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FORMATION OF RESERVE GALACTOMANNAN IN THE SEEDS OF *TRIGONELLA FOENUM-GRAECUM*

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Abstract—Seeds at different stages of ripeness taken from a single plant of *Trigonella foenum-graecum* have been analysed to determine the quantity and chemical composition of the reserve galactomannan and to identify the low molecular weight carbohydrates. The galactomannan begins to be formed at an early stage of seed development and its amount increases throughout the growth of the seed; its chemical composition is invariable. At the same time as the polysaccharide is laid down, considerable quantities of stachyose appear in the seeds. It is probably also a reserve carbohydrate. The galactomannans of the Leguminosae appear to be synthesized by a specific mechanism involving simultaneous deposition of both galactosyl and mannosyl residues. This contrasts with the known mode of their degradation in germinating seeds and with earlier suggestions concerning their biosynthesis. Because of the simultaneous formation of stachyose and galactomannan it is suggested that galactinol, which is known to be implicated in the biosynthesis of stachyose, also might play a role in the formation of the galactomannan.

INTRODUCTION

THE SEEDS of many leguminous plants have mucilaginous endosperms.[1] Treatment of either the endosperm[2] or the whole seed with water or dilute alkali leads to the extraction of polysaccharide material which can readily be purified to give a galactomannan in a yield of up to 38 per cent of the seed.[2] The galactomannans, with one reported exception,[3] have a common basic structure. To chains of β,1→4-linked D-mannopyranosyl residues are attached, at the 6-positions of certain of the latter, single α-D-galactopyranosyl residues. The galactomannans from the seeds of different species differ with respect to their ratios of galactose to mannose residues. Small differences in the proportion of galactose to mannose residues in galactomannans from plants of the same species have been reported by different groups of workers, and it has been suggested that this may be due to varietal or environmental factors,[4,5] or to metabolism of galactose residues during the life of the seed.[6] The breakdown of galactomannans during the germination of the seeds of *Gleditschia ferox* and *G. tricanthos* has been studied by Courtois and Le Dizet.[7,8] They demonstrated that there is first of all a removal of most of the galactose residues and then a scission of the mannan chain to give mannooligosaccharides. In germinated seeds they found galactose, mannose, mannobiose, mannotriose and mannotetraose.[8]

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[1] A. TSCHIRCH, *Angewandte Pflanzenanatomie* Vol. 1, p. 193, Urban and Schwarzenberg, Vienna (1889).
[2] E. ANDERSON, *Ind. Eng. Chem.* **41**, 2887 (1949).
[3] E. L. HIRST, J. K. N. JONES and W. O. WALDER, *J. Chem. Soc.* 1443 (1947).
[4] F. SMITH and R. MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, p. 330, Reinhold Publishing Corporation, New York (1959).
[5] E. L. HIRST and J. K. N. JONES, *J. Chem. Soc.* 1278 (1948).
[6] P. ANDREWS, L. HOUGH and J. K. N. JONES, *J. Amer. Chem. Soc.* **74**, 4029 (1952).
[7] J.-E. COURTOIS and P. LE DIZET, *Bull. Soc. Chim. Biol.* **45**, 731 (1963).
[8] J.-E. COURTOIS and P. LE DIZET, *Bull. Soc. Chim. Biol.* **48**, 190 (1966).

The biosynthesis of legume seed galactomannans has received relatively little attention. It has been proposed that galactose residues may be stored by being attached in random fashion to a main chain of mannose residues.[5,6] When sucrose and raffinose were found in the seeds of *Medicago sativa* it was suggested[9] that a plausible pathway for the synthesis and degradation of its galactomannan might be by transglycosylation of D-galactopyranosyl units, in the first case from raffinose to a mannose polymer and in the second from the galactomannan to sucrose. This paper reports a study of the galactomannans and low-molecular-weight carbohydrates in the seeds of *Trigonella foenum-graecum* at different stages of seed maturation. The purpose of the investigation was threefold; to find at what stage of seed development the galactomannan was synthesized, to determine whether any change in the proportion of galactose to mannose residues took place during its formation and to check whether any of the low-molecular-weight carbohydrates in the seeds could possibly be precursors of the polysaccharide.

