction with resorcinol, and the Bredereck reaction with ammonium molybdate. Positive tests were also secured with fructose and trimethyl methylfructofuranoside as controls, but negative results were obtained from galactose, glucose, 2 : 4 : 6-trimethyl galactose, and methyl lævulate. Agar too on mild hydrolysis gave the Bredereck reaction strongly. The syrupy ketoside was treated at 100° with 6% sulphuric acid (2 hours); esterification and distillation then gave more methyl lævulate (30%), indicating at least one probable source of this ester.

When the crystalline 2 : 4 : 6-trimethyl methylgalactoside was hydrolysed with sulphuric acid and submitted to glucoside formation and esterification exactly as for methylated agar, no methyl lævulate could be detected in the final product, so the trimethyl galactose must be discounted as a source of lævulic acid.

The Hydrolysis of Methylated Agar with Methyl-alcoholic Hydrogen Chloride.—Methylated agar (3 g.), dissolved in methyl-alcoholic hydrogen chloride (130 c.c., 5%), was heated at 80° for 6 hours until the rotation was constant. After neutralisation with silver carbonate and evaporation a syrup was isolated which on distillation gave crystalline 2 : 4 : 6-trimethyl methylgalactoside (1·9 g.) at 140—150°/0·03 mm. and a syrup (0·8 g.) at 190°/0·02 mm.; the latter appeared to be identical with the syrup (C), except that the rotation, $[\alpha]_D^{15°}$ + 13° in chloroform (*c*, 0·4), was somewhat lower. It was also found possible to hydrolyse methylated agar with 1% methyl-alcoholic hydrogen chloride.

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