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Polysaccharides in Germination

XYLOGLUCANS ('AMYLOIDS') FROM THE COTYLEDONS OF WHITE MUSTARD

BY SHEILA E. B. GOULD, D. A. REES* AND N. J. WIGHT†
Department of Chemistry, The University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K.

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Two xyloglucan fractions have been isolated from the cotyledons of resting white-mustard seeds, the first by extraction with hot EDTA, and the second by subsequent extraction with alkali or lithium thiocyanate. Although both appear to have the 'amyloid' type of structure in which chains of (1→4)-linked β-D-glucopyranose residues carry D-xylose-rich side chains through position 6, these side chains are rather different in structure in the two polysaccharide fractions, and the second or 'insoluble' xyloglucan has fewer of them. The side chains in both polysaccharides are also different from those in other seed amyloids, especially in having xylose linked through positions 3 and 4 (instead of through position 2 as usual) and in containing fucose residues. Both polysaccharides show the characteristic blue 'amyloid' colour with iodine in the presence of sodium sulphate, and it is suggested that this arises by the interaction of iodine molecules and possibly iodide ions within the interstices between aggregated xyloglucan chains. 'Soluble' xyloglucan is metabolized during germination and is presumed to have a reserve function. 'Insoluble' xyloglucan is metabolized less completely over the period studied but its lack of turnover during cell-wall differentiation indicates that it also is a reserve. These and other β-(1→4)-linked reserve polysaccharides of seeds might also have a structural function which is of particular value for the survival of the dormant seed.

The object of our investigation of seed germination is to obtain information about the structure and biological changes of cell-wall polysaccharides to correlate with parallel investigations of chain conformation and interactions (Rees & Skerrett, 1968, 1970; Rees, 1969; Rees & Wight, 1971; Rees & Scott, 1971; Anderson, Campbell, Harding, Rees & Samuel, 1969; Rees, Steele & Williamson, 1969; McKinnon, Rees & Williamson, 1969), hoping to work towards an understanding of polysaccharide function in molecular terms. In earlier papers (Rees & Wight, 1969; Rees & Richardson, 1966; Rees & Steele, 1966; Gould & Rees, 1965; Hirst, Rees & Richardson, 1965) we have described the chemical composition of the cotyledons of white mustard and the way this changes with germination, with special reference to the carbohydrate components. The major polysaccharides of the cell wall are cellulose, pectic materials (Rees & Richardson, 1966; Rees & Steele, 1966; Rees & Wight, 1969) and substances which give xylose, glucose, and smaller amounts of other sugars, on acid hydrolysis. More information is now given about the latter polysaccharides, which, following Aspinall (1969), we propose to call 'xyloglucans'. Two types are present, which differ in structure and solubility properties and perhaps to some extent in biological function. The 'soluble' xyloglucan is extracted together with pectic substances by hot aqueous EDTA, whereas the 'insoluble' xyloglucan remains in the residue from which it is brought into solution with alkali or lithium thiocyanate.

* Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, U.K.

† Present address: Courtaulds Ltd., Acetate and Synthetic Fibres Laboratory, P.O. Box 16, Matlock Road, Coventry CV6 5AE, U.K.

MATERIALS AND METHODS

Starting materials. The mustard seeds, the separated organs, and other starting materials for the isolation of polysaccharides, were obtained as described elsewhere (Hirst *et al.* 1965; Rees & Wight, 1969; S. E. B. Gould, D. A. Rees, I. W. Steele & N. J. Wight, unpublished work).

Paper chromatography. The descending method was used, with Whatman no. 1 paper for analysis or 3MM paper for preparative separations, and the following solvent systems: A, butan-1-ol–propionic acid–water

(4:1:5, by vol., upper phase); B, aq. NH_3 (sp.gr. 0.88)–water–butan-2-one (1:17:200, by vol.). Spots were detected with *p*-anisidine hydrochloride spray (Hough, Jones & Wadman, 1950).

