42. *The Constitution of Agar.*

By W. G. M. JONES and S. PEAT.

Evidence is presented that agar is the sulphuric acid ester of a linear polygalactose. The unit-chain is composed of nine residues of *d*-galactose mutually combined by 1 : 3-glycosidic linkages. This chain is terminated at the reducing end by a residue of *l*-galactose, which is united with the rest of the chain by a glycosidic linkage engaging C_4 and not C_3. The *l*-galactose member is esterified at C_6 with sulphuric acid.

This conception of the structure of agar explains the isolation of *l*-galactose from it, the separation of 2 : 4-dimethyl 3 : 6-anhydro-*l*-galactose and 2 : 5-*dimethyl* 3 : 6-*anhydro*-l-*galactonic acid* from methylated agar and also satisfactorily accounts for the absence of sulphuric acid residues from acetylated and methylated agar.

The probable course of the biological synthesis of agar is discussed.

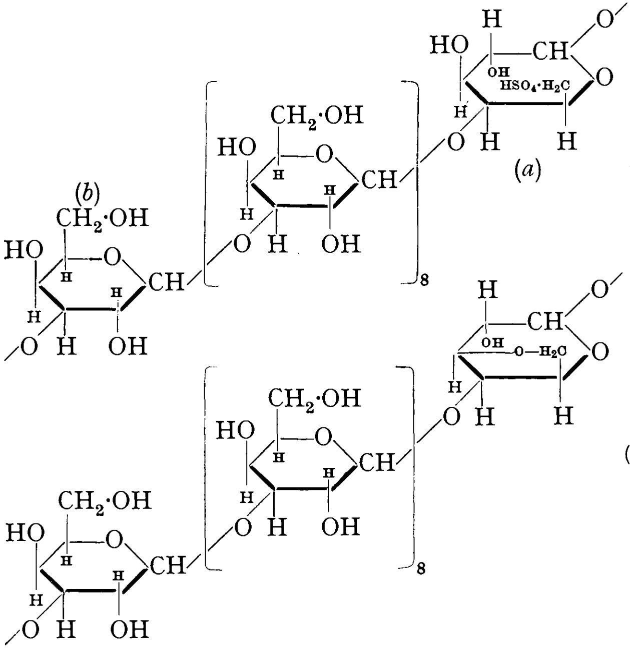
IN the past decade progress has been made in the elucidation of the constitution of agar, for which is now proposed a simple structure conforming with the known facts and new directions are suggested along which further investigation may be pursued.

The agar polysaccharide, as it occurs in the plant, is to be envisaged as composed entirely of galactose residues. These are arranged in chains which are themselves repeating units. Each unit-chain consists of approximately nine *d*-galactopyranose residues mutually combined by 1 : 3-glycosidic linkages;

but a further fact of exceptional significance is that this chain is terminated at the reducing end not by a member of the *d*-series but by one residue of *l*-galactopyranose, the glycosidic link in this case involving C_4, and not C_3. Furthermore, the primary alcohol group (C_6) of each *l*-galactose residue is esterified with sulphuric acid. The complete repeating unit is shown in (I).

If the structure (I) were not a repeating unit, but represented the whole molecule of agar, it is clear that methylated agar would contain tetramethyl *d*-galactopyranose as the non-reducing end group. Except in special circumstances detailed below, tetramethyl galactose has not been detected hitherto as a constituent of methylated agar (cf. Percival *et al.*, J., 1937, 1615; 1939, 1844). It is a reasonable assumption, therefore, that (I) is a repeating chain-unit in which the reducing group of the *l*-galactose member, (*a*), provides the point of attachment to a second unit-chain, and this end-to-end attachment is established with the terminal non-reducing *d*-galactose residue, (*b*), of the second chain. The degree of aggregation of the unit-chain is not yet known, but information on this point may be forthcoming from determination of the molecular weight of agar.

Commercial agar, as was established by Neuberg and Ohle (*Biochem. Z.*, 1921, **125**, 311) and confirmed subsequently by others, has sulphuric acid residues present in organic combination. The sulphuric ester is easily hydrolysed by acid or alkali. The formulation suggested for agar, (I), requires a sulphur content of 1·8%. Figures varying from 0·5 to 1·5% are given in the literature for the sulphur content of samples of agar, a variability which may easily be due to the greater or lesser extent of the hydrolysis of sulphate effected during the technical purification of agar, a process which often involves bleaching with chlorine (cf. Lüdtke, *Biochem. Z.*, 1929, **212**, 419).