RESULTS

The structure of the galactomannan of *Trigonella foenum-graecum* is known;[10] it has galactose and mannose residues in the ratio of 1:1[11] or 1:1·2[10] and conforms to the general pattern of structure of legume-seed galactomannans. The plant itself is an annual one with a relatively long flowering period. Flowering only occurs at the tip of the plant and a single plant may therefore have pods at all stages of ripeness arranged in order of increasing maturity down the stem. The plant used in the comparative studies had 32 pods at various stages of maturity. The average dry weight of a seed in pods at different stages of ripeness is given in Fig. 1 and the seeds are described in Table 4. The pods are numbered in the reverse order to that in which they were formed; that is, pod No. 32 is fully mature and No. 1 is at a very early stage of development. In all subsequent tables and figures, pod number is used as a scale of seed maturity.

The seeds from a number of pods at different stages of ripeness were processed to deactivate enzymes and then treated with hot water to extract galactomannans and low-molecular-weight carbohydrates. The average weight of galactomannan, purified via the insoluble copper complex, per seed at different stages of development is shown in Fig. 2.

Galactomannans were obtained from all but the very youngest seeds, the amount per seed increasing to a maximum at about the stage of ripeness represented by pod No. 19 and then falling off slightly. Pod No. 19 was about 9–10 weeks old. The results of quantitative estimation of the products of hydrolysis of the galactomannans are given in Table 1.

No change in the chemical composition of the galactomannan was observed either during or after its formation. It has been pointed out that the exact chemical composition of a highly purified hemicellulosic polysaccharide may be dependent upon the method of fractionation used.[12] It might therefore be argued that the constancy of composition of the galactomannans is due to their being fractionated via the copper complex. This was considered unlikely as galactomannans with both high[5] and low[6] mannose/galactose ratios are precipitated as the copper complex. But to remove all doubt the ethanol precipitated non-purified polysaccharide materials were examined by hydrolysis and chromatography; no evidence was obtained for the chemical composition of the galactomannans being different before and after purification.

[9] M. E. HENDERSON, L. HOUGH and T. J. PAINTER, *J. Chem. Soc.* 3519 (1958).
[10] P. ANDREWS, L. HOUGH and J. K. N. JONES, *J. Chem. Soc.* 2744 (1952).
[11] K. M. DAOUD, *Biochem. J.* **26**, 255 (1932).
[12] J. S. G. REID and K. C. B. WILKIE, *Phytochem.* **8**, 2059 (1969).

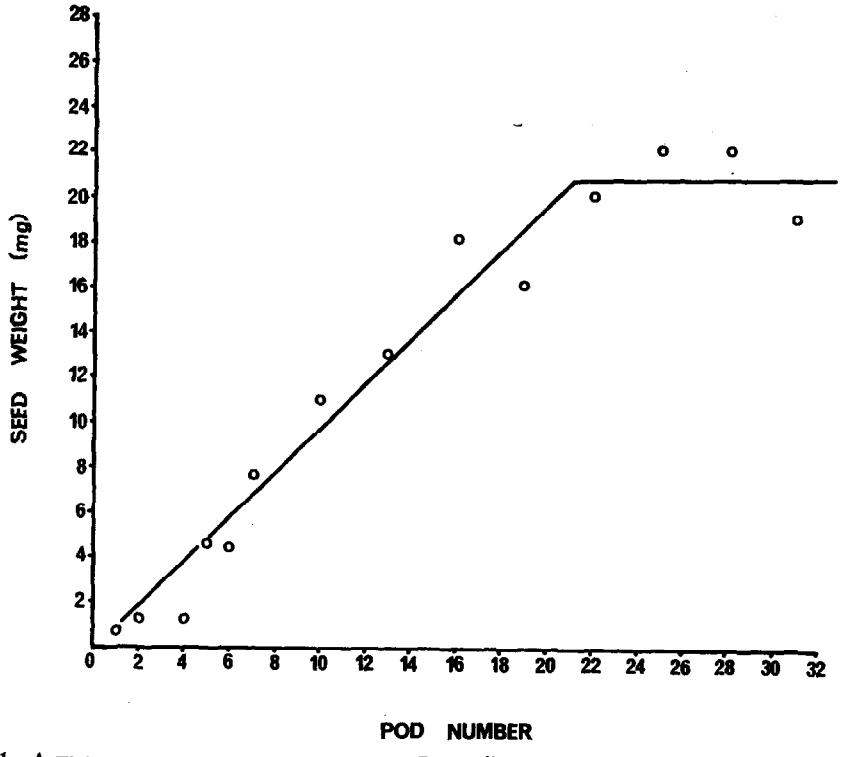

FIG. 1. AVERAGE WEIGHT OF A SINGLE SEED OF *Trigonella foenum-graecum* IN PODS AT DIFFERENT STAGES OF MATURITY.