Gas–liquid chromatography. The systems used were: *A*, the Pye Argon Chromatograph with the polyethylene glycol adipate column at 175°C as described by Rees & Wight (1969); *B*, the same instrument and conditions with butane-1,4-diol succinate polyester (10% on Celite); *C*, the same instrument and conditions with *m*-bis-(*m*-phenoxyphenoxy)benzene ('polyphenyl ether') (10% on Celite); *D*, the Pye 104 Chromatograph with dual flame-ionization detectors and neopentyl glycol (2,2-dimethylpropane-1,3-diol) adipate polyester (10% on Gas Chrom P) as stationary phase, with nitrogen as carrier gas and a column temperature of 175°C. The polyester columns *A* and *D* were the most useful and gave slightly different separations, as shown below, and were therefore complementary. Conversion of methylated sugars and polysaccharides into suitable mixtures of methyl glycosides for analysis was done by the method already given (Rees & Wight, 1969).

Iodine colour reaction with polysaccharides. This is essentially the procedure of Kooiman (1960*b*). To the aqueous polysaccharide solution (1.00ml, containing 0.05–0.30mg of xyloglucan), was added an aqueous solution (0.50ml) containing iodine (0.5%, w/v) and potassium iodide (1%, w/v; AnalaR). Sodium sulphate solution (20%, w/v; AnalaR; 5.0ml) was added with shaking and the colour was allowed to develop for 1h at room temperature before measurement of the extinction at 640nm or recording of the entire spectrum in 1cm cells, by using the Perkin–Elmer model 137 spectrophotometer. Measurements were against a reagent blank. For quantitative analysis, a calibration curve was drawn of extinction at 640nm against concentrations of *Tamarindus* 'amyloid' to 0.30mg/ml. The graph was linear and 1mg of 'amyloid' gave an extinction of 3.78 at 640nm.

EXPERIMENTAL AND RESULTS

Isolation of 'soluble' xyloglucan. This was the material that was described previously as 'fraction A' (Rees & Wight, 1969), and which remained in solution when the crude mixture of EDTA-soluble polysaccharides was treated to precipitate pectic substances as the calcium salts.

Isolation of 'insoluble' xyloglucan. The residue (100g) that remained after non-germinated cotyledons had been extracted with EDTA (Rees & Wight, 1969) was suspended in water (1.6litres) and stirred at room temperature with lithium thiocyanate (2kg) for 10days. Solid material was removed by centrifugation, washed with water and dried *in vacuo* (69g). To the solution was added acetone (45litres, 9vol.), with stirring, to precipitate a mixture of polysaccharide and protein and leave lithium thiocyanate in solution. This product was collected by centrifugation, washed briefly with water and freeze-dried (25g). Much of the protein could be removed by stirring for 10 days with phenol (2.2kg) and water (250ml), followed by centrifugation, washing with ethanol (thrice) followed by diethyl ether (thrice) and drying *in vacuo*. Final purification was achieved by solution in water (750ml) and slow addition of cupric acetate solution (7%, w/v) until precipitation occurred. The precipitate was removed by centrifugation and stirred with a mixture of ethanol and concentrated hydrochloric acid (20:1, v/v) to remove copper salts, then washed with ethanol, dissolved in water and isolated by freeze-drying. This is fraction 1 (0.4g).

To the solution remaining after the first precipitate was added methylated spirit (2vol.), to give a second precipitate, which was worked up in the same way to give fraction 2 (2.1g).

Carbohydrate was present in the residue after extraction with lithium thiocyanate, in fraction 1 and in fraction 2. A sample of each was therefore hydrolysed (45% formic acid at 100°C for 16h); the residue gave mainly glucose with some arabinose, xylose, galactose and galacturonic acid, suggesting that the polysaccharides present were cellulose, residual pectic substances and probably some residual xyloglucan. Fraction 1 gave galacturonic acid, glucose, xylose and fucose in similar amounts and analysis showed N 5.1% (Kjeldahl); hence it contains much protein and pectic material as well as some xyloglucan. Fraction 2, however, showed xylose and glucose as the main sugar components with smaller amounts of fucose, and analysis showed N 0.1%; it therefore contains the main part of the extracted xyloglucan and is essentially free from protein and pectic materials. On analytical ultracentrifugation at 110000*g*, it showed a single sharp peak.

