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α-GLUCAN SYNTHESIS IN THE COTYLEDONS OF GERMINATING LUPINS

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Key Word Index—*Lupinus lupeus*; Leguminosae; lupins; α-glucan; starch; cotyledons; germination.

Abstract—On germination, both in the dark and light, a glucan, that can be extracted with DMSO and precipitated as the I_2 complex, is synthesized in the cotyledons of lupins. In the dark, it can be labelled with radioactivity from U-^{14}C-labelled D-glucose, D-fructose, D-galactose, L-arabinose and sucrose. Enzymic hydrolysis of this material was consistent with it being α (1 → 4)(1 → 6) glucan. The iodine absorption spectra and gel chromatographic behaviour on Sepharose CL-2B of extracts at 4 days and later after imbibition indicated that the polysaccharide was starch-like. The amylose percentage increased with time of germination. The material extracted at 2 days contained no significant amount of amylose and the gel chromatographic behaviour differed somewhat from that of amylopectin.

INTRODUCTION

There have been several observations of starch formation during germination in the cotyledons of legume seeds that contain no starch in the ungerminated mature seeds. In soybeans, it appears as up to several % of the dry wt of seedlings, and granules have been detected after homogenizing [1]. Formation of starch in germinating fenugreek cotyledons has also been found [2]. This starch can be extracted with perchloric acid. None is present until 24 hr after imbibition and the maximum content is *ca* 0.4 mg seed from 48 to 60 hr and after 72 hr it starts to decline. Synthesis also occurs in germinating seeds that already contain starch at maturity. [^{14}C]-D-Glucose was incorporated into starch in pea cotyledons during germination [3].

In germinating lupin cotyledons, a temporary, glucose-containing polysaccharide that gives a colour with iodine can be extracted by hot water and alkali [4]. No material of this nature can be detected in the mature ungerminated seed. This paper reports on an examination of this glucan and its formation from monosaccharides that would be available during germination.

RESULTS AND DISCUSSION

Attempts to isolate granules by maceration of cotyledons in salt solution with toluene, followed by centrifugation [5], gave I_2-staining material heavily contaminated with cell debris, and further purification by washing and centrifugation caused major losses. Extraction with DMSO [6], followed by extraction with hot NaCl solution containing Hg^{2+} and I_2 precipitation was used but, to further minimize any risk of amylolytic degradation during extraction, Hg^{2+} was also added to the DMSO. For complete extraction of glucan, fine maceration of the sample was required.

Seeds were germinated, both in the dark and in the light, and sampled at different times and the α (1 → 4) (1 → 6) glucan content estimated (Fig. 1) by treatment of the extracted samples with amyloglucosidase and measuring the D-glucose released with glucose oxidase [7]. Duplicate experiments were made. No α (1 → 4) (1 → 6) glucan was detected before germination but there was then a rapid increase, after which the amount declined. The rate of decline was less rapid in the light indicating a contribution from photosynthesis. Although the chlorophyll content increased up to 12 days [4], a major source of the starch even in the light was apparently endogenous carbohydrate. At 1 day glucan was just detectable. One hundred ungerminated lupin seeds weighed 11 g and the time of maximum starch accumulation varied from 2 to 4 days with different batches of seed. Some other *Lupinus* spp. examined had small

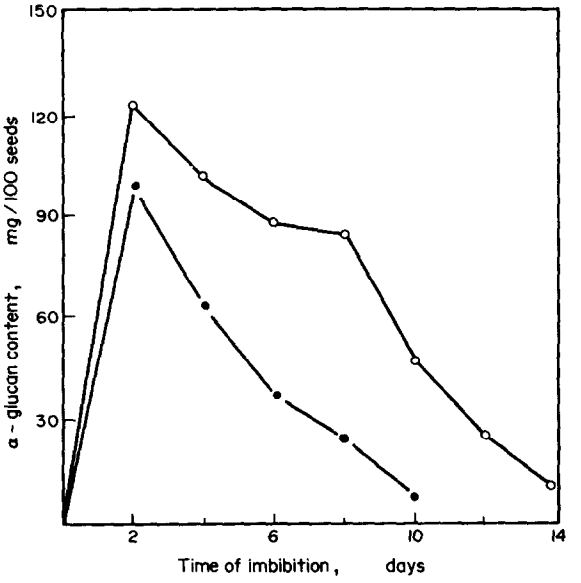

Fig. 1. Changes in α-glucan content of cotyledons of germinating lupins. ●—●, in dark; ○—○, in light.

