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THE CONSTITUTION OF THE AMYLOID FROM SEEDS OF *ANNONA MURICATA* L.

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Abstract—The preparation of amyloid, an alkali-soluble polysaccharide, from seeds of *Annona muricata* L. is described. The gross structure of the polysaccharide was studied by methylation and fragmentation techniques. Partial hydrolysis of the polysaccharide by *Myrothecium*-cellulase, with or without cellobiase, liberated a tetra- and a penta-saccharide together with glucose, cellobiose and a small quantity of a xylosyl-cellobiose. Comparison of the molar amounts of the products of hydrolysis by cellulase led to the conclusion that the molecule of *Annona*-amyloid is essentially a polymer of *O*-β-D-galactopyranosyl-(1 → 2)-*O*-α-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucose resulting in a linear chain of 1,4-β-D-glucosyl units with side chains of galactosyl–xylosyl residues attached at regular intervals. Periodate oxidation data were consistent with this structure. The molecular weight was found to be *ca.* 10,000. In its properties *Annona*-amyloid is intermediary between cellulose and *Tamarindus*-amyloid.

INTRODUCTION

IN A RECENT study on the occurrence of amyloids* in plant seeds,[3] species of *Annonaceae* were found to be anomalous in that their endosperm cell-walls were stained brownish-violet by the iodine reagent instead of blue, which is the normal color for amyloids. Since there are no reports in the literature concerning the chemical composition of the cell-walls of seeds of *Annonaceae*, the amyloid from seeds of one species of this plant family was isolated and a study of its chemical structure carried out. It seemed worth while to compare its structure with that of the classical amyloid, viz. *Tamarindus*-amyloid[4] in order to be able to explain the observed difference in staining by the iodine reagent.

RESULTS AND DISCUSSION

The starting material for the preparation of *Annona*-amyloid were the decorticated seeds of *Annona muricata* L. which were first ground and defatted. The powder was extracted with water, 0·2 N sodium hydroxide, and finally 2 N sodium hydroxide. The aqueous extract contained no appreciable amounts of carbohydrate, while the 0·2 N sodium hydroxide extract contained material which on acid hydrolysis and subsequent paper chromatographic examination was found to contain glucose, xylose, galactose, mannose, uronic acid and presumably some rhamnose. In this extract it therefore seemed probable that an amyloid and a mannan (or a galactomannan) were present as the main polysaccharides. The 2 N sodium hydroxide extract was neutralized with acetic acid and the precipitate formed was the

* As early as 1839 Vogel and Schleiden[1] introduced this designation for cell-wall substances which are stained blue by iodine. Much afterwards it was found that the blue-staining materials are polysaccharides composed of glucose, xylose, and galactose residues.[2]

[1] TH. VOGEL and M. J. SCHLEIDEN, *Pogg. Ann. Phys. Chem.* **46**, 327 (1839).
[2] P. KOOIMAN, *Onderzoek van amyloid uit zaden*, Diss. Delft (1959).
[3] P. KOOIMAN, *Acta Botan. Neerl.* **9**, 208 (1960).
[4] P. KOOIMAN, *Rec. trav. chim.* **80**, 849 (1961).

crude amyloid (yield 27 per cent of defatted seeds). It was purified by precipitation as the copper complex and after regeneration had $[\alpha]_D + 32{\cdot}7^\circ$ (C 1, 0·1 N NaOH). The purified amyloid was stained violet-red by the usual iodine reagent for amyloids, and violet-blue by stronger iodine solution. The absence of starchy material was demonstrated by the inability of salivary amylase to hydrolyze the substance responsible for the color reaction.

After hydrolysis of the purified amyloid in dilute mineral acid glucose, xylose and galactose were present in a ratio of 4·00 : 1·09 : 1·01.

The polysaccharide was methylated and the fully methylated product had $[\alpha]_D + 18{\cdot}3^\circ$ (C 1, $CHCl_3$). After hydrolysis of the product, a mixture of methyl ethers of the constituent sugars was obtained; this mixture was resolved into its components by paper chromatography. The methyl sugars were identified as 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, and 3,4-di-*O*-methyl-D-xylose; they were present in a molar ratio of 0·05 : 1 : 3 : 1 : 1.