Preliminary comparison of 'soluble' and 'insoluble' xyloglucans. In addition to the same sugars that were formed from 'insoluble' xyloglucan (see above), 'soluble' xyloglucan gave substantial amounts of galactose, small amounts of arabinose, and traces of galacturonic acid; the last two sugars were presumed to have arisen from contaminating pectic substances.

On analytical ultracentrifugation, the 'soluble' and 'insoluble' xyloglucans were very similar in band shape and sedimentation.

Methylation of 'insoluble' xyloglucan. Polysaccharide (0.86g) was dissolved in dimethyl sulphoxide (60ml) and stirred under nitrogen with the addition of sodium hydroxide pellets (43g) and dimethyl sulphate (30ml) over a period of 8h by the method of Srivastava, Singh, Harshe & Virk (1964). After further stirring at room temperature for 16h, the mixture was worked up as usual (Srivastava *et al.* 1964), to give a product (0.73g), which was dissolved in *NN*-dimethylformamide (20ml) and remethylated by stirring with methyl

iodide (10ml) and silver oxide (5g), first in ice (2h) and then at room temperature (16h). The methylated polysaccharide was isolated in the usual way (Kuhn, Trischmann & Löw, 1955) (yield, 0.62g; Found: $-OCH_3 = 41.6\%$). Hydrolysis in formic acid (45%, v/v) at 100°C for 16h followed by paper chromatography (solvent B), showed that glucose and xylose and their monomethyl ethers were absent, confirming that methylation was complete. The sugars in this mixture were identified tentatively by paper-chromatographic comparison with standards (solvent B). They were then isolated by preparative paper chromatography (also in solvent B), and each individual fraction was examined by methanolysis and g.l.c. against standards (systems *B*, *C*, *D*). Eventually, it was possible to identify the major peaks in the gas–liquid chromatogram of the methanolysate of the polysaccharide methyl ether (Fig. 1).

Comparison of the 'soluble' and 'insoluble' xyloglucans by methylation analysis. The 'soluble' xyloglucan (1.06g) was dissolved in water (25ml) and stirred vigorously under nitrogen for 5 days with daily slow additions of sodium hydroxide (30%, w/v; 20ml) and dimethyl sulphate (6.6ml) over 6h. On the first day, the flask was kept in an ice bath. After dialysis against running tap water, the solution was concentrated and methylation was repeated in the same way. Finally, the partly methylated product (1.11g), which showed strong OH absorption in the infrared, was isolated by dialysis and freeze-drying. It was dissolved in *NN*-dimethylformamide (20ml) and methylated with methyl iodide (10ml) and silver oxide (5g), in the same way as for the 'insoluble' xyloglucan, except that a second quantity of reagents was added after stirring for several hours. The product was still undermethylated, as judged by the hydroxyl absorption in the i.r. spectrum, and was treated again with silver oxide and methyl iodide in *NN*-dimethylformamide. The final product (0.57g) was still not fully methylated (Found: $-OCH_3 = 33.4\%$). It was compared with the product from 'insoluble' xyloglucan by methanolysis and g.l.c. (Fig. 2).

Iodine-staining reactions of xyloglucans. The 'soluble' and 'insoluble' xyloglucans from mustard both gave a positive response to this test, and the quantitative procedure was therefore applied to a variety of polysaccharide extracts from mustard seeds. The results are given in Table 1. The colour which developed with mustard polysaccharides was visually identical with that for *Tamarindus* 'amyloid'. The absorption maximum for both xyloglucans from mustard was at 650nm.