(I.)

(II.)

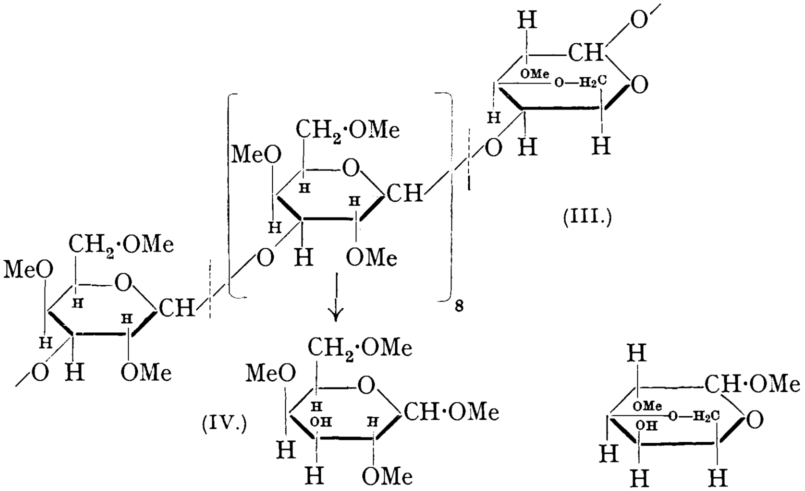
There is reason to affirm that the hydrolysis of a sugar sulphuric ester follows the same course as the hydrolysis of a sugar toluenesulphonic ester, namely, that an anhydro-ring is developed in the sugar residue if a suitably situated hydroxyl group is available. Thus, Percival and Soutar (J., 1940, 1475) have shown that the alkaline hydrolysis of α-methylglucoside sulphate and of α-methylgalactoside sulphate leads, in each case, to the formation of an anhydro-methylhexoside.* In agar, the *l*-galactose sulphate residue is united with the rest of the chain through position 4, and consequently the hydroxyl at C_3 is available for anhydro-ring formation.

It is to be expected, therefore, that the hydrolytic removal of the sulphate group at C_6 would be attended by the formation of a 3 : 6-anhydro-bridge in the *l*-galactose residue, and the repeating unit of agar would assume the form (II).

Neither acetylated nor methylated agar contains sulphur and it is in keeping with the above considerations to assume that during the methylation process the hydrofuranol (*i.e.*, 3 : 6-anhydro) ring appears in the *l*-galactose residue (II) in place of the sulphate grouping (I). On this conception, agar

* (*Note added in proof, March 20th,* 1942.) Duff and Percival have now shown (J., 1941, 830) that the anhydro-methylhexosides formed as described are 3 : 6-anhydro-α-methylglucoside and 3 : 6-anhydro-α-methylgalactoside respectively.

methylated entirely in alkaline solution will have the constitution represented by (III) and the methanolysis of methylated agar will give rise to 2 : 4 : 6-trimethyl *d*-methylgalactoside (IV) and 2-methyl 3 : 6-anhydro-*l*-methylgalactoside (V), the latter representing *ca.* 9% of the methylated polysaccharide.



(III.)

(IV.)

(V.)

2 : 4 : 6-Trimethyl *d*-galactose was first isolated from methylated agar by Percival and Somerville (J., 1937, 1615), who showed that this sugar derivative was the chief constituent of the methylated agar hydrolysate, amounting to at least 65% of the whole (see Forbes and Percival, J., 1939, 1846). It is demonstrated thereby that the predominant glycosidic linkages along the chain are at positions 1 and 3 in each pair of such residues which are mutually combined.