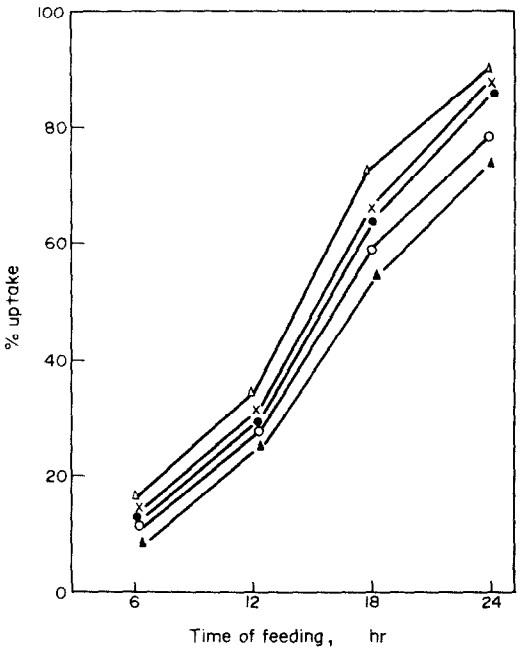

Fig. 2. Uptake of various sugars by germinating lupin cotyledons. △—△, D-glucose; ●—●, D-fructose; ▲—▲, L-arabinose; ×—×, D-galactose; ○—○, sucrose.

amounts of starch present prior to germination but all the samples of *Lupinus luteus* cv Weiko III used contained no starch at maturity.

Possible monosaccharide sources for the synthesis of glucan in dark grown seeds are: the D-glucose, D-fructose and D-galactose, formed by hydrolysis of sucrose and the raffinose series of oligosaccharides, which are present as *ca* 20% of the dry wt in ungerminated seeds; and the L-arabinose and D-galactose released, along with smaller amounts of other monosaccharides, on hydrolysis of intercellular and wall polysaccharides [8]. When these sugars and sucrose were supplied to seedlings that had imbibed for 48 hr, they were all readily taken up by the seedlings (Fig. 2). If sugar were supplied from imbibition, the glucan content, measured at 12, 48 and 96 hr after imbibition, increased (Table 1) over controls that had imbibed only water. D-Galactose and sucrose gave larger increases than D-glucose. Triplicate experiments were made.

Table 1. % increase in starch content of lupin cotyledons supplied with sugars, compared with water controls

	Time after imbibition (hr)		
Sugar supplied	12	48	96
D-Glucose	18	40	26
Sucrose	54	60	44
D-Galactose	64	68	46

Seeds were germinated for 12 or 48 hr in water and then supplied with ^{14}C-labelled sugars. The $^{14}CO_2$ respired was collected and after 24 hr the cotyledons were macerated and extracted with hot ethanol, filtered and the glucan extracted from the residue. Incorporation into α-glucan was estimated after hydrolysis with amyloglucosidase and separation by PC of the D-glucose released. In Table 2 the incorporation of label into these fractions is shown. All the sugars, including L-arabinose and D-galactose, were metabolized and converted in part into α-glucan. The lower conversion of D-glucose to α-glucan, accompanied by the higher conversion to CO_2 than with the other sugars, was found consistently. There was more incorporation into glucan in the period 12–36 hr after imbibition than in the later period (48–72 hr) when there was a nett decrease in starch content and more epicotyl and hypocotyl growth. Experiments were duplicated.

Thus, lupin seeds on germination appear to hydrolyse sucrose and galactosylsucrose oligosaccharides rapidly

Table 2. Uptake and metabolism of ^{14}C-labelled sugars by germinating lupin cotyledons

	Labelled sugar supplied				
	D-Glc	D-Fru	L-Ara	D-Gal	Sucrose
Seeds imbibed 12 hr prior to supply of labelled sugar					
% uptake	96	87	70	97	86
% incorporated* into:					
α-Glucan	1.0	6.8	8.3	7.5	6.0
CO_2	20.0	6.8	5.1	10.5	6.8
EtOH-soluble	8.8	10.0	17.1	19.5	14.0*
Seeds imbibed 48 hr prior to supply of labelled sugar					
% uptake	90	90	90	90	80
% incorporated* into:					
α-Glucan	0.7	1.7	1.6	0.4	0.5
CO_2	13.0	8.8	5.5	5.5	8.6
EtOH-soluble	7.8	6.5	9.0	5.5	6.6

* % of total supplied activity.