Partial acid hydrolysis of the polysaccharide liberated a mixture of mono- and oligosaccharides, one of which behaved on the paper chromatograms as *O*-β-D-galactopyranosyl-(1 → 2)-D-xylose. The ethanol-insoluble product of partial acid hydrolysis consisted practically exclusively of glucose-residues as was found by hydrolyzing the material and examining the hydrolyzate by means of paper chromatography (solvent A). An X-ray diagram of the ethanol-insoluble product was very similar to that of cellulose II.

Annona-amyloid, when subjected to hydrolysis by the fungal enzyme preparation Luizym, yielded glucose, galactose and *O*-α-D-xylopyranosyl-(1 → 6)-D-glucose in a molar ratio of 1·00 : 0·32 : 0·46, corresponding to a molar ratio of 4·00 : 1·2 : 0·9 for glucose, xylose and galactose respectively. These results indicate a structure for the polysaccharide which is highly related to that of *Tamarindus*-amyloid; the polysaccharide molecule from *Annona* consists of a linear backbone chain of 1,4-β-D-glucosyl residues with side chains of 2-*O*-β-D-galactosyl-α-D-xylosyl residues attached at C_6 of one-fourth of the glucosyl residues. Probably a minor part of the xylosyl residues is not substituted by galactosyl residues.

By the action of *Myrothecium*-cellulase (either the untreated preparation containing both cellulase and cellobiase, or the heated enzyme solution containing only cellulase) *Annona*-amyloid was hydrolyzed, glucose and some oligosaccharides being liberated. The composition of the sugar mixture liberated by untreated cellulase was different from the same liberated by heated cellulase; this is indicated in Table 1. Apart from a small quantity of undissolved material removed at the end of the period of hydrolysis, some 5–10 per cent of the material was sacrificed for locating the sugars on the chromatograms. Within the experimental error the total amount of sugars in Table 1 accounts for all of the starting material.

TABLE 1. SUGARS LIBERATED FROM *Annona*-AMYLOID (250 mg) BY *Myrothecium*-CELLULASE

	Method of hydrolysis			
	Heated cellulase		Untreated cellulase	
	(mg)	(mmoles)	(mg)	(mmoles)
Glucose	29	0·16	65	0·36
Oligosaccharide *A*1	16	0·05	7	0·02
Oligosaccharide *A*2	7	0·02	12	0·03
Oligosaccharide *A*3	22	0·04	104	0·16
Oligosaccharide *A*4	146	0·18	39	0·05

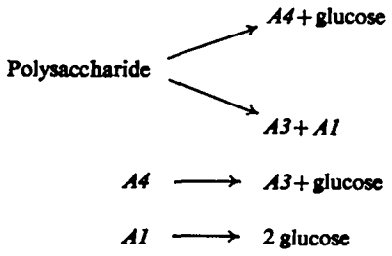
Oligosaccharide *A*1 had the same R_f value as cellobiose and after hydrolysis by dilute acid paper chromatographic examination showed the presence of only glucose. The acetylated product could be identified as cellobiose octaacetate.

Oligosaccharide *A*2 behaved on the paper chromatograms as either a di- or a tri-saccharide, and moved with the same speed as oligosaccharide 2 from *Tamarindus*-amyloid; the latter has been shown to be *O*-α-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucose.[4] *Annona*-oligosaccharide *A*2 was hydrolyzed by Luizym into approximately equal molar quantities of D-glucose and *O*-α-D-xylopyranosyl-(1 → 6)-D-glucose; therefore the structure of *A*2 is probably identical with *Tamarindus*-oligosaccharide 2.

Oligosaccharide *A*3 could be formed from *A*4 by the action of untreated cellulase, glucose being liberated. Both *A*3 and *A*4 could be degraded by Luizym; the end-products of hydrolysis were D-glucose, D-galactose and *O*-α-D-xylopyranosyl-(1 → 6)-D-glucose in molar ratios of 1·00:0·98:1·00 for *A*3, and of 2·00:0·97:1·12 for *A*4. In *A*3 and in *A*4 the glucose contents were therefore 50 and 60 mole per cent respectively. After reduction of the oligosaccharides with potassium borohydride the glucose contents were 36 and 49 per cent respectively, as determined in the hydrolyzates using the glucose oxidase method. These results proved that the reducing ends of the oligosaccharide molecules consisted of glucose residues.