The monomethyl anhydro-*l*-methylgalactoside (V) has not yet been isolated, but its presence in the methylated agar hydrolysate has been detected, inasmuch as when the higher-boiling fractions of the hydrolysate are further methylated, crystalline 2 : 4-dimethyl 3 : 6-anhydro-β-*l*-methylgalactoside is obtained (Hands and Peat, *Chem. and Ind.*, 1938, 937; *Nature*, 1938, **142**, 797; Percival, Somerville, and Forbes, *ibid.*, p. 797; Forbes and Percival, *loc. cit.*). The increase of methoxyl content of these higher-boiling fractions by further methylation corresponded with the conversion of a monomethyl into a dimethyl anhydromethylhexoside.

The linkage of the anhydro-*l*-galactose unit to the rest of the chain (II) is regarded as occurring at C_4, since it cannot take place at C_3 or C_6, these positions being occupied by the anhydro-ring, nor yet at C_2 for reasons which will now be outlined.

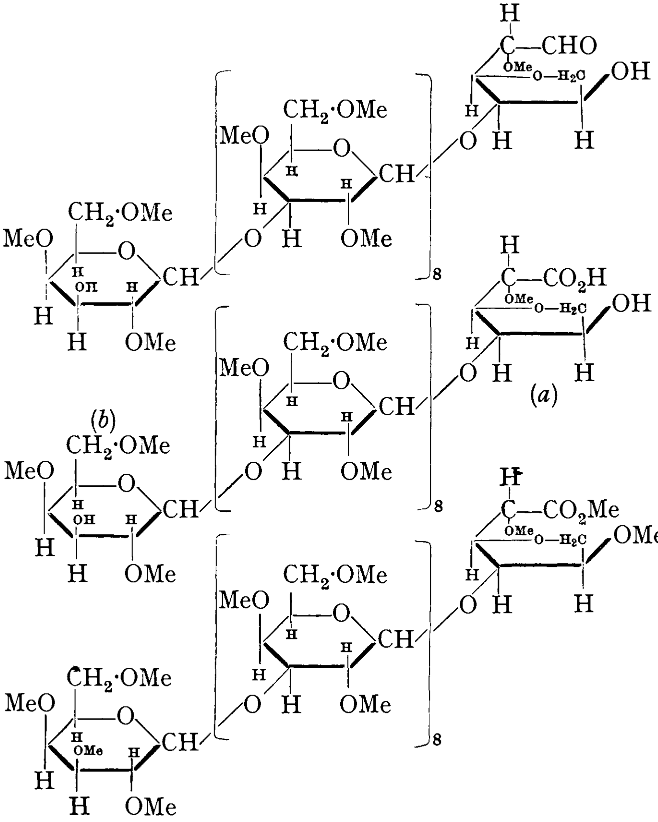
Commercial agar was exhaustively methylated by the direct action of methyl sulphate in alkaline solution. Fractional precipitation of the methylated agar separated it into two main fractions (A and B) distinguished by their ash contents. The fraction (A) with relatively high ash content was dissolved in dilute sulphuric acid and submitted to prolonged dialysis against running tap-water. Although the product of dialysis contained no sulphuric acid residues, its aqueous solution was strongly acid in reaction. The presence of acidic groups was further demonstrated by the fact that the treatment of the dialysed product with diazomethane led to its esterification and a consequent increase in methoxyl content. The extent of this increase, considered together with the results of equivalent determination by alkali titration, indicated the presence in the dialysed product of one carboxyl group to every ten hexose residues.

The question now arose as to the origin of this acidic polysaccharide. It might have been an original constituent of agar, or on the other hand a factitious product formed during the technical preparation of agar or during the processes of methylation and purification of the methylated agar. There are many reasons for adopting the second view.

It has been shown by Haworth, Jackson, and Smith (J., 1940, 625) that the presence together in a hexose of hydrofuranol and pyranose rings confers exceptional properties on the compound, particularly in regard to its sensitivity to acid. The normal stability of the pyranose ring is markedly diminished and it is found, for example, that 2 : 4-dimethyl 3 : 6-anhydro-*d*-methylgalactopyranoside is converted by contact with cold N/10-sulphuric acid into 2 : 4-dimethyl 3 : 6-anhydro-*aldehydo*-*d*-galactose.

Now the *l*-galactose residue in (II) is constituted on precisely this model, and it is to be expected that the effect of acid would be to cause the fission of the glycosidic linkage between two repeating units, the disappearance of the pyranose ring from the *l*-galactose, and the appearance of a free aldehyde group in its place.