Xyloglucans from germinated cotyledons. Full details of the extraction with EDTA after germination of mustard seeds will be given elsewhere, in the context of structural investigations of pectic substances (S. E. B. Gould, D. A. Rees, I. W. Steele & N. J. Wight, unpublished work); the procedure was broadly similar to the method which has been published for extraction before germination (Hirst *et al.* 1965; Rees & Wight, 1969). The mixture of soluble polysaccharides thus isolated from the cotyledons of seedlings germinated for 4 days in the dark, unlike the equivalent product before germination, gave no reaction for 'amyloid' with iodine–sodium sulphate. Even after chromatography on DEAE-cellulose, the fraction in which

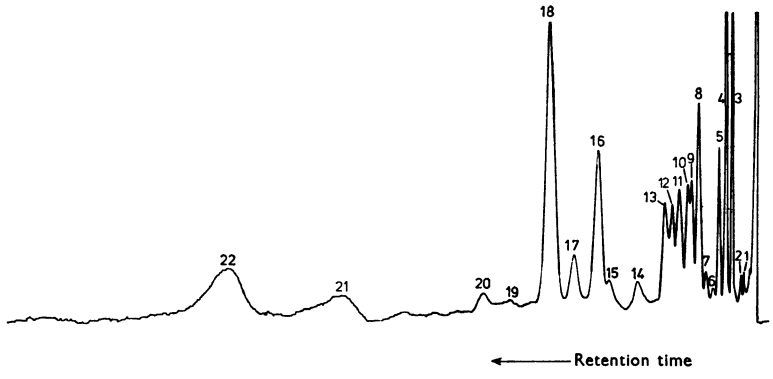

Fig. 1. Methanolysis products of the methylated 'insoluble' xyloglucan from mustard cotyledons, analysed by g.l.c. with system *D*. The derivatives identified are: 2,3,4-tri-*O*-methylxylosides (peaks 3 and 4), 2,3-di-*O*-methylxylosides (7 and 10), 2,4-di-*O*-methylxylosides (9 and 12), 2-*O*-methylxylosides (15 and 18), 2,3,4-tri-*O*-methylfucosides (peak 5), 2,3,4,6-tetra-*O*-methylglucosides (8 and 11), 2,3,6-tri-*O*-methylglucosides (16 and 18), 2,3-di-*O*-methylglucosides (21 and 22), 2,3,4,6-tetra-*O*-methylgalactosides (peak 13). This separation was achieved in 50min.

any xyloglucan would have emerged gave a negative test. Details are given in Table 1. We conclude that 'soluble' xyloglucan is absent from germinated cotyledons.

To examine the content of 'insoluble' xyloglucan after germination, mustard seeds (2.5g) were germinated in the dark (Gould & Rees, 1965) for 4 days, then dropped into liquid nitrogen and freeze-dried. Cotyledons were separated after coarse grinding in a hand mill and extracted with aqueous ethanol (80%, v/v; 10×100ml) at boiling point, then with aqueous EDTA (2%, w/v; 10×150ml, at 100°C for 3h) until the extract gave a negative phenol–sulphuric acid test for carbohydrate. The residue was dried by washing with ethanol and diethyl ether, then dried *in vacuo* (yield 0.27g). Glucose, xylose and fucose were the main products of hydrolysis with traces of arabinose, showing that xyloglucan is the main hydrolysable polysaccharide in this fraction of germinated cotyledons.

In an attempt at quantitative comparison of the amounts of 'insoluble' xyloglucan before and after germination, part of each residue after EDTA extraction (50g) was stirred under nitrogen with aqueous potassium hydroxide (5%, w/v; 1litre). After centrifugation, extraction was repeated a further three times in the same way and the residue was washed in turn with water, ethanol and diethyl ether, then dried *in vacuo* (yields: 4.1g and 4.0g from non-germinated and germinated cotyledons respectively). Precipitable protein was removed by the gradual addition of hydrochloric acid until a further addition gave no turbidity, collected by centrifugation, washed with water and dried *in vacuo* (yields, 24.0 and 26.6g respectively). After neutralization with sodium hydroxide, the