The molecular weights of *A*3 and *A*4 were determined using the hypoiodite method. Values of 708 and 865 respectively were obtained; calculated values for *A*3 and *A*4 as a tetrasaccharide and a pentasaccharide respectively were 636 and 798.

With the aid of the respective calculated molecular weights the amounts of sugars liberated from amyloid by the action of cellulase were converted to millimoles (Table 1). On a molar basis the quantities of glucose and *A*4 in the digest with heated cellulase were very similar, while in the digest with untreated cellulase the quantity of glucose was about twice that of *A*3. This suggested that *Annona*-amyloid consists mainly of repeating units of *A*4 with a glucose residue attached to it, or, in other words, of *A*3 with two glucose residues attached. The stoichiometry was more evident when the liberated cellobiose (oligosaccharide *A*1) was also considered. From the equations



it is inferred that the ratio $\frac{\text{glucose} + 2 \times A1}{A4 + 2 \times A3}$ should be 1; the values obtained were 1·00 in the experiment with heated, and 1·08 with untreated cellulase. The repeating unit accounted for the major part of the molecule of *Annona*-amyloid, the only other oligosaccharide found (*A*2) occurring in the digest in amounts of approximately 5 per cent.

The difference between heated and untreated cellulase—in so far as it is of interest in this connexion—was that the latter enzyme preparation had an active cellobiase while the former had not. While cellulase itself (as exemplified in the digest with heated cellulase) liberated 3–4 times more glucose and *A*4 than cellobiose and *A*3, the combination, cellulase and cellobiase, liberated practically only glucose and *A*3. The small quantities of cellobiose

and *A*4 in the latter instance may be considered as intermediate products, which would be convertible into glucose and *A*3 on prolonged incubation with the enzyme preparation.

The production of glucose and *A*3 by heated cellulase may be explained by assuming the presence of traces of cellobiase. Alternately it is conceivable that the way of cleavage of the repeating unit by cellulase is influenced by a steric factor, viz. the galactosylxylosyl side-chain, resulting in the preference for the production of *A*4 and glucose over *A*3 and cellobiose.

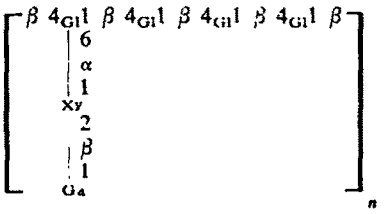
Determination of the molecular weight of *Annona*-amyloid using the hypoiodite method yielded a value of 11,600, corresponding to a degree of polymerization of 74.*

Periodate oxidation of reduced *Annona*-amyloid and subsequent determination of the amount of formaldehyde formed led to a molecular weight value of 8800 (corresponding to a degree of polymerization of 56), assuming the production of 2 molecules of formaldehyde per polysaccharide molecule (from carbon atoms 1 and 6 of the reduced glucose end-group).

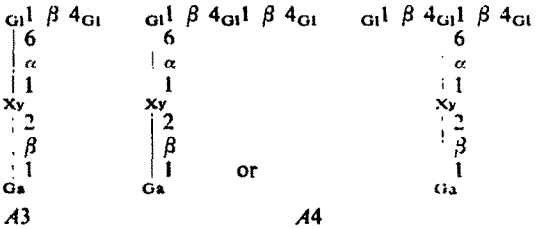
Periodate oxidation experiments showed that *Annona*-amyloid consumed 1·16 moles of periodate per mole of anhydrosugar, while 0·20 moles of formic acid per mole of anhydrosugar were produced. A polymer of *O*-β-D-galactopyranosyl-(1 → 2)-*O*-α-D-xylopyranosyl-(1 → 6)-cellotetraose joined as to give a polysaccharide with a cellulosic main chain and a molecular weight of approximately 10,000 should consume 1·21 moles of periodate and produce 0·21 moles of formic acid per mole of anhydrosugar. The correspondence between the experimental and the expected data is rather close.