If acid conditions are avoided before and during methylation, methylated agar will have the constitution represented by (III), and this is probably a true representation of the fraction (B) with low ash content. On the other hand, if acid conditions supervene after methylation, then the sequence of events just related will occur and the methylated agar will assume the form (VI), which represents it as a single chain of nine 2 : 4 : 6-trimethyl *d*-galactose residues terminated at the reducing end by monomethyl 3 : 6-anhydro-*aldehydo*-*l*-galactose.



(VI.)

(VII.)

(VIII.)

If, concurrently, oxidation conditions are present, the reactive aldehydo-group of the *l*-galactose will readily be converted into carboxyl. Such oxidation might conceivably be effected by atmospheric oxygen during the dialysis in acid solution of the methylated agar, which would now be represented by (VII).

If the acid (VII) were submitted to further methylation with methyl iodide and silver oxide, the carboxyl group would be esterified but, in addition, the hydroxyl groups at C_5 of the *l*-galactose residue (*a*) and at C_3 of the terminal *d*-galactose unit (*b*) would be methylated and (VIII) would be formed. This indeed appears to be the effect of methylation with these reagents of the dialysed product (A), for there occurs an increase in methoxyl content (from 33 to 38%) which is larger than can be accounted for by the simple esterification of a carboxyl group, such as diazomethane effects. The complete hydrolysis of the ester (VIII) would yield mainly 2 : 4 : 6-trimethyl *d*-galactose, but there would also be produced tetramethyl *d*-galactose from (*b*), and 2 : 5-dimethyl 3 : 6-anhydro-*l*-galactonic acid from (*a*), the last two in equimolecular proportion.

These predictions were fulfilled in the actual results of our experiments. From the hydrolysate of the ester derived from (A) by the procedure described, there were separated tetramethyl *d*-galactose and a dimethyl anhydrohexonic acid, the latter being isolated as the crystalline amide (m. p. 173°). The constitution of the anhydrohexonic acid was established in the following manner.

The anhydro-ring in this substance has survived repeated treatment with acid and alkaline reagents and is therefore clearly not of the ethylene oxide type. Its stability in fact recalls that of the hydrofuranol type of anhydro-ring and in the absence of evidence to the contrary the description of the substance as a 3 : 6-anhydro-hexonic acid would be justified. Furthermore, since the presence of 3 : 6-anhydro-*l*-galactose in agar has already been proved, the hexonic acid may be particularised as dimethyl 3 : 6-anhydro-*l*-galactonic acid. This assumption finds support in that the rotation of the amide

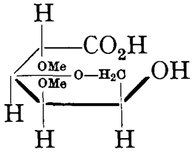
($[\alpha]_D^{12^\circ}$ − 76°) is of the same order and sign as that of the amide derived from 2 : 4-dimethyl 3 : 6-anhydro-*l*-galactonic acid ($[\alpha]_D$ − 74°. Forbes and Percival, *loc. cit.*). The disposition of the methoxyl groups is inferred from the following considerations. There are three possible structures to examine, namely, 2 : 4-dimethyl (IX), 2 : 5-dimethyl (X), and 4 : 5-dimethyl 3 : 6-anhydro-*l*-galactonic acid (XI).

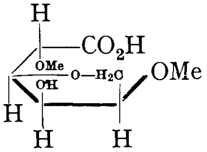
The amide in question gave a negative Weerman test for α-hydroxy-amides, on which ground (XI) is excluded. The amide of (IX) has been shown by Forbes and Percival (*loc. cit.*) to melt at 151°, as does the *d*-isomer prepared by Haworth, Jackson, and Smith (*loc. cit.*). This melting point is 20° lower than that of the amide now isolated and described, and it is improbable that the two are identical. The conclusion must therefore be drawn that the acid isolated in the form of its amide from the acidic polysaccharide constituent of methylated agar is 2 : 5-*dimethyl* 3 : 6-*anhydro*-l-*galactonic acid* (X).