[4] and to convert the monosaccharides produced in excess of synthetic and respiratory requirements to polymeric glucan. This can be compared to germinating fat-storing seeds where the corresponding storage carbohydrate is sucrose [9].

Some properties of the extracted samples were then studied. They contained substantial amounts of protein which were only slightly reduced by re-precipitation with I_2. Hydrolysis with acid or amyloglucosidase showed that glucose was the only monosaccharide produced and that the same amounts were produced by either treatment. All the polysaccharide was completely excluded on Bio-gel P-10 when carbohydrate was detected with anthrone. After treatment with amyloglucosidase all the carbohydrate was completely retarded, indicating complete hydrolysis by this enzyme. α-Amylase treatment also removed all excluded polysaccharide and gave a series of malto-oligosaccharides on PC. β-Amylase produced maltose. On long incubation with β-glucosidase, which was prepared from germinating lupin cotyledons by DEAE-cellulose chromatography [8] and which was free of amylolytic and α-glucosidase activities, no reducing sugar was released. These results are consistent with the polysaccharide being an α-glucan of the starch type. The isopotential absorption of I_2 was then measured by potentiometric titration [6] (Table 3). Absorption increased with time after imbibition, indicative of an increase in the % amylose content of total starch. The sample from seeds germinated for 2 days in the dark may have been devoid of, or contained insignificant amounts of, amylose, as at the temperature of measurement amylopectin shows a slight absorption [10]. The absorption spectra of the iodine–glucan complexes were then measured for dark- and light-grown samples (Figs. 3 and 4), as well as for samples of amylopectin (waxy maize starch), phytoglycogen and bean leaf starch (Fig. 4), under the same conditions. The wavelength of A_{max} and the A unit wt of glucan increased in the lupin samples, in agreement with the results from potentiometric I_2 absorption, indicating an increase in the % amylose content with time or germination. The wavelength of A_{max} in the dark-grown samples increased from 540 nm at 2 days to 560 nm at 4 days and in the light-grown to 580 nm at 8 days. At similar I_2 and starch concentrations, bean leaf starch had a maximum at 580 nm and potato starch at 590 nm. The spectrum of the 2 day dark sample was very similar to waxy maize starch indicating that it contained very little or no amylose.

When the extracted material, prepared after 8 days in the light, was chromatographed on Sepharose CL-2B (Fig. 5), the elution pattern was similar to that found with starch, i.e. an excluded fraction with an A_{max} with I_2 at a similar wavelength to that shown by amylopectin and an included fraction corresponding to amylose [11]. Also, after treatment with *n*-BuOH and centrifugation of the amylose–BuOH complex, chromatography of the supernatant indicated removal of the included amylose peak.

Table 3. Isopotential I_2 absorption of α-glucans isolated from the cotyledons of lupins germinated in the dark and light

Time after imbibition (days)	I_2 absorption (g/100 g)	
	Dark	Light
2	0.4	n.d.*
4	0.92	1.20
6	1.44	2.30
8	n.d.	2.52

* n.d. = not determined.

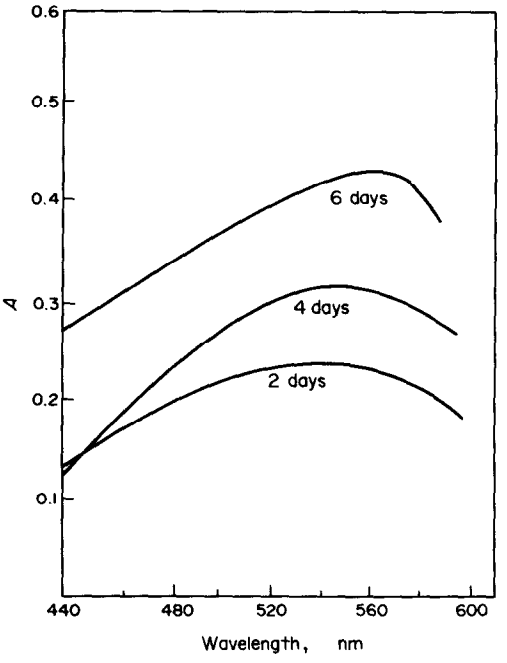

Fig. 3. Glucan–I_2 absorption spectra of α (1 → 4)(1 → 6) glucans isolated from germinating lupin cotyledons in the dark. Numbers refer to days after imbibition.