The evidence presented favors the conception that *Annona*-amyloid is a polysaccharide built from repeating units of *O*-β-D-galactopyranosyl-(1 → 2)-*O*-α-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucose with a minor (*ca.* 5%) contribution of *O*-α-D-xylopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucose units to form a linear chain of 1,4-β-D-glucosyl residues with side chains of galactosylxylosyl residues attached at regular intervals.

Schematically this structure is:



Taking into account the specificity of *Myrothecium*-cellulase towards substituted celluloses[4,5] the most probable structures for the oligosaccharides *A*3 and *A*4 are:



* With the same method the molecular weight of *Tamarindus*-amyloid was found to be 10,750, sharply contrasting to the value of 115,000 given by the dinitrosalicylic acid method.

[5] W. KLOP and P. KOOIMAN, *Biochim. Biophys. Acta* **99**, 102 (1965).

As compared to cellulose and to *Tamarindus*-amyloid, the constitution of *Annona*-amyloid is intermediary.

This is reflected by the behavior of *Annona*-amyloid towards iodine to which it has less affinity than *Tamarindus*-amyloid, while the iodine affinity of *Annona*-amyloid is larger than that of cellulose. Also the solubility of *Annona*-amyloid is intermediate, *Tamarindus*-amyloid being soluble in water, while *Annona*-amyloid, once isolated from the cell-walls, can only be dissolved in 0·1 N sodium hydroxide, but not in water. Again, the specific optical rotation reflects the intermediary structure of the investigated polysaccharide (*Annona*-amyloid + 32·7°, *Tamarindus*-amyloid + 79·6°, cellulose ± 0°). The intermediate behavior is conceivable since the main chain (being cellulosic in amyloids) of *Annona*-amyloid is less substituted than in *Tamarindus*-amyloid.

Since the endosperm cell-walls of all *Annonaceae* tested for the amyloid reaction[3] gave the same brownish-violet color with the iodine reagent it is likely that the amyloids from species of this plant family all have similar intermediary constitutions.

EXPERIMENTAL

All specific rotations are equilibrium values, melting points are corrected, and all evaporations were done at 35° or less.

Chromatographic separations were made by the descending method on either Whatman No. 1 or No. 3 MM filter paper using the following solvents (v/v): (A) *n*-butanol, pyridine, water—6:4:3; (B) butanone, water—2:1; (C) *n*-butanol, ethanol, water—5:1:4.

Sugars were located on the papers by the *p*-anisidine phosphate spray reagent.[6]

The color reagent for amyloid was a solution of iodine (0·3 g) and potassium iodide (1·6 g) in 100 ml water. A stronger solution was composed of iodine (1 g) and potassium iodide (2 g) dissolved in 100 ml water.

Preparation of Crude Annona-*Amyloid*

The kernels of decorticated seeds of *Annona muricata* L. were finely ground in a hammer-mill and exhaustively extracted with benzene–ethanol (1:1) in a Soxhlet apparatus. The dried residue (90 g) was stirred in water (1 l.) at room temperature for 2 days with some toluene and thymol added to prevent microbial contamination. The undissolved material was separated by centrifugation and extracted with 0·2 N NaOH (1 l.) at room temperature for 1 day; the residual material was subjected to two similar extractions. Then the residue was extracted three times with 2 N NaOH (1 l.), each extraction being continued for 2 days. The 2 N NaOH extracts were pooled, and neutralized with acetic acid; part of the dissolved material precipitated and was separated by centrifuging. The precipitate was washed successively with 50% ethanol (three times) then with 96% ethanol (three times), and finally with ethyl ether (three times). Ether was removed on the steam bath and the product was dried *in vacuo* at room temperature. The yield was 24·3 g of crude amyloid.

Hydrolysis of a sample (5 mg) in a sealed tube with N H_2SO_4 (0·5 ml) for 4 hr at 105°, neutralization of the hydrolyzate with $BaCO_3$, removal of insoluble salts by filtration, and examination of the sugars by paper chromatography in solvent (A) showed the presence of glucose, xylose, galactose and a trace of mannose.