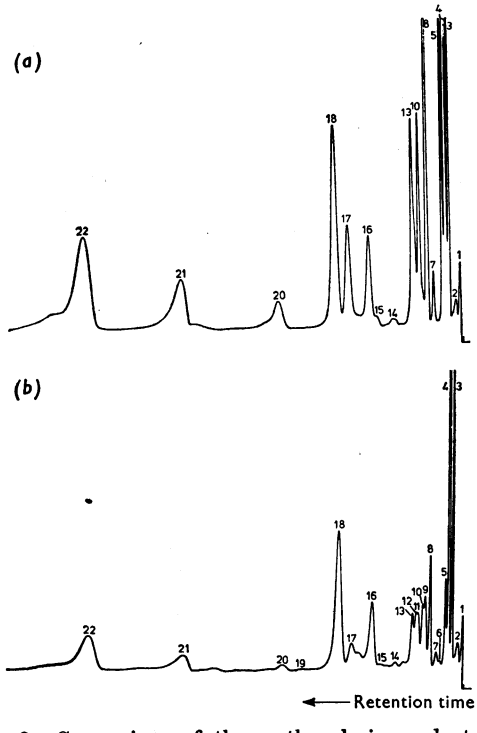

Fig. 2. Comparison of the methanolysis products of methylated 'soluble' xyloglucan (*a*) and methylated 'insoluble' xyloglucan (*b*), by g.l.c. with system *A*. The derivatives identified are: 2,3,4-tri-*O*-methylxylosides (peaks 3 and 4), 2,3,4-tri-*O*-methylfucosides (peak 5), 2,3-di-*O*-methylxylosides (peaks 7 and 10), 2,4-di-*O*-methylxylosides (9 and 12), 2,3,4,6-tetra-*O*-methylgalactosides (peak 13), 2-*O*-methylxylosides (15 and 18), 2,3,6-tri-*O*-methylglucosides (16 and 18), 2,3-di-*O*-methylglucosides (21 and 22). These separations were achieved in 40min each.

Table 1. *'Amyloid' contents of polysaccharide fractions from mustard seeds*

This was estimated colorimetrically by the iodine–sodium sulphate reaction and calibrated with 'amyloid' from *Tamarindus indica*. Polysaccharide preparations are those described in the present paper or an earlier one (Rees & Wight, 1969), or were prepared by strictly analogous methods. DEAE-cellulose chromatography was on columns that had been conditioned with 0.5M-NaH_2PO_4, pH6.2, then washed with 0.005M-NaH_2PO_4, and with use of solutions that were 7M with respect to urea (Applegarth & Dutton, 1964).

Sample no.	Description	Apparent 'amyloid' content (%)
1	Total extract of non-germinated mustard embryos with EDTA at pH7.5, before deproteinization	Nil
2	Similar material to sample 1, but deproteinized with phenol	5.5
3	Similar material to sample 2, but isolated by extraction at pH4.9 instead of pH7.5	4.0
4	'Soluble' xyloglucan from sample 1	71
5	Pectic fraction from sample 1	3.7
6	Similar material to sample 2, but isolated from the cotyledons of 4-day-old mustard seedlings	Nil
7	Neutral polysaccharide fraction from sample 2 (i.e. that part which was not retained on a DEAE-cellulose column)	29.5
8	Neutral polysaccharide fraction from sample 6 (isolated as for sample 7)	Nil
9	'Insoluble' xyloglucan, isolated by extraction with lithium thiocyanate and purified with copper acetate, as described in this paper	90.5

solution was dialysed, concentrated under reduced pressure and freeze-dried (yields, 12.2 and 11.7g). The product was dissolved in water (1litre) and extracted with phenol (4×250g) in the usual way (Hirst *et al.* 1965), to remove most of the remaining protein. After dialysis, the solution was concentrated and freeze-dried [yields, 2.9 and 1.2g respectively; Found: N = 5.25 and 3.40% respectively (Kjeldahl)].