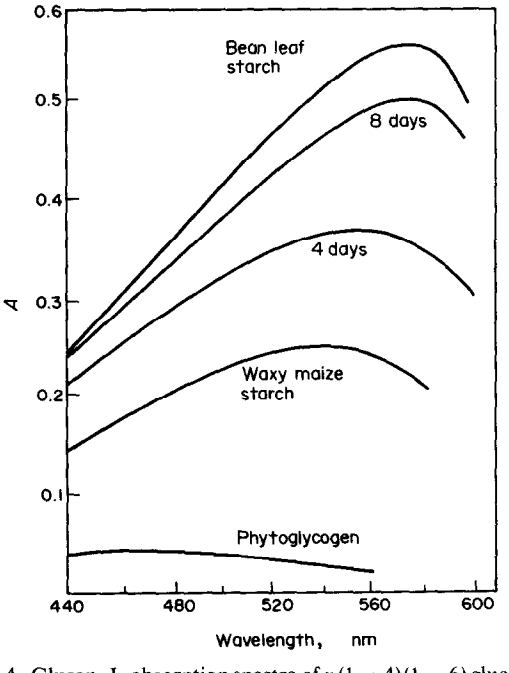

Fig. 4. Glucan–I_2 absorption spectra of α (1 → 4)(1 → 6) glucans isolated from germinating lupin cotyledons in the light, corn phytoglycogen, waxy maize starch and bean leaf starch. Numbers refer to days after imbibition.

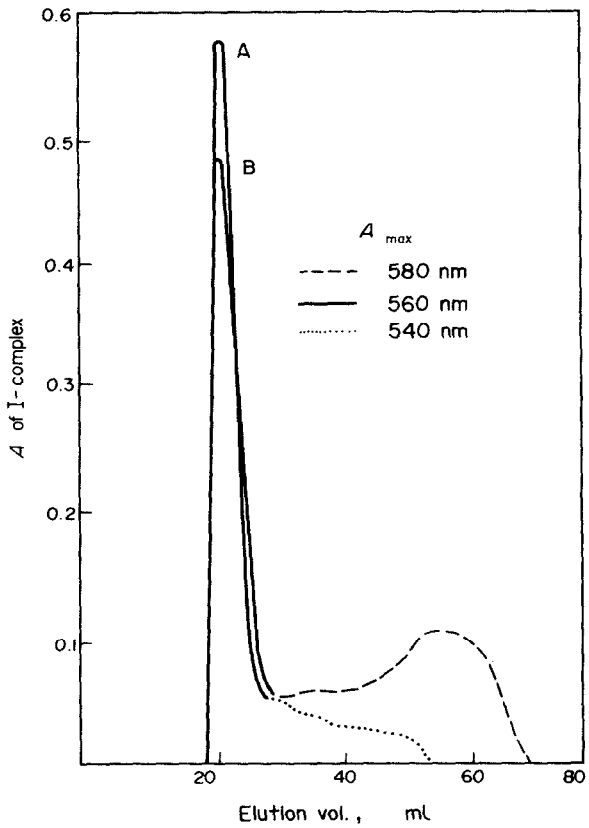

Fig. 5. Gel chromatography on Sepharose CL-2B of α (1 → 4) (1 → 6) glucan isolated from lupin cotyledons at 8 days in the light. A, before *n*-BuOH treatment; B, after *n*-BuOH treatment.

However, when the sample from 2 days in the dark was chromatographed, although it showed two elution peaks (Fig. 6), the included was larger than the excluded and the A_{max} with I_2 of the included fraction was at lower wavelength. Also, it was unchanged by treatment with *n*-BuOH and centrifugation. Fractions were also assayed for carbohydrate with anthrone and the peaks obtained corresponded with the I_2-absorbing fractions. The results show that the α-glucan extracted from seeds germinated in the dark for 2 days contains little or no unbranched fraction. Also, a major portion of it is a branched component that is included on Sepharose CL-2B chromatography and hence probably has a lower MW than is usually found for amylopectin [6, 11]. Chromatography on Sepharose CL-6B showed a wide range of MW for this fraction. The lower wavelength of A_{max} suggests a possible minor difference in structure to amylopectin for the included fraction, e.g. shorter average chain length.