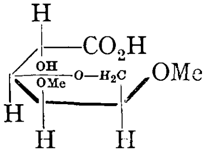
The reason for the description of the mode of union of the *l*-galactose residue in agar with the rest of the chain as a 1 : 4-glycosidic linkage (I and II) is now apparent. The ultimate isolation of 2 : 5-dimethyl 3 : 6-anhydro-*l*-galactonic acid is only explicable on the assumption that the hydroxyl at C_4 of the original *l*-galactose is involved in the chain linkage.

In the foregoing analysis, it has been assumed that the changes leading to the oxidation of agar to an acid polysaccharide occur during the dialysis of the methylated agar, and this is possibly the stage at which oxidation is most marked. But it is obvious that the requisite conditions of acidity and oxidation might be present also in the preparation of commercial agar, and even in its methylation with methyl sulphate, should local acidity develop during this process. The heterogeneity observed in methylated agar might therefore be due to a combination of these causes.

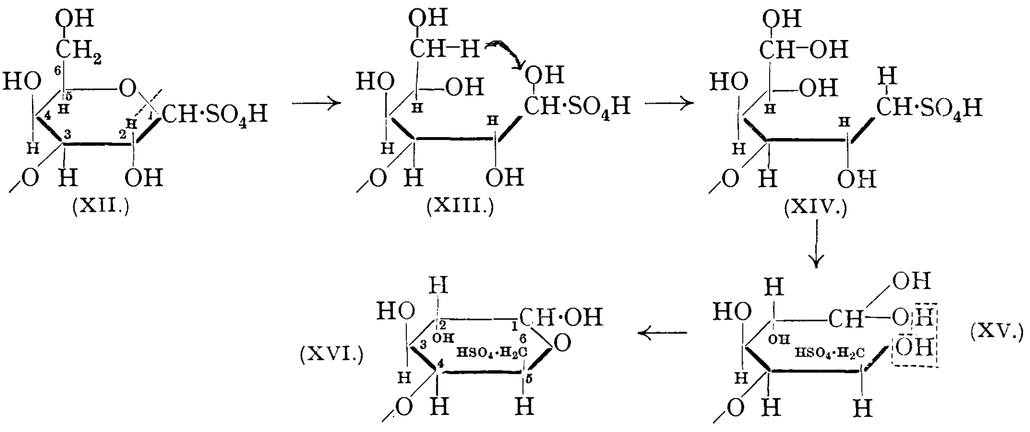
It is known that when a sulphuric ester of a hexose is hydrolysed with alkaline reagents an anhydro-ring is invariably formed if the disposition of the free hydroxyl groups is appropriate. The effect of acid hydrolysing agents has not however been so fully investigated and it is conceivable that acid agents effect normal hydrolysis of the ester without the formation of the anhydro-ring. If that be the case, agar (I) will yield a mixture of *d*- and *l*-galactose when it is resolved into its monosaccharide constituents by hydrolysis with acid. It would thus be possible to explain the isolation of *dl*-galactose acetate from the products of acetolysis of agar (Pirie, *Biochem. J.*, 1936, **30**, 369).


(IX.)


(X.)


(XI.)

It may well be significant in considering the mode of the biological synthesis of agar that *d*-galactose 1-sulphate substituted at C_3 (XII) is convertible by a simple intramolecular oxidation-reduction change into *l*-galactose 6-sulphate substituted at C_4 (XVI) and the sequence of events involved in such a change could follow as illustrated in (XII)—(XVI).


(XII.) (XIII.) (XIV.) (XV.) (XVI.)

It is not unreasonable, therefore, to suppose that a sulphate fulfils a similar function in the biological synthesis of agar to that of a phosphate in the enzymatic synthesis of starch from glucose 1-phosphate (Hanes, *Proc. Roy. Soc.*, 1940, *B*, **128**, 421; **129**, 174). On this supposition, the initial material in the biological synthesis of agar is *d*-galactose 1-sulphate, from which is formed a chain of *d*-galactose residues combined by 1 : 3-glycosidic linkages. When ten such *d*-galactose members have been united, a change in the synthetic procedure becomes operative, and the tenth member, bearing its sulphuric acid group, undergoes the intramolecular transformation just described, whereby it is converted into the *l*-galactose residue. In the case of starch, the phosphoric acid, having fulfilled its function, is not retained by the starch, but with agar the sulphuric acid remains, with its orientation readjusted, as an integral part of the molecule.