The aqueous extract contained 8·9 g of solid material the carbohydrate content of which was negligible. The 0·2 N NaOH extract and the filtrate from the neutralized 2 N NaOH extract contained a polysaccharide mixture (together 16·8 g); acid hydrolysis and subsequent paper chromatographic analysis (solvent A) showed the presence of glucose, xylose, galactose, mannose, uronic acid, and probably some rhamnose. From the undissolved residue (24·3 g) a small portion was hydrolyzed and the hydrolyzate subjected to paper chromatographic analysis; glucose was present as the major component together with traces of xylose, mannose, uronic acid and probably rhamnose.

Purification of Crude Annona-*Amyloid*

Crude *Annona*-amyloid (5 g) was dissolved in 0·1 N NaOH (500 ml) and the solution was centrifuged to remove a small quantity of brown insoluble material. Fehling solution (20 ml diluted to 100 ml) was added, upon which a gel formed which was filtered on cloth and rinsed with water. The gel particles were suspended in water (500 ml), and 2 N HCl (10 ml) was added to decompose the copper complex. The regenerated polysaccharide was separated, washed with water and again dissolved in 0·1 N NaOH (500 ml). The copper complexing was repeated and after decomposing the complex with HCl an equal volume of ethanol was added.

[6] S. MUKHERJEE and H. C. SRIVASTAVA, *Nature* **169**, 330 (1952).

The precipitated material was separated by centrifuging and washed successively with 50% ethanol (three times), 96% ethanol (three times), and ethyl ether (three times). Ether was removed on the steam bath and the product was dried *in vacuo* at room temperature. The yield was 2·1 g.

The polysaccharide was dissolved in dilute alkali to give a clear solution $[\alpha]_D^{20}+32·7°$ (C 1, 0·1 N NaOH). Chromatographic inspection of a hydrolyzate in solvent (A) showed that the purified polysaccharide contained glucose, xylose and galactose.

Determination of Sugar Composition of the Hydrolyzate

A sample of the neutralized hydrolyzate was applied to Whatman No. 1 filter paper and subjected to chromatography in solvent (A). The sugar spots were located with the aid of guide strips and eluted with 50% ethanol. After evaporating the solvent the amounts of sugars were determined using a modification of the hypoiodite method of Willstätter and Schudel.[7]

Methylation of Annona-*amyloid and Hydrolysis of the Methylated Product*

The purified polysaccharide (5·0 g) was methylated in the usual way by the Haworth procedure followed by the Purdie method until the product (4·4 g) had 38·6% methoxyl and did not show hydroxyl absorption in the i.r. The methylated polysaccharide (2·5 g) was methanolyzed in 5% methanolic HCl at 95° for 18 hr. After removing the solvent the residue was hydrolyzed in N HCl (25 ml) at 95° for 7 hr. Excess HCl was neutralized with Ag_2CO_3 and insoluble salts were removed. The filtrate was concentrated to a small volume and applied to sheets of Whatman No. 3 MM filter paper. The methyl ethers of the sugars were separated by chromatography using solvent (B) as the developing phase. The components were eluted with ethanol. The material in the five fractions thus obtained was compared with known methyl sugars using paper chromatography in solvents (B) and (C); the presence of the following components was indicated (Table 2). The mixture with R_f 0·55 in solvent (B) was resolved by chromatography in solvent (C).

TABLE 2. COMPOSITION OF HYDROLYZATE OF METHYLATED *Annona*-AMYLOID (2·50 g)

Fraction	R_f*	R_{TMG}†	Methyl sugar	Amount (g)
1	0·80	1·00	2,3,4-tri-*O*-Me-D-xylose	0·012
2	0·65	0·96	2,3,4,6-tetra-*O*-Me-D-galactose	0·39
3	0·55	0·92	2,3,6-tri-*O*-Me-D-glucose	1·12
4		0·84	3,4-di-*O*-Me-D-xylose	0·30
5	0·25	0·75	2,3-di-*O*-Me-D-glucose	0·36

* In solvent (B).
† In solvent (C).