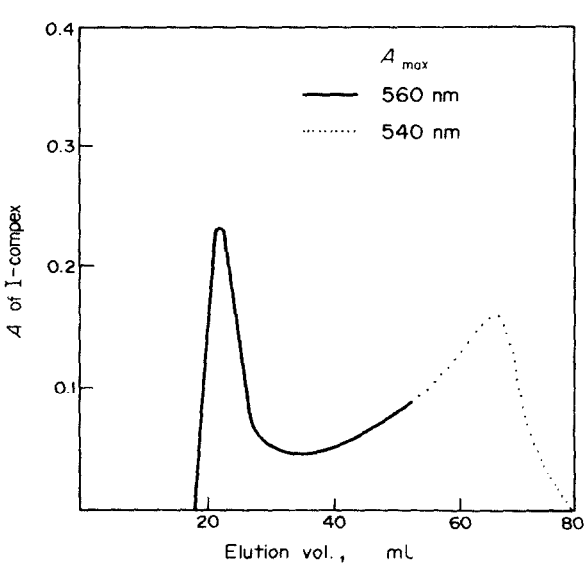

Fig. 6. Gel chromatography on Sepharose CL-2B of α (1 → 4) (1 → 6) glucan isolated from lupin cotyledons at 2 days in the dark.

In germinating lupin cotyledons, production of this α-glucan in quantity may precede the usual mixture of amylopectin and amylose or it may be partly degraded material. The function of cotyledons to provide carbohydrate for translocation to the developing hypocotyl and epicotyl suggests the latter. However, it is produced during a period when cotyledons are expanding. It is also a period of rapid accumulation of glucan, when adequate carbohydrate reserve is still present as sucrose [4]. Also, at later times of imbibition both in the dark and the light, the isolated glucan resembles starch. It is characteristic of starch accumulation in plant tissues over a longer time period for the amylose percentage in the starch to increase as starch content increases, e.g. in potato tubers [12], tobacco leaves [13], pea seeds [14] and wheat grains [15].

The present data suggest that the first insoluble polymer to accumulate may be branched. The synthesis of this presumably involves a branching enzyme and a soluble unbranched fraction, which may be attached to protein [16]. Thus, the early stages of starch synthesis may resemble glycogen synthesis, followed by increasing amylose deposition as starch accumulates. Germinating lupin cotyledons may provide a useful system for examining the *de novo* synthesis of starch.

EXPERIMENTAL

Seed germination. Lupinus luteus cv Weiko III was germinated as previously described [4].

Extraction of glucan. Cotyledons (1000) were washed with H_2O and macerated in EtOH. The mixture was boiled and then further macerated in an Ultra-turrax, filtered and washed with Me_2CO and Et_2O and dried. 48 Hr after imbibition 29 g of flour was obtained. The flour was wetted with DMSO (Merck) containing 0.01 M $HgCl_2$ (5 ml/g flour) and kept at 35° for 24 hr. EtOH (4 vols) was added and the mixture centrifuged. It was washed with Me_2CO and peroxide-free Et_2O, and heated 15 min in a steam bath under N_2 with 0.1 M NaCl soln containing 0.01 M $HgCl_2$ (10 ml/g flour). The mixture was centrifuged and the residue extracted (× 2) with cold NaCl soln using an Ultra-turrax blender. The supernatants were combined, 3% I_2 in 30% KI soln was added and the mixture stored at 2°. The ppt. was collected after centrifugation (14 000 *g*, 15 min, 2°) and the supernatant discarded. This ppt. was dispersed in 0.1 M $NaAsO_2$ in 0.2 M Pi buffer (pH 6) and dialysed for 18 hr. NH_4OAc was added to 0.001 M, the soln stirred for 30 min and centrifuged (14 000 *g*, 15 min, 25°). The supernatant was poured into 3 vols of EtOH. The ppt. was collected by centrifugation, washed with EtOH, Me_2CO and Et_2O and dried in a desiccator.

Protein estimation. The biuret method [17] was used.

Estimation of glucan content of extracts. Samples were redissolved by heating in a boiling water bath, cooled and a suitable aliquot was hydrolysed with amyloglucosidase (0.4 μkat/mg glucan; Sigma, 1.7 μkat/mg) in 0.025 M acetate buffer (pH 5) at

37°. Another aliquot was hydrolysed with 0.5 M H_2SO_4 for 3 hr. Glucose released was estimated using glucose oxidase (Sigma, 4.1 μkat/mg) and the colorimetric method with 4-amino-antipyrine [18].

Uptake of unlabelled sugars by germinating lupin seeds. The seed coats were removed from 15 seeds that had imbibed H_2O for 48 hr and were incubated at 30° with the sugar soln (15 mg in 5 ml) in a container inside a sterile chamber through which a stream of filtered air was passed. Uptake was determined by estimation of the remaining carbohydrate in soln with anthrone [19].