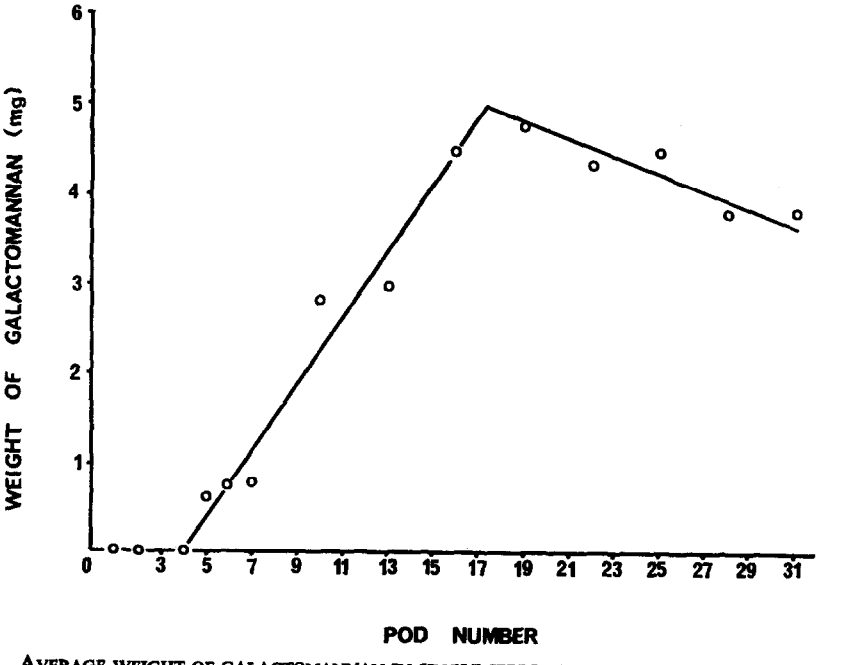

FIG. 2. AVERAGE WEIGHT OF GALACTOMANNAN IN SINGLE SEEDS AT DIFFERENT STAGES OF MATURITY.

TABLE 1. QUANTITATIVE ESTIMATION OF THE HYDROLYSIS PRODUCTS OF GALACTOMANNANS FROM SEEDS AT DIFFERENT STAGES OF MATURITY

	Pod number*													
	1	2	4	5	6	7	10	13	16	19	22	25	28	31
Per cent galactose	No galactomannan			46	46	47	47	48	47	46	47	48	47	48
Per cent mannose	Isolated			54	54	53	53	52	53	54	53	52	53	52

* An increase in pod number is equivalent to an increase in seed maturity.

Only small amounts of low-molecular-weight carbohydrates were obtained from the seeds, especially at the earliest stages of growth (see Table 2). It was therefore evident that comparison of the various sugar mixtures would have to be based on paper chromatography alone and that foreknowledge of the compounds likely to be present was necessary. For this reason a study of the low-molecular-weight carbohydrates in a larger quantity of mature seeds was undertaken with particular emphasis on the identification of compounds containing residues of galactose or mannose and which might be precursors of the polysaccharide. No free mannose was present nor were mannose-containing oligosaccharides detected. A trace of free galactose was found and raffinose, galactinol (1-*O*-α-D-galactopyranosyl-D-myoinositol) and stachyose were identified. Two further galactose-containing compounds were tentatively identified as verbascose and digalactosylmyoinositol.[13] Sucrose, myoinositol, glucose, fructose and trace amounts of other carbohydrates were also present.

TABLE 2. AMOUNT AND COMPOSITION OF THE LOW-MOLECULAR-WEIGHT CARBOHYDRATES IN THE SEEDS OF *Trigonella foenum-graecum* AT DIFFERENT STAGES OF RIPENESS

		Relative amounts of †					
Pod No.	Wt. of sugar mixture (mg/seed)	Fructose	Glucose	Sucrose	Raffinose and myoinositol	Galactinol	Stachyose
1	*ca.* 0·2	1	2	3	4	3	—
2	*ca.* 0·2	1	2	3	4	3	—
5	0·6	tr.	2	3	4	3	tr.
6	>0·3*	tr.	2	3	4	3	1
7	0·65	tr.	2	3	3	2	1
10	1·3	tr.	1	3	3	1	1
13	1·6	tr.	1	3	3	1	2
16	1·9	tr.	1	3	3	1	3
19	2·5	tr.	1	2	2	1	3
22	2·9	tr.	tr.	1	2	1	4
25	2·9	tr.	tr.	1	1	1	4
28	>1·5*	tr.	tr.	1	1	1	4
31	2·7	tr.	tr.	1	1	1	4

* Handling losses incurred.