Identification of the Methyl Sugars

2,3,4-Tri-*O*-methyl-D-xylose. The component of fraction 1 had the same chromatographic mobility as 2,3,4-tri-*O*-methyl-D-xylose in solvents (B) and (C). On seeding with a crystal of authentic material a small amount of the substance crystallized, insufficient for recrystallization and further characterization.

2,3,4,6-Tetra-*O*-methyl-D-galactose. The anilide was prepared in the usual way and had m.p. 196–197° (lit.[8] 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine 197°).

2,3,6-Tri-*O*-methyl-D-glucose. The melting point could not be raised over 116° by recrystallization (lit.[9] 2,3,6-tri-*O*-methyl-D-glucose 121–123°). The substance had $[\alpha]_D$ +86° (1 hr), +66° (20 hr) (C 2·6, H_2O). The 1,4-bis-*p*-nitrobenzoate was prepared and had m.p. 190–191° (lit.[10] 2,3,6-tri-*O*-methyl-D-glucose-1,4-bis-*p*-nitrobenzoate 189–190°).

2,3-Di-*O*-methyl-D-glucose. The methyl sugar was crystallized from ethanol–ethyl ether–light petroleum and had m.p. 110–111° (lit.[11] 2,3-di-*O*-methyl-D-glucose 109°, 110°). The anilide had m.p. 135–136° (lit.[12] 2,3-di-*O*-methyl-*N*-phenyl-D-glucosylamine 134°).

[7] R. WILLSTÄTTER and G. SCHUDEL, *Ber. Deut. Chem. Ges.* **51**, 780 (1918).
[8] W. CHARLTON, W. N. HAWORTH and W. J. HICKINBOTTOM, *J. Chem. Soc.* 1527 (1927).
[9] J. C. IRVINE and E. L. HIRST, *J. Chem. Soc.* **121**, 1213 (1922).
[10] P. A. REBERS and F. SMITH, *J. Am. Chem. Soc.* **76**, 6097 (1954).
[11] J. C. IRVINE and J. P. SCOTT, *J. Chem. Soc.* **103**, 575 (1913).
[12] E. SCHLÜCHTELER and M. STACEY, *J. Chem. Soc.* 776 (1945).

3,4-Di-*O*-methyl-D-xylose. The compound had $[\alpha]_D$ +21·8° (C 0·97, H_2O) (lit.[13] 3,4-di-*O*-methyl-D xylose +20·5°). The corresponding 1,5-lactone was prepared in the usual way and had m.p. 68° (lit.[14] 3,4-di-*O*-methyl-D-xylono-1,5-lactone 68°).

Partial Acid Hydrolysis

Purified *Annona*-amyloid (250 mg) was dissolved in water (7·5 ml), and N H_2SO_4 (0·1 ml) was added. The mixture was heated in a sealed tube at 80° for 4 days. The precipitate formed was separated by centrifugation and the H_2SO_4 was removed from the filtrate with the aid of $BaCO_3$. The filtrate was treated with an equal volume of ethanol; the precipitated material was separated and subjected to X-ray examination. The clear solution was concentrated to approximately 1 ml and then applied to a column of Darco G-60 and Celite 535 (2 g of each). The monosaccharides were removed by elution with water (25 ml). With 10% ethanol (10 ml) the disaccharide fraction was eluted. Paper chromatographic inspection using solvent (A) of the concentrated eluate demonstrated the presence of principally one sugar; the latter had the same R_{Gl}-value (0·76) as *O*-β-D-galactopyranosyl-(1 → 2)-D-xylose, and did not react with triphenyltetrazolium reagent.

Hydrolysis by Luizym

Hydrolysis by Luizym (Luitpoldwerke, München) was performed as described for *Tamarindus*-amyloid.[4]

Determination of Component Sugars in Enzymic Hydrolyzates

Annona-amyloid (15 mg) was hydrolyzed by Luizym and the hydrolyzate was applied to a column containing Darco G-60 (2 g) and Celite 545 (2 g). The sugars were eluted with 10% ethanol (50 ml); the eluate was concentrated to dryness and then dissolved in water (exactly 2 ml).