Incorporation of labelled sugars into fractions of lupin cotyledons. 15 seedlings (with seed coats removed) that had imbibed H_2O for either 12 or 48 hr were incubated at 30° with 5 ml of soln containing unlabelled sugar (15 mg) and [U-^{14}C]-sugar (2 μCi; 50–200 mCi/mmol) in a container inside a sterile chamber (painted black to maintain dark conditions) through which was passed a stream of filtered air from which CO_2 had been removed. The gas leaving the chamber was then passed through aq. NaOH to trap the CO_2. After 24 hr incubation, unabsorbed soln and seed washings were made up to a standard vol. for counting. Cotyledons were separated, macerated in EtOH and the mixture was boiled for 5 min, cooled and filtered. The filtrate was made up to standard vol. for counting as the EtOH-soluble fraction. The residue was washed with Me_2CO and Et_2O, dried, and the glucan extracted as described previously. The product was re-dissolved, incubated with amyloglucosidase, de-ionized and glucose separated by PC and counted on filter paper in toluene using the scintillation method.

Determination of I_2 absorption spectra. Glucan samples (25 mg) were incubated with DMSO (0.15 ml) for 18 hr at 37°. H_2O was added, the mixture shaken and warmed if necessary until the material was in soln, and the vol. made up to 50 ml. An aliquot was added to a soln of I_2 (0.1% in 1% KI; 2 ml) and made up to a suitable vol. with H_2O.

Gel chromatography on Sepharose CL-2B. A 1% soln of the glucan in 0.1 M NaCl–0.005 M EDTA was prepared by heating samples under N_2 in a steam bath for 10 min. After cooling, an aliquot (1.0 ml) was applied to a column at 25°. I_2 absorption spectra and carbohydrate content by anthrone [19] were measured for each fraction. A portion of the glucan soln was also treated with *n*-BuOH (0.75 ml/10 ml) while still hot and after 48 hr at room temp., was centrifuged (14 000 *g*, 15 min, 25°) to remove any amylose–BuOH complex and an aliquot (1 ml) chromatographed.

Gel chromatography on Bio-Gel P-10 of reaction products of enzymic hydrolysis of glucans. Glucan solns (0.5% in 0.001 M NaCl, 5 ml) were incubated at 30° with salivary α-amylase (0.4 μkat; Sigma, 15 μkat/mg), crystalline sweet potato β-amylase (2 μkat; Sigma, 14 μkat/mg), or amyloglucosidase (1 μkat) at 30° for 30 min. Reaction was stopped by heating the tube in a steam bath for 1 min. An aliquot (1 ml) was applied to a Bio-Gel P-10 column and the fractions assayed with anthrone [19]. Another aliquot was examined by PC irrigated with *n*-BuOH–pyridine–H_2O–C_6H_6 (5:3:3:1 upper phase) as solvent and developed with *p*-anisidine–HCl or $AgNO_3$. TLC was also performed on Si gel–kieselguhr with *n*-PrOH–$MeNO_2$–H_2O (5:2:3) [20]. The developed plates were sprayed with 5% conc H_2SO_4 in EtOH and heated at 100° for 5–10 min.

Measurement of isopotential I_2 binding of glucans. This was performed as previously described [6]. Readings were reproducible to ±0.5 g I_2/100 g starch.

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REFERENCES

1. Hizukuri, S., Fujii, M. and Nikuni, Z. (1961) *Nature* **192,** 239.
2. Reid, J. S. G. (1971) *Planta* **100,** 131.
3. Juliano, B. O. and Varner, J. E. (1969) *Plant Physiol.* **44,** 886.
4. Matheson, N. K. and Saini, H. S. (1977) *Phytochemistry* **16,** 59.
5. Cowie, J. M. G. and Greenwood, C. T. (1957) *J. Chem. Soc.* 2658.
6. Matheson, N. K. (1975) *Phytochemistry* **14,** 2017.
7. Libby, R. A. (1970) *Cereal Chem.* **47,** 273.
8. Matheson, N. K. and Saini, H. S. (1977) *Carbohydr. Res.* **57,** 103.
9. Bewley, J. D. and Black, M. (1978) *Physiology and Biochemistry of Seeds in Relation to Germination,* Vol. 1, p. 212. Springer, New York.
10. Banks, W. and Greenwood, C. T. (1975) *Starch and Its Components,* p. 110. Edinburgh University Press, Edinburgh