The Structure of the Oligosaccharides produced by the Enzymic Breakdown of Pectic Acid. Part I.

By J. K. N. Jones and W. W. Reid.

[Reprint Order No. 4859.]

Pectic acid is degraded by the enzymes of *Aspergillus fœtidus*, Thom and Raper, with the formation of D-galacturonic acid, digalacturonic acid, and trigalacturonic acid. Constitutional studies on the trisaccharide and the disaccharide are described. A summary of part of the work now described has appeared earlier (Jones and Reid, *Chem. and Ind.*, 1953, 303).

Pectic acid is believed to be composed of a large number of D-galactopyruronic acid residues, linked together by α-glycosidic linkages, with the resultant formation of a linear polysaccharide of high molecular weight (cf. Hirst and Jones, *Adv. Carbohydrate Chem.*, 1946, **2**, 235, for a review).

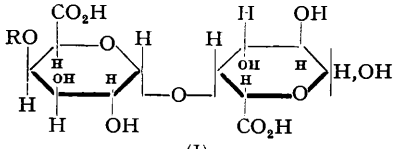
It is well known that pectic acid is rapidly degraded by enzymes which are produced by certain varieties of fungi. One of us (W. W. R.) has observed that *Aspergillus fœtidus*, Thom and Raper, under defined cultural conditions produces a (D)-type enzyme which degrades pectic acid to a mixture of D-galacturonic acid and two oligosaccharides only (Ayres, Dingle, Phipps, Reid, and Solomons, *Nature*, 1952, **170**, 834). From the mixture, by fractional precipitation, it is possible to obtain pure samples of a disaccharide and a trisaccharide. The efficiency of the fractionation of these galacturonic acid derivatives was followed on paper chromatograms. The di- and the tri-saccharide were purified as the calcium salts; that of the trisaccharide was difficult to obtain in a crystalline form. It was assumed that no resynthesis by enzymic action occurred during the formation of these oligosaccharides.

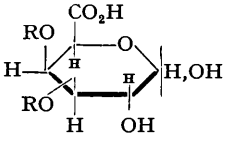
In order to determine the structure of the trisaccharide (I; R = III or II; R = III) the free acid was converted into the methyl glycoside of the methyl ester, the carbomethoxy-groups of which were then reduced in aqueous solution with sodium borohydride. The resulting galactose derivative was then methylated with sodium hydroxide and methyl sulphate, Purdie's reagents, and thallous ethoxide and methyl iodide, and the product was hydrolysed. There resulted a mixture of three sugars which were identified on paper chromatograms and separated cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511). The sugars were identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose, 2 : 3 : 6-tri-*O*-methyl-D-galactose and crystalline 2 : 6-di-*O*-methyl-D-galactose. The yields of sugars approximated to a ratio of 1 : 1·3 : 1. The isolation of these sugars suggests either (*a*) that the trisaccharide is of the unbranched-chain type (I; R = III), which on hydrolysis should yield one mole of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose and two moles of 2 : 3 : 6-tri-*O*-methyl-D-galactose, or (*b*) that the trisaccharide is of the branched-chain type (II; R = III), which should yield on hydrolysis two molecules of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose and one mole of di-*O*-methyl-D-galactose.

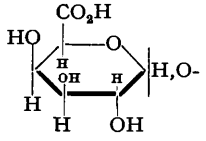
In either case the deviation of the ratios of the respective sugars from 1 to 2 and 2 to 1 may be due to incomplete methylation perhaps owing to formation of esters of boron acids (cf. experimental section) or perhaps to some steric factor. In view of the results of Hough, Jones, and Richards (*Chem. and Ind.*, 1953, **40**, 1064) further discussion is deferred until more information is available as to the effect of sodium borohydride on

oligosaccharides of this type. These reactions prove for the first time that some at least of the uronic acid residues in pectic acid are in the pyranose form. These results were reported in brief earlier (cf. Jones and Reid, *loc. cit.* *Note:* their formula I is incorrect).