Although hydrolysis and paper chromatography showed that these products consist largely of xyloglucan, neither gave a positive reaction with iodine–sodium sulphate. Since a more highly purified preparation of 'insoluble' xyloglucan from non-germinated cotyledons did give a strong reaction (sample 9 in Table 1), it would appear that the test is inhibited by residual protein. A similar conclusion is suggested by comparison of the results for samples 1 and 2 in Table 1. The weights and protein contents of the products, when taken with the changes in cotyledon weight (Gould & Rees, 1965) and yield of EDTA-extracted residue during germination, suggest that the content of 'insoluble' xyloglucan has diminished to about one-half.

DISCUSSION

The xyloglucan fractions from mustard cotyledons are similar to the 'amyloids' that have been detected by their characteristic blue stain with iodine (Kooiman, 1960*a*) in many species of seeds and which have been isolated and characterized from seeds of tamarind (*Tamarindus indica*: White & Rao, 1953; Kooiman, 1961; Srivastava & Singh, 1967), nasturtium (*Tropaeolum majus*; Hsu & Reeves, 1967) and *Annona muricata* L. (Kooiman, 1967). Similar polysaccharides have been isolated from bark (of *Picea engelmann*; Ramalingam & Timell, 1964), red-spruce compression wood (Schreuder, Côté & Timell, 1966) and sycamore callus cells (Aspinall, Molloy & Craig, 1969). They have a cellulose-like main chain of (1→4)-linked β-D-glucopyranose residues to which are attached, through position 6, α-D-xylopyranose residues, some of which, in turn, carry β-D-galactopyranose as a substituent on position 2. The evidence that our mustard xyloglucans are of this type is (i) they show the characteristic iodine reaction, provided they are first deproteinized, and (ii) the hydrolysis products of their methylated derivatives include large amounts of 2,3-di-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, and 2,3,4-tri-*O*-methylxylose and substantial amounts of 2,3,4,6-tetra-*O*-methylgalactose (Figs. 1 and 2).

In the presence of calcium chloride, iodine gives a blue or black colour (sometimes a precipitate) with a variety of polysaccharides which have a sequence of β-(1→4)-linked glucose, glucosamine, xylose or mannose residues (Gaillard & Bailey, 1966; Gaillard, Thompson & Morak, 1969). For the α-(1→4)-glucan, amylose, which also gives a coloured complex, the mechanism is known to involve inclusion of iodine and perhaps iodide in the core of a polysaccharide helix (Rundle, 1947). However, the conformational properties of α- and β-linked polysaccharides are so very different (Rees & Scott, 1971) that a similar explanation is unlikely to hold for the β-series. In our opinion, there is an important clue to the mechanism in the observation (Gaillard *et al.* 1969) that the 'amyloid' reaction proceeds faster at lower temperature; this is characteristic of processes for which the rate is determined by a nucleation step (Mandelkern, 1956, 1964), such as crystallization or a co-operative transition to an ordered conformation. Taking into account the known conformational properties of β-(1→4)-linked polysaccharides (Rees & Scott, 1971) and the known aggregation behaviour of cellulose and derivatives (Warwicker & Wright, 1967; Rees, 1969), the most likely mechanism would be as follows. Ribbon-like chains pile on top of each other to form stacks in which apolar surfaces are in contact and 'foreign' ions and molecules are sandwiched in layers between the stacks. The colour develops by the interaction of iodine molecules and perhaps anions within such layers (compare Morawetz, 1965). The lack of preferred stoicheiometry (Gaillard *et al.* 1969) is explained by irregularities in polysaccharide structure that would lead to microcrystallites of continuously varying stability. The function of sodium sulphate or calcium chloride would primarily be to 'salt out' the aggregate, although certain salts could also be complexed into the aggregate. This model, based as it is on known behaviour in other cellulose-type systems, is also supported in that, as well as proceeding faster at low temperature, the amyloid reaction proceeds further (Gaillard *et al.* 1969), just like the aggregation of *O*-methylcellulose (Rees, 1969) and of cellulose itself (Rees & Skerrett, 1968).