The following determinations were carried out, in all cases with appropriate blanks.

(*a*) Reducing groups were estimated using a modification of the hypoiodite method of Willstätter and Schudel.[7] To the sugar solution (0·5 ml) N Na_2CO_3 (0·1 ml) and 0·1 N I_2 (0·5 ml) were added. The solution was kept at 25° for 20 min and was subsequently acidified with 2 N H_2SO_4 (0·4 ml). Unreacted I_2 was then titrated with 0·1 N $Na_2S_2O_3$.

(*b*) Glucose was removed from the sugar solution (0·5 ml) by incubating the latter with glucose oxidase (D-glucose aerodehydrogenase) "Boehringer" (0·05 mg in 0·1 ml McIlvaine buffer solution at pH 5·5) at 30° overnight with free access of air. Then reducing groups were determined as under (*a*), the difference yielding the amount of free glucose in the sugar solution.

(*c*) Sugar solution (0·4–0·8 ml) was diluted to 25 ml in a volumetric flask and a portion (1 ml) of the diluted solution was reduced with $NaBH_4$ and subsequently treated with phenol–sulphuric acid reagent, according to Timell's modification[15] of the method of Peat *et al.*[16] The absorptivity of the solution was determined at 480 nm and the amount of xylose was read from a standard curve prepared from pure xylosyl-glucose. Since xylose occurs in the sugar solution as xylosyl-glucose the amount of xylose found yielded also the equivalent quantity of bound glucose. The amount of galactose in the sugar solution was calculated by difference.

Fragmentation by Cellulase

Amyloid (250 mg) was suspended in water (8 ml) and a heated (3 min at 100°) solution of cellulase (β-1,4-glucan 4-glucanohydrolase) from *Myrothecium verrucaria*[17] (2 ml) was added. Another portion of amyloid (250 mg) was suspended in water (9·5 ml) and an untreated solution of *Myrothecium*-cellulase (0·5 ml) was added. Both mixtures were incubated at 37° for 2 weeks with a few drops of toluene added to prevent microbial contamination. Small quantities of undissolved material (15 mg and 7 mg respectively) were removed by centrifuging and the solutions were concentrated to small volumes. The sugar mixtures were resolved on sheets of Whatman No. 3 MM filter paper using solvent (A) and the components eluted with 50% ethanol. The eluates were concentrated to dryness, dried *in vacuo* and weighed.

Identification of D-Glucose

The glucose fraction from the hydrolyzate with Luizym was purified with charcoal and crystallized from 90% ethanol. After recrystallization the product had m.p. 144° (lit.[18] 146°) and $[\alpha]_D^{19}$ +52·3° (C 2·6, H_2O) (lit.[19] +52·7°). The *p*-nitroanilide was prepared and had m.p. 183° (lit.[20] 184°).

[13] G. J. Robertson and T. H. Speedie, *J. Chem. Soc.* 824 (1934).
[14] S. P. James and F. Smith, *J. Chem. Soc.* 739, 746 (1945).
[15] T. E. Timell, *Svensk Papperstid.* **63**, 668 (1960).
[16] S. Peat, W. J. Whelan and J. G. Roberts, *J. Chem. Soc.* 2258 (1956).
[17] For properties of this enzyme see P. Kooiman, P. A. Roelofsen and S. Sweeris, *Enzymologia* **16**, 237 (1953).
[18] O. Hesse, *Ann. Chem. Liebigs* **277**, 302 (1893).
[19] H. S. Isbell and W. W. Pigman, *J. Res. Natl Bur. Std.* **18**, 141 (1937).
[20] F. Weygand, W. Perkow and P. Kuhner, *Chem. Ber.* **84**, 594 (1951).

Identification of D-*Galactose*

The galactose fraction from the hydrolyzate with Luizym was purified with charcoal and crystallized from 75% ethanol. After recrystallization the product had m.p. 165° (lit.[21] 167°) and $[\alpha]_D^{19}$ +81° (C 1, H_2O) (lit.[21] +80·2°; +81·1°).