Sufficient of the crystalline diuronide was available for its analysis, and the determination of its physical properties and rate of movement on the chromatogram. Since the isolation of this crystalline substance is a long and tedious procedure it was decided to convert apple pectic acid, by enzymic degradation with the (D)-type enzyme, into the resulting mixture of triuronide (60%), diuronide (20%), and galacturonic acid (20%), and then to fractionate this mixture after it had been converted into a mixture of methyl ester methyl glycosides. Accordingly the crude mixture of the calcium salts of these three galacturonic acid derivatives (Ayres *et al.*, *loc. cit.*) was shaken with cold methanolic hydrogen chloride until a sample no longer reduced Fehling's solution. The resulting methyl ester methyl glycosides were then fractionated on charcoal (Darco G 60) (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677), and the syrupy uronides of lower molecular weight which were eluted with water were isolated and methylated first with sodium hydroxide and methyl sulphate and then with Purdie's reagents. The carbomethoxy-groups in the mixture of methylated galacturonic acid derivatives were then reduced with lithium aluminium hydride and the resultant mixture of methylated galactose-containing oligosaccharides was again methylated with Purdie's reagents. With this procedure no complications due to the formation of boron derivatives are possible and the methylated derivatives may be further fractionated by distillation. Fractional distillation of this product gave two main fractions and a still residue. The first fraction was identified after


(I)


(II)


(III)

hydrolysis as mainly 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose, and the second fraction, after hydrolysis and separation on cellulose, yielded a mixture of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose, 2 : 3 : 6-tri-*O*-methyl-D-galactose, and 2 : 3 : 4-tri-*O*-methyl-D-galactose. The ratio of "tetra and 2 : 3 : 4-tri" to "2 : 3 : 6-tri" was about 1 : 1). Evidently some of the hydroxyl groupings on C_6 had escaped methylation. Accordingly the still residue (fraction 3) was remethylated and the product distilled and hydrolysed. Only two sugars resulted: 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose and 2 : 3 : 6-tri-*O*-methyl-D-galactose. It is evident that the original product consisted of a D-galactopyranose residue linked to a second D-galactose residue through C_4 or C_5. The linkage joining the two sugar residues is very probably of the α-glycosidic type since it results from the reduction of a disaccharide with a high positive optical rotation (+160°).

It follows that there was present in the enzymic hydrolysate a disaccharide (I; R = H) composed of a D-galacturonic acid residue in the pyranose form, linked very probably by an α-link to a second D-galacturonic acid residue, and that the linkage was very probably through $C_{(4)}$ because the disaccharide possesses a high positive rotation.

Experimental

The following solvents were used in chromatographic separation on Whatman No. 1 paper: (*a*) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4); (*b*) *n*-butanol–pyridine–water (10 : 3 : 3); (*c*) *n*-butanol–ethanol–water (40 : 11 : 19); and (*d*) ethyl acetate–acetic acid–water (3 : 1 : 3) (top phase); all v/v. Ammoniacal silver nitrate (Partridge, *Biochem. J.*, 1948, **42**, 238) and *p*-anisidine hydrochloride solution were used as spray reagents to detect glycosides and reducing sugars, respectively. With solvent mixture (*d*), bromophenol blue (0·2% of the sodium salt in 50% v/v ethanol) was used to detect acids. Optical rotations were determined at 20° ± 2° in water, unless otherwise stated. Microanalyses are by Mr. B. S. Noyes of Bristol. Evaporation of solutions was carried out under reduced pressure.

By the action of a (D)-type enzyme preparation from *Aspergillus fœtidus*, Thom and