The products of methylation analysis show that mustard 'amyloids' differ from other known seed amyloids in having fucose end groups and in having xylose residues linked through positions 3 and 4 rather than position 2 only. They are more similar to the 'amyloid' from sycamore callus cells (Aspinall *et al.* 1969). The callus polysaccharide gave no blue colour with iodine but the authors did not report on the protein content and therefore the possibility cannot be excluded that the negative reaction was caused by inhibition. Of the two mustard polysaccharides, the 'soluble' xyloglucan is more branched because it gives more 2,3-di-*O*-methylglucose relative to 2,3,6-tri-*O*-methylglucose derivatives, as well as more methylated sugars derived from residues that must be terminal in the

structure. It also seems to be structurally simpler than the 'insoluble' xyloglucan because it gives little if any 2,3-di-*O*-methylxylose (Fig. 2), suggesting that xylose residues are present in only two instead of three types of combination. As with other families of polysaccharides, such as hemicelluloses and pectic substances, these xyloglucans therefore show structural variation within the same plant tissue.

Histochemical studies (Kooiman, 1957) have shown that, in general, 'amyloids' in plant seeds occur as thickenings of the cell walls. Their disappearance during germination and the very large deposits which occur in some seeds (40–50% of *Tamarindus* seeds), suggest the role of energy reserves. Since polysaccharides are bound into cell walls largely by non-covalent forces, it is not surprising that the more highly branched xyloglucan fraction was the more easily extracted from mustard cotyledons and, further, that an effective method could be devised for bringing residual xyloglucan into solution by treating the process as a denaturation, i.e. by dissociating non-covalent forces with lithium thiocyanate (cf. Grant, McNab, Rees & Skerrett, 1969).

The reserve function of 'soluble' xyloglucan was confirmed by the failure to detect it in the cotyledons after germination, showing that it had been metabolized (Table 1). Some 'insoluble' xyloglucan persisted after germination. From labelling experiments, it is already known that there is no synthesis of xyloglucan under germination conditions in which significant synthesis occurs of the other polysaccharides of cotyledon cell walls (Gould & Rees, 1965). Thus the 'insoluble' xyloglucan is not turned over in the cell-wall differentiation and is also likely to be a reserve deposit. It is possible that the xyloglucans are biologically related to each other and to cellulose, either in being interconvertible or perhaps in sharing some stages of their biosynthesis.

The hexopyranose polymers which occur as reserve materials in plants (and indeed in animals and in other forms of life) are usually of a flexible conformational type for which any regular conformation is likely to be helical rather than extended, and for which the solution dimensions are likely to be expanded (Rees & Scott, 1971; see also Brant & Dimpfl, 1970). Examples are starch, glycogen, laminarin, protozoan glucans and snail galactan. Although starch is found in many seeds, it is also common for seed reserves to be more closely related to the stiff, extended polysaccharides which normally characterize cell walls. 'Cell-wall type' seed reserves include mannans, arabinoxylans, galactomannans, glucomannans, cereal β-glucans and xyloglucans, and do indeed generally occur as actual cell-wall thickenings rather than separate deposits (Roelofsen, 1959; MacLeod & Sandie, 1961). In some species, starch is the reserve polysaccharide during the whole period of maturation and is only replaced by 'amyloid' in the final phase of seed ripening. This 'amyloid' is then replaced again by starch at the beginning of germination (Kooiman, 1957). We suggest that certain seed polysaccharides may have a dual function; as well as acting as reserves for germination, they have a survival value in that they reinforce the wall (and hence reinforce a tissue structure that might otherwise become brittle by dehydration) against crushing or cracking by hazards in dormancy. This second function is reflected in a molecular structure and hence conformation that is not normally found in reserves (Rees & Scott, 1971), and indeed that is not conserved after dormancy. It remains to be shown whether 'amyloids' in non-seed sources have a reserve function, or are (like mannans, glucomannans and xylans from non-seed sources) purely structural.

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