† Key: Reaction with $AgNO_3$/NaOH on chromatogram; tr. = trace, 1 = weak, 2 = medium, 3 = strong, 4 = very strong. It should be noted that reducing sugars and myoinositol react much more strongly with the $AgNO_3$/NaOH reagent than equimolar quantities of galactinol, sucrose and the sugars of the raffinose series.

[13] F. PETEK, E. VILLARROYA and J.-E. COURTOIS, *Compt. Rend Acad. Sci. Paris*, Ser. D **263**, 195 (1966).

Approximately equal amounts of the mixtures of low-molecular-weight carbohydrates from seeds at different stages of development were compared chromatographically with one another and with reference compounds. The results are shown in Table 2. The visual estimations of the compounds were based on the reduction of silver nitrate on the chromatogram.

The young seeds contain small amounts of low-molecular-weight carbohydrates consisting predominantly of sucrose, myoinositol, galactinol, glucose and possibly raffinose. With increasing seed maturity the amount of free carbohydrates per seed increases and the composition of the mixture changes markedly. The proportion of stachyose in the mixture increases and then remains constant whereas the proportions of sucrose, galactinol and the hexoses decrease. Myoinositol and raffinose were not separated in the irrigant used but the type of reaction with silver nitrate showed that in the more mature seeds very little myoinositol was present. The trends with respect to fructose, sucrose, raffinose and stachyose were confirmed by quantitative determinations carried out on these four sugars in the mixtures of carbohydrates from the seeds from pods Nos. 7, 13, 22 and 31. The results are presented in Table 3.

TABLE 3. PROPORTIONS OF FRUCTOSE, SUCROSE, RAFFINOSE AND STACHYOSE IN SEEDS AT DIFFERENT STAGES OF MATURITY

Pod No.	Percentage molar composition*			
	Fructose	Sucrose	Raffinose	Stachyose
7	6·9	77	8·0	8·0
13	6·1	70	12·2	11·4
22	5·5	18·5	20·4	55
31	8·0	24·2	12·6	55

* Assuming fructose + sucrose + raffinose + stachyose = 100 per cent.

The results demonstrate that the galactomannan begins to be laid down in the seeds at an early stage of development and continues to be formed until the seeds reach their full size and begin to become yellow (compare Fig. 2 and Table 4). Thereafter the amount of galactomannan per seed appears to decrease, but this is probably due to decreasing solubility of the polysaccharide as the seeds dry out. Were it due to metabolism of the galactomannan a change in the galactose/mannose ratio would almost certainly have been observed.[7,8] No change in the proportion of galactose to mannose residues was observed during the formation of the polysaccharide; it is therefore unlikely that it is formed by random attachment of galactosyl residues to a pre-formed mannan chain.[9] Galactose and mannose residues are probably laid down at the same time by a specific mechanism.

The spectrum of low-molecular-weight carbohydrates in the seeds undergoes a remarkable change during the period of formation of the galactomannan. When the polysaccharide begins to be formed the seeds contain mainly sucrose, myoinositol, glucose and galactinol; only a trace of stachyose is present. When deposition of the galactomannan is complete the most abundant sugar in the seeds is stachyose; it is accompanied by smaller amounts of raffinose, sucrose, galactinol and the hexoses. Stachyose is probably also a reserve carbohydrate in the seeds as it is synthesized in considerable amounts at the same time as the galactomannan. Because of the synthesis of stachyose it is difficult to determine which of the

sugars in the seeds might play a part in the biosynthesis of the polysaccharide. The biosynthesis of stachyose in the seeds of *Phasaeolus vulgaris*, a legume whose seeds do not contain a reserve galactomannan, has been shown to proceed by the transfer of an α-D-galactopyranosyl residue from galactinol to raffinose.[14] In *Trigonella foenum-graecum*, stachyose and the galactomannan are synthesized in the seeds at the same time and it is not impossible that galactinol may be involved in the biosynthesis of the D-galactopyranosyl residues in the latter, especially as the galactosidic linkage in stachyose and the galactomannan is of the same type (α, 1→6).