Identification of O-α-D-*xylopyranosyl*-(1 → 6)-D-*Glucose*

The xylosylglucose fraction obtained by chromatographic separation of the components present in a digest of *Annona*-amyloid with Luizym was purified with charcoal and crystallized from 85% ethanol. After several recrystallizations the product had m.p. 201° (lit.[22] 200·5–201·5°) and $[\alpha]_D^{19}$ +122° (C 4, H_2O) (lit.[22] +121·3°).

Identification of Oligosaccharide A1

Oligosaccharide *A1* (34 mg) was heated with anhydrous $ZnCl_2$ (15 mg) and acetic anhydride (3 ml) on a boiling water bath for 3 hr. After cooling the solution was poured into ice-cold water and the precipitate formed was washed with water and then dried. The product was crystallized from ethanol and had m.p. 227° (lit.[23] α-octaacetylcellobiose 229·5).

Tentative Identification of Oligosaccharide A2

The oligosaccharide had the same R_{Gl}-value (0·53) as oligosaccharide 2 from *Tamarindus*-amyloid.[4] A small portion (10 mg) of the product was hydrolyzed by Luizym as described below and the hydrolyzate was subjected to paper chromatography. The components were eluted and the eluates concentrated to dryness. Glucose and xylosylglucose were obtained in yields of 3·5 and 4·5 mg respectively.

Characterization of Oligosaccharides A3 *and* A4

Oligosaccharide *A3* had R_{Gl}=0·27 and $[\alpha]_D^{19}$ +72° (C 0·35, H_2O); oligosaccharide *A4* had R_{Gl}=0·11 and $[\alpha]_D^{19}$ +48° (C 1·4, H_2O). The compositions of *A3* and *A4* were determined by the following method. The oligosaccharide (150 mg) was dissolved in water (5 ml), and an extract (10 ml) of Luizym (50 mg) in water (20 ml) was added. The solution was incubated at 37° for 2 weeks with a few drops of toluene added to prevent microbial contamination. The solution was concentrated to a small volume and subjected to paper chromatography on sheets of Whatman No. 3 MM using solvent (A). The components were eluted from the chromatograms with 50% ethanol, and the eluates were concentrated, dried and weighed.

Determination of Molecular Weight

The hypoiodite method described by Jansen and MacDonnell[24] was used.

Periodate Oxidation of Annona-*Amyloid*

Annona-amyloid (100 mg) was dissolved in 0·1 N NaOH (10 ml) and KBH_4 (100 mg) was added to the solution. After 2 days at room temperature the solution was filtered to remove a trace of undissolved material. The filtrate was acidified by the addition of a slight excess of acetic acid. Reduced amyloid was then precipitated by adding ethanol (20 ml) to the solution. The precipitated amyloid was separated by centrifugation, washed with ethanol (three times) and with ethyl ether (three times) respectively, and then dried *in vacuo*.

Reduced *Annona*-amyloid (90 mg) was dissolved in 0·05 N NaOH hydroxide (10 ml), and the solution was neutralized with dilute HCl. $NaIO_4$ (1.065 g) dissolved in water (50 ml) was added and the mixture adjusted to 100 ml. The solution was kept at room temperature in the dark, and samples were taken in the course of several weeks for determination of periodate consumption,[25] formic acid production,[25] and formaldehyde production.[26] An appropriate blank solution was also prepared. In Table 3 the results are given in moles per mole of anhydromonosaccharide, the mean molecular weight of the latter being 157. Extrapolation of periodate consumption and formic acid production to zero time yielded values of 1·16 and 0·20 molar equivalents respectively.

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TABLE 3. PERIODATE CONSUMPTION, FORMIC ACID PRODUCTION, AND FORMALDEHYDE PRODUCTION DURING PERIODATE OXIDATION OF *Annona*-AMYLOID

Time (days)	1	2	4	8	15	22	35	38	42
Periodate consumption	0·84	—	0·89	1·04	1·19	—	1·23	—	1·25
Formic acid production	0·19	0·20	0·21	0·23	0·24	0·27	0·30	0·32	0·33
Formaldehyde production	0·038	0·037	0·031	—	0·034	0·038	—	—	—

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