EXPERIMENTAL.

Methylation of Agar.—Commercial agar (20 g.), dissolved in 15% sodium hydroxide solution (100 c.c.), was stirred at room temperature with methyl sulphate (430 c.c.) and 30% sodium hydroxide solution (1150 c.c.), added during 10 hours. After being heated for 1 hour on a boiling-water bath, the solution was cooled in ice, neutralised with 15% sulphuric acid, and made faintly alkaline. The methylated product was precipitated thereby as a grey powder, which was collected on a linen filter. The methylation was repeated under the same conditions. The third and subsequent methylations were conducted at 45° in the presence of acetone or dioxan. After eight methylations, crude methylated agar was obtained in a granular form.

Fractional precipitation of the methylated agar was carried out on batches of 40 g. Each batch was dissolved by being stirred for 48 hours with 1500 c.c. of alcohol–chloroform (1 : 10). The viscid solution was dried with anhydrous magnesium sulphate (which was removed in the centrifuge) and then treated with increasing volumes of light petroleum. The properties of the fractions so obtained are given in Table I.

TABLE I.

Batch.	Fraction.	Weight, g.	Ash, %.	OMe, % (corrected for ash).	Batch.	Fraction.	Weight, g.	Ash, %.	OMe, % (corrected for ash).
A	1	2·5	25·8	31·5	C	1	6·5	21·8	30·0
	2	18·5	3·9	33·2		2	4·9	14·8	31·6
	3	6·5	0·02	34·8		3	15·4	2·7	33·7
	4	0·3	0·0	38·4		4	3·3	0·5	34·6
B	1	12·6	17·4	31·0	D	1	9·6	15·6	31·8
	2	2·4	11·5	33·0		2	3·6	11·4	32·4
	3	25·0	2·7	34·1		3	9·8	1·7	32·3
	4	0·8	0·0	35·0		4	3·5	0·0	34·8

Dialysis of Methylated Agar.—All the fractions (Table I) having an ash content of more than 2% were combined (methylated agar A; 86 g.) and submitted to dialysis in batches of 20 g. Similarly, the fractions of low ash content were combined (methylated agar B; 11 g.). The batch for dialysis was dissolved in 2% sulphuric acid by being shaken for 48 hours. A parchment paper membrane was used, and the dialysis against running tap-water continued for 72 hours. The solution was maintained slightly acid by the addition of sulphuric acid in drops until near the end of the dialysis, which was continued until all sulphate ion had been removed. The dialysed solution was then evaporated at 50° and the residue was purified by precipitation from its solution in chloroform–alcohol with light petroleum. The dialysed material had OMe 33·6%, was acid in reaction, and had equivalent (by titration), 1870. The latter figure corresponds to one carboxyl group in ten hexose units.

A small portion of fraction B3 (Table I) was dialysed separately in sulphuric acid and yielded a sample of methylated agar A, which had OMe, 31·6% (ash corrected). Esterification of this sample with diazomethane resulted in a product with a methoxyl content of 32·5% (ash corrected). This increase in methoxyl content corresponds again to one carboxyl group in ten hexose units.

Further Methylation of Methylated Agar A.—The whole of the dialysed material was methylated in batches of 20 g. with methyl iodide and silver oxide. After four treatments the product had OMe, 38·2% (constant value) and showed $[\alpha]_D^{21^\circ} - 56{\cdot}85^\circ$ (*c*, 0·67 in chloroform). The increase in methoxyl was therefore *ca.* 5%, compared with the increase of 1% produced by diazomethane.

Methanolysis of the Fully Methylated Ester A.—The material (44·0 g.; OMe, 38·2%) was boiled with 2% methyl-alcoholic hydrogen chloride (616 c.c.) until no further change in rotation occurred ($[\alpha]_D - 51{\cdot}8^\circ \longrightarrow + 37{\cdot}8^\circ$ in 20 hours). After neutralisation with silver carbonate, the solution was evaporated and the syrupy residue (47·5 g.) was submitted to high-vacuum distillation. It thus became apparent that the methanolysis was far from complete and it was necessary repeatedly to treat with acid methyl alcohol the fraction of the product which did not distil. After four repetitions of this procedure, the undistillable residue was very small and four fractions of distillate were separated, each consisting of a mixture of ester and glycoside. The ester was separated by the treatment of each fraction with saturated barium hydroxide at 80° for 3 hours. The solution was then diluted, and the excess of barium hydroxide removed with carbon dioxide. The residue obtained after evaporation of the solution was dried and exhaustively extracted with boiling dry ether. The extract (N) contained no acidic constituents and the residue (R) was composed chiefly of barium salts.