EXPERIMENTAL

The Plant Material

The plants were sown under glass in late December 1967 and first flowered in early April 1968. Flowering continued until July 1968 when a single plant, unbranched and having thirty-two pods at all stages of maturity, was chosen for investigation. Each pod contained fourteen to twenty seeds. The seeds in the pods selected for investigation are described in Table 4.

TABLE 4. DESCRIPTION OF THE *Trigonella* SEEDS STUDIED

Pod No.	Number of seeds per pod	Max. dimension of a typical seed (cm)	Colour of seeds
1	14	0·35	Green
2	18	0·4	
4	19	0·4	
5	17	0·5	
6	16	0·5	
7	19	0·55	
10	20	0·6	
13	20	0·6	
16	17	0·7	Yellow–green
19	17	0·65	
22	15	0·7	Green–yellow
25	15	0·7	Yellow
28	15	0·65	
31	15	0·7	

General Methods

Solvents were removed using a rotary evaporator at or below 40°. Polysaccharide samples were dried by lyophilization. Polysaccharide materials (*ca.* 1 mg) were hydrolysed by the 72 per cent H_2SO_4–3 per cent H_2SO_4 method.[15] The acid was neutralized by adding $BaCO_3$ and the solids removed by centrifugation. Neutralized hydrolysates were taken to dryness and the sugars dissolved in water (0·5 ml) prior to chromatographic analysis. Small amounts of oligosaccharides (*ca.* 1 mg) were hydrolysed in 1 N HCl (3 drops) by heating for 1 hr at 110° in an autoclave. The HCl was removed by evaporation and the residue dissolved in a little water before chromatography. Chromatographic solvents were (v/v)—A, EtOAc-pyridine–H_2O (8:2:1); B, EtOAc–pyridine–H_2O (2:1:2); C, *n*-BuOH–EtOAc–HOAc–H_2O (4:3:2·5:4); D, Me_2CO–H_2O (17:3). Carbohydrates were detected on paper chromatograms using $AgNO_3$ and NaOH[16] or 2,3,5-triphenyltetrazolium chloride.[17] Galactose and mannose in hydrolysates were determined after separation by paper chromatography in solvents A or B by a modification of the method of Fischer and Dörfel.[17] The method was modified in that chromatograms were heated in a dry atmosphere and no operation was carried out in direct daylight. All analyses were carried out in triplicate and when values differed by more than 1·5 per cent from

[14] W. TANNER and O. KANDLER, *Plant Physiol.* **41**, 1540 (1966).
[15] J. F. SAEMAN, J. L. BUHL and E. E. HARRIS, *Ind. Eng. Chem. Anal. Ed.* **17**, 35 (1945).
[16] W. E. TREVELYAN, D. P. PROCTER and J. S. HARRISON, *Nature* **166**, 444 (1950).
[17] F. G. FISCHER and H. DÖRFEL, *Z. physiol. Chem.* **297**, 164 (1954).

the mean the determination was repeated. Fructose, sucrose, raffinose and stachyose were determined by the resorcinol–thiourea method.[18] The standard curve was constructed using sucrose. Dry weights were determined by heating the plant tissue for 24 hr at 105°.

Isolation of the Polysaccharides and Low-Molecular-Weight Carbohydrates

The procedure is described for a single pod; all others were treated identically. The seeds were removed from the pod and, except for those required for dry weight determination, immediately dropped into water (10 ml) at 90°. The temperature was maintained for 15 min to ensure deactivation of the enzymes. The liquid was retained and the seeds transferred to a 20 ml vial containing 10 ml of water. Ultrasonic energy was led into the mixture for *ca.* 10 min by means of the Sonifier ultrasonic probe model S75 (Branson Instruments, Inc.). During the treatment the water became very hot (*ca.* 90°) and the seed coats were broken. The supernatant liquid was sucked off and combined with the water used for enzyme deactivation. At this stage the embryos, which were apparently undamaged, were removed and the seed coats, to which the endosperm remained attached, were subjected to a further ultrasonic treatment (3–5 min). This caused dissolution or dispersion of most of the endosperm. The combined extracts were clarified by centrifugation, diluted to 50 ml and divided in two. One-half of the solution was treated with Fehling's solution to precipitate the galactomannan as its copper complex.[19] The complex was dissolved in water (15 ml), acidified with acetic acid (10 drops) and the galactomannan precipitated by the addition of ethanol (30 ml). The other half of the extract was concentrated to *ca.* 5 ml and polysaccharide material precipitated by the addition of two vols. of ethanol. The precipitate was retained for further examination and the supernatant concentrated to remove the alcohol and passed successively through short columns of Dowex 50W (H^+) and Dowex 3 (free base). The deionized solution was shaken twice with an equal volume of ether, concentrated and freeze-dried. The mixtures of soluble carbohydrates were examined by chromatography in solvent B.