Raper, on apple pectic acid at pH 3·5 a mixture of mono-, di-, and tri-galacturonic acids was obtained (Ayres, Dingle, Phipps, Reid, and Solomons, *loc. cit.*). The calcium salts of these acids were repeatedly precipitated from 60% v/v ethanol in which the calcium salt of galacturonic acid is soluble. The calcium salts of the di- and tri-galacturonic acids, which were insoluble in 60% ethanol, were fractionated from aqueous solution with ethanol. The fractions from the lower ethanol concentrations were pure samples of the calcium trigalacturonate, and those from the higher alcohol concentrations were pure calcium digalacturonate. The intermediate fractions (about 80% of the starting material) were mixtures of the two compounds. The samples of the calcium salts of the di- and tri-galacturonic acids did not crystallise from water. An aqueous solution of each sample was therefore treated with ZeoCarb 225 to remove calcium ions. The filtrate and washings were then evaporated to dryness under reduced pressure, and the dry acid mixed with the equivalent amount of dry calcium carbonate. On the addition of a few drops of water, the mass dissolved with effervescence and in each case crystallised immediately. The crystalline calcium di- and tri-galacturonates so obtained, were used to inoculate aqueous solutions of the mixed calcium di- and tri-galacturonates. The mixtures were left to crystallise in desiccators over sulphuric acid; after 2—3 weeks a crystalline mush was formed in each case. The crystals were collected and washed with water. The nearly pure samples so obtained were suspended in water, and ZeoCarb 225 was added with stirring until the crystals had dissolved. The resin was filtered off and washed with water, and the filtrates and washings were neutralised with calcium carbonate, filtered, and concentrated. The thin syrups so obtained were inoculated with the appropriate seed crystals and left over sulphuric acid for 2—3 weeks to crystallise. The white crystalline mush was filtered off (sintered glass), washed with water, ethanol, and ether, and dried at room temperature. The crystalline calcium salts were chromatographically pure [solvent (*d*)]. In some experiments the calcium trigalacturonate set to a stiff gel. When this was ground in a mortar with water, an amorphous, chromatographically pure calcium salt was obtained. It was not soluble in water but dissolved readily in dilute mineral acids.

Trigalacturonic Acid.—The *calcium salt* crystallised from water as rosettes of minute needles, and was dried at room temperature. It had $[\alpha]_D$ +154° (*c* 1·44, in N-HCl) (Found: C, 29·5; H, 5·2; Ca, 8·35. $C_{18}H_{23}O_{19}1\frac{1}{2}Ca,7H_2O$ requires C, 29·6; H, 5·1; Ca, 8·2%). When dried to constant weight, the sample lost weight (Found: loss, 10·3. Calc. for $4H_2O$: loss, 9·7%). The residual *trihydrate* (Found: C, 33·3; H, 4·4. $C_{18}H_{23}O_{19}1\frac{1}{2}Ca,3H_2O$ requires C, 33·2; H, 4·3%) was hygroscopic. A sample of the calcium salt (equivalent to 1 mmol. of anhydrous trisaccharide) gave, on complete hydrolysis with an (S)-type pectic enzyme preparation (Ayres *et al.*, *loc. cit.*), only D-galacturonic acid which was detected on paper chromatograms [solvents (*c*) and (*d*)] (Found, by hypoiodite oxidation: 2·96 mmol.; galacturonic acid requires 3·00 mmol.).