The barium salts (R) from the four fractions were combined (4·9 g.) and esterified by being boiled for 9 hours with methyl-alcoholic hydrogen chloride (150 c.c.) of concentration sufficient to remove barium as chloride and to leave a 1% solution of hydrogen chloride. After neutralisation with silver carbonate the solution was evaporated, and the residual ester (3·6 g.) extracted with dry ether. Fractional distillation of the ester was only partly successful, and it was necessary to reconvert the ester into the barium salt, which was again extracted exhaustively with ether. A small amount (0·27 g.) of a non-acidic constituent was thus removed. The residual barium salt was again esterified by treatment with cold methyl-alcoholic hydrogen chloride and subsequently with diazomethane. The ester so obtained (1·4 g.) was separated into three fractions by distillation at 0·03 mm. pressure. The main fraction (2) (0·7 g.) had $[\alpha]_{D}^{17^\circ}$ ± 0° (*c*, 0·978 in methyl alcohol); OMe, 44·0% and equivalent, by titration, 253. (Methyl dimethyl anhydrohexonate, $C_9H_{18}O_7$, requires OMe, 39·1% and equivalent 238.)

2 : 5-*Dimethyl* 3 : 6-*Anhydro*-l-*galactonamide*.—The ester fraction 2 (49 mg.) was dissolved in dry methyl alcohol (5 c.c.) and the solution after saturation with ammonia was kept at 0° for 3 days. Evaporation of the alcohol left a syrup which crystallised. The crystals were separated by trituration with dry acetone and the product was recrystallised from acetone (yield of amide, 20% of weight of ester). The *amide* had m. p. 173° and $[\alpha]_{D}^{12^\circ}$ − 75·7° (*c*, 1·56 in water) (Found: C, 47·2; H, 7·4; N, 7·4; OMe, 30·5. $C_8H_{15}O_5N$ requires C, 46·8; H, 7·3; N, 6·8; OMe, 30·2%).

The amide gave a negative Weerman test for α-hydroxy-amides.

Examination of the Neutral Glycoside Mixture (*N*).—The material was fractionally distilled at 0·02—0·03 mm. over a range of 96—180° (bath temp.), and the following fractions were collected:

Fraction	1	2	3	4	5	6	7	8	9	10
Weight (g.)	3·2	3·5	2·5	6·6	3·8	6·4	0·7	3·6	1·5	0·8
OMe, %	60·4	57·2	52·4	—	—	—	—	48·0	43·1	41·4
$[\alpha]_{D}^{20^\circ}$	+114·5°	—	—	—	—	+52·8°	—	+34·5°	+1·0°	—

Fractions 2 to 6 were crystalline or partly crystalline.

Fraction 1 was not hydrolysed by 0·1N-sulphuric acid at 100°, but hydrolysis took place when the acid strength was increased to N ($[\alpha]_D$ + 124° ⟶ + 100° in 9 hours). The solution was neutralised and evaporated, and the residue extracted with ether. The extract yielded crystalline tetramethyl *d*-galactopyranose, m. p., alone and in admixture with an authentic specimen, 73—74° ($[\alpha]_{D}^{20^\circ}$ + 119·3°, equilibrium value in water).

Fractions 2—6 consisted mainly of 2 : 4 : 6-trimethyl αβ-methyl-*d*-galactoside (cf. Percival *et al.*, *loc. cit.*), which was also found in fraction 8. It was separated in crystalline form, m. p. 80° (softening at 65°) alone or in admixture with an authentic specimen. It had $[\alpha]_{D}^{20^\circ}$ + 57·6° in water (*c*, 0·64) (Found: C, 50·5; H, 8·3; OMe, 53·2. Calc. for $C_{10}H_{20}O_6$: C, 50·8; H, 8·5; OMe, 52·5%).

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