Examination of the Ethanol Precipitated Polysaccharides Materials

On treatment of the crude seed extracts with ethanol the amounts of polysaccharide materials precipitated corresponded closely to those in Fig. 2 which shows the amounts of the Fehling-precipitated galactomannans. From the seeds from pods Nos. 1–4 very small amounts of polysaccharide material were obtained which, on hydrolysis, released mainly galactose, arabinose and glucose along with small amounts of mannose; they probably consisted mainly of arabinogalactan and glucan. On visual inspection of chromatograms (solvent A) treated with triphenyltetrazolium chloride no difference in galactose/mannose ratio between Fehling-purified and non-purified polysaccharide materials could be detected. All of the non-purified materials from pods 5–31 consisted mainly of galactomannan but were contaminated by glucan.

Examination of the Free Carbohydrates in the Mature Seeds

Milled seeds (10 g) were dropped into boiling water (200 ml) and the mixture allowed to cool to room temperature and filtered through cloth. The filtrate was treated with ethanol to precipitate the polysaccharides, filtered and deionized as for the young seeds and freeze-dried. A portion (*ca.* 400 mg) of the mixture was separated by filter sheet chromatography (irrigant B) into seven fractions, each corresponding to a major $AgNO_3$-positive band; no part of the paper was rejected. In the irrigant used the major non-carbohydrate impurities flowed off the end of the paper, but some of the weights given below must be regarded as approximate as fractions 1–4 were deliquescent.

Fraction 1 (*ca.* 10 mg). It was chromatographically homogeneous (solvents A, B, C) and indistinguishable from mannose and fructose. After paper electrophoresis in borate buffer, under conditions where fructose and mannose were completely separated, only fructose could be detected.

Fraction 2 (*ca.* 6 mg). It was chromatographically homogeneous and indistinguishable from glucose.

Fraction 3 (*ca.* 10 mg). Chromatography revealed two components. One was present only in trace amounts and indistinguishable from galactose; the major component was indistinguishable from sucrose.

Fraction 4 (*ca.* 10 mg). It was contaminated by non-carbohydrate material and contained at least three carbohydrate components, none of which has yet been identified. On hydrolysis it released galactose and glucose (ratio *ca.* 2:1).

Fraction 5 (6 mg). It contained two components (ratio *ca.* 15:1) chromatographically indistinguishable from raffinose and myoinositol. On hydrolysis it released galactose, glucose and fructose in the same proportions as did authentic raffinose under the same conditions.

Fraction 6 (54 mg). It contained two components (ratio *ca.* 20:1) chromatographically indistinguishable from stachyose and galactinol; they were separated by filter sheet chromatography (solvent C). On hydrolysis the major component released galactose, glucose and fructose in the same proportions as did a sample of

[18] J. H. Roe, J. H. Epstein and N. P. Goldstein, *J. biol. Chem.* **178**, 839 (1949).

[19] J. K. N. Jones and R. J. Stoodley, in *Methods in Carbohydrate Chemistry*, Vol. 5, p. 37, Academic Press, New York (1965).

stachyose under the same conditions. The minor component on hydrolysis gave galactose and myoinositol (ratio *ca.* 1:1).

Fraction 7 (14 mg). It contained two components (ratio *ca.* 5:1) both of which were non-reducing; they were separated by paper chromatography. The major component gave the same type of reaction on chromatograms treated with the $AgNO_3$/NaOH reagents as did the members of the raffinose series. On hydrolysis it released galactose and glucose (ratio *ca.* 3:1) and fructose. It was probably verbascose. The minor component released galactose and myoinositol (ratio *ca.* 2:1) on hydrolysis. It was possibly digalactosylmyoinositol [α-D-galactopyranosyl-(1→6)α-D-galactopyranosyl-(1→1)D-myoinositol].[13]

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