The free *acid* (1·30 g.) was prepared from the calcium salt by deionisation with ZeoCarb 225 [Found: equiv., 191 (by titration); *M*, 557 (after oxidation with hypoiodite). $C_{18}H_{26}O_{19},H_2O$ requires equiv., 188; *M*, 564)]. The dried *acid* (1·23 g.) (Found: C, 39·6; H, 4·9. $C_{18}H_{26}O_{19}$ requires C, 39·5; H, 4·8%) was dissolved in dry methanolic hydrogen chloride (1·5% w/v; 100 c.c.), and the solution set aside in a stoppered vessel at room temperature until a sample no longer reduced Fehling's solution (24 hr.). The solution was then neutralised (Ag_2CO_3), filtered, and evaporated. The residual glassy solid (1·2 g.) was dissolved in water (50 c.c.), and reduced with sodium borohydride (0·4 g.). The solution was left for 7 days at room temperature, it was then acidified with acetic acid and filtered, and the filtrate deionised by a column of Amberlite resins IR120 and IR4B. The neutral effluent was concentrated to a solid (0·98 g.) which was dried by dissolution in methanol followed by removal of the solvent. It had $[\alpha]_D$ +190° (*c*, 9·8) (Found: OMe, 13·1. Calc. for $C_{19}H_{28}O_{19}$: OMe, 5·6%). The reason for this high methoxyl value is obscure but it may be due to contamination of the product with some non-reduced methyl ester of the trigalactosiduronic acid or with boric esters. A portion (0·89 g.) was methylated with sodium hydroxide and methyl sulphate (twice), silver oxide and methyl iodide, thallium ethoxide and methyl iodide, and finally with silver oxide and methyl iodide. The product (0·73 g.) (Found: OMe, 46·3. Calc. for $C_{29}H_{54}O_{16}$: OMe, 52·1%) was a thick yellow syrup which could not be distilled without decomposition. A portion (0·69 g.) was hydrolysed with boiling N-hydrochloric acid (10 c.c.) for 8 hr. ($[\alpha]_D$ +190° ⟶ +101°, constant value). The cooled solution was neutralised (Ag_2CO_3) and filtered before and after the passage of hydrogen sulphide, and the filtrate concentrated to a syrup (0·65 g.). Paper-chromatographic examination of a portion of the syrup [solvent (*b*)] indicated the presence of three sugars with R_G * 0·93, 0·84, and 0·64, in approximately equal proportions. The syrup

* R_G is the rate of movement relative to tetra-*O*-methylglucopyranose.

was fractionated on cellulose, the top layer of benzene–ethanol–water (34 : 10 : 3 v/v) being used as mobile phase. Concentration of the appropriate fractions of the effluent from the column gave: Fraction 1 (0·166 g.), $[\alpha]_D$ +104° (*c*, 1·4), identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose by its rate of movement on the chromatogram [solvents (*a*), (*b*), and (*c*)] and characterised as the *N*-phenylgalactosylamine, m. p. and mixed m. p. with an authentic specimen 194°. Fraction 2 (0·219 g.), $[\alpha]_D$ +89° (*c*, 3·6) (Found: OMe, 42·1. Calc. for $C_9H_{18}O_6$: OMe, 41·9%), moved at the same rate as 2 : 3 : 6-tri-*O*-methyl-D-galactose on the chromatogram [solvents (*a*), (*b*), and (*c*)] and after oxidation with bromine water under the usual conditions yielded 2 : 3 : 6-tri-*O*-methyl-D-galactofuranolactone, m. p. 99° not depressed in admixture with an authentic specimen (Found: OMe, 40·4. Calc. for $C_9H_{16}O_6$: OMe, 42·3%). Fraction 3 (0·19 g.), $[\alpha]_D$ +82° (*c*, 3·8), slowly crystallised. The substance was recrystallised from acetone–ether–light petroleum (b. p. 40—60°) and then had m. p. 131°, not depressed on admixture with an authentic specimen of 2 : 6-di-*O*-methyl-D-galactose (Found: C, 46·1; H, 7·5; OMe, 31·3. Calc. for $C_8H_{16}O_6$: C, 46·1; H, 7·7; OMe, 29·8%). After the crystals had been kept in air, the m. p. fell, owing to formation of a hydrate of 2 : 6-di-*O*-methyl-D-galactose.

Digalacturonic Acid.—(*a*) A small amount of the *calcium salt* of the disaccharide was crystallised from water and dried over calcium chloride; the sheaves of needles had $[\alpha]_D$ +119° (*c*, 1·30 in N-HCl) (Found: C, 29·4; H, 5·3; Ca, 8·1. $C_{12}H_{16}O_{13}Ca,5H_2O$ requires C, 29·0; H, 5·2; Ca, 8·2%). When dried the pentahydrate lost weight (Found: loss, 13·1. Calc. for $4H_2O$: loss, 14·4%). The residual *monohydrate* (Found: C, 33·8; H, 4·9. $C_{12}H_{16}O_{13}Ca,H_2O$ requires C, 33·4; H, 4·2%) was extremely hygroscopic. Part of the crystalline calcium salt was converted into the free acid with ZeoCarb 225 and concentrated to a glassy solid (Found: equiv., by titration, 202; *M*, after oxidation with hypoiodite, 390. $C_{12}H_{18}O_{13},H_2O$ requires equiv., 199; *M*, 398). A sample of the calcium salt (equivalent to 1 mmole of anhydrous disaccharide) gave, on complete hydrolysis with an (S)-type pectic enzyme preparation (Ayres *et al.*, *loc. cit.*), D-galacturonic acid only, detected on paper chromatograms [solvents (*c*) and (*d*)] (Found: by hypoiodite oxidation, 2·02 mmole. Required, galacturonic acid, 2·00 mmole). The oxidation of a sample of this material with bromine water gave inconclusive results; similarly its reduction with sodium borohydride or sodium amalgam did not give a crystalline product.

(*b*) A mixture of oligosaccharides obtained by the action of a (D)-type pectic enzyme [from *Aspergillus fœtidus*, Thom and Raper, (Ayres *et al. loc. cit.*)] on apple pectic acid was used as starting material. Chromatographic examination [solvent (*a*)] of this material showed that it contained D-galacturonic acid, much di- and tri-saccharides, and traces of oligosaccharides of high molecular weight. The rates of movement relative to galactose in solvent (*a*) were galacturonic acid, 0·96 (weak); disaccharide, 0·29 (strong), 0·18 (faint); trisaccharide, 0·12 (very strong).

The crude mixture of calcium salts (20 g.) was shaken with methanolic hydrogen chloride (2% w/v; 250 c.c.); much dissolved rapidly, and after 48 hr. at 20° the solution was non-reducing. Hydrogen chloride was removed by addition of silver carbonate, and the solution was filtered and concentrated to a syrup (14 g.). Chromatographic examination [solvent (*b*)] on paper showed at least seven components moving at the following rates [relative to rhamnose (1·0)]: 1·7, 1·5, 1·0, 0·7, 0·55, 0·35, and 0·08. The picture had been complicated by the formation of the glycoside anomers. Accordingly the syrup was fractionated on Celite–charcoal (22 × 5 cm. diam.) (Whistler and Durso, *loc. cit.*). Elution with water and concentration of the effluent gave a syrup (9·1 g.) which contained five components [solvent (*b*)] whose rate of movement relative to rhamnose was 0·7, 0·55, 0·4, 0·16, and 0·18, the second and the third component predominating. The mixture of non-reducing glycosides was methylated with sodium hydroxide (30%) and methyl sulphate six times, by the usual procedure; the reaction mixture was concentrated between each operation. The solution was then acidified with sulphuric acid and extracted exhaustively with chloroform. Concentration of the extract gave a syrup (5 g.) (Found: OMe, 25·0%), which was again methylated with Purdie's reagents, and the product (4·18 g.), n_D^{20} 1·4780, $[\alpha]_D$ +120° (*c*, 1·8 in MeOH) (Found: OMe, 47·1%), was reduced with lithium aluminium hydride in dry ether. Excess of lithium aluminium hydride was destroyed by water, the slurry of aluminium hydroxide, ether, and water was filtered, the residue was well washed with water, and the filtrate was concentrated to dryness. Extraction of the residue with chloroform gave a syrupy galactose derivative (3·08 g., from 3·4 g. of galacturonic acid derivative). This product was again methylated with Purdie's reagents (yield 2·76 g.) and then distilled *in vacuo* giving: fraction 1 (0·4 g.), b. p. 120°/0·5 mm., n_D^{20} 1·4482 (Found: OMe,

59·7%); fraction 2 (0·17 g.), b. p. 120—150°/0·5 mm., n_D^{20} 1·4518 (Found: OMe, 57·6%); fraction 3 (0·91 g.), b. p. 225—240°/0·5 mm., n_D^{19} 1·4740 (Found: OMe, 50·0%); and a residue (1·3 g.) (Found: OMe, 41·3%).

Fractions 1 and 2 were combined and hydrolysed with boiling N-sulphuric acid. Chromatographic examination [solvent (*b*)] indicated that 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose was the major component, together with some 2 : 3 : 5 : 6-tetra-*O*-methyl-D-galactose and traces of trimethyl-D-galactose. The sugars were isolated in the usual way and heated with a solution of aniline in ethanol whereupon 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m. p. and mixed m. p. 194°, was produced.

Fraction 3 (0·88 g.) was hydrolysed with boiling N-hydrochloric acid (25 c.c.) for 6 hr. The optical rotation was not observable owing to the darkening of the solution. The solution was neutralised by addition of silver carbonate and filtered before and after the passage of hydrogen sulphide. Concentration of the filtrate gave a syrup (0·8 g.), $[\alpha]_D$ +89° (*c*, 2·5), examination of which on the paper chromatogram showed presence of three components [R_G 0·93, 0·83, and 0·77, in solvent (*c*)]; the first two gave a red colour with the *p*-anisidine hydrochloride spray, the third a brown colour. The syrup was fractionated on cellulose, benzene–ethanol (19 : 1 v/v) being used as eluent. By concentration of the appropriate portion of the effluent three main fractions were obtained and were identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose (0·29 g.), $[\alpha]_D$ +107° (*c*, 5·0) [the *N*-phenylgalactosylamine derivative had m. p. 193°]; 2 : 3 : 6-tri-*O*-methyl-D-galactose (0·203 g.), $[\alpha]_D$ +57° ⟶ +90° (*c*, 3·3 in water) (2 : 3 : 6-tri-*O*-methyl-D-galactofuranolactone, m. p. and mixed m. p. 90°); and 2 : 3 : 4-tri-*O*-methyl-D-galactose (0·10 g.), m. p. (hydrate) 80°, $[\alpha]_D$ +113° (*c*, 1·7) (the *N*-phenyl-D-galactosylamine derivative had m. p. 169°, not depressed in admixture with an authentic specimen). The rates of movement of these sugars on paper chromatograms were not distinguishable from those of authentic specimens. After separation of these sugars, the cellulose column was eluted with alcohol. Concentration of the solvent left a syrupy mixture of di-*O*-methylgalactoses (0·04 g.).

The still residue (1·3 g.) was dissolved in water and methylated three times with 30% sodium hydroxide and methyl sulphate and the product isolated by chloroform extraction. Concentration of the extract gave a syrup (0·81 g.) which was again methylated (Purdie's reagents) and distilled. The product (0·75 g.) had b. p. 210°/0·3 mm., $[\alpha]_D$ +150° ± 20° (*c*, 0·12, in MeOH) (micropolarimeter tube) (Found: OMe, 54·4. Calc. for $C_{20}H_{38}O_{11}$: OMe, 54·6%). This product was hydrolysed with boiling N-hydrochloric acid for 6 hr., and the methylated sugars isolated in the usual way. Chromatographic analysis of the syrup (0·71 g.) indicated that it contained two sugars only. These were separated on cellulose, benzene–ethanol (19 : 1 v/v) being used as mobile phase, and were identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose (0·33 g.), $[\alpha]_D$ +112° (*c*, 3·3) (Found: OMe, 52·0. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%) (the *N*-phenylgalactosylamine derivative had m. p. 194°, not depressed on admixture with an authentic specimen), and 2 : 3 : 6-tri-*O*-methyl-D-galactose (0·3 g.), $[\alpha]_D$ +88° equilibrium (*c*, 3·0) (Found: OMe, 42·0. Calc. for $C_9H_{18}O_6$: OMe, 41·9%), which was identified after oxidation with bromine water as 2 : 3 : 6-tri-*O*-methyl-D-galactofuranolactone, m. p. 96° not depressed on admixture with an authentic specimen.

CHEMISTRY DEPARTMENT, BRISTOL UNIVERSITY.
RESEARCH DEPARTMENT, H. W. CARTER & CO., LTD., COLEFORD, GLOUCESTERSHIRE.
[Present address (J. K. N. J.): QUEEN'S UNIVERSITY, KINGSTON, ONTARIO, CANADA.]

[*Received, December 2nd,* 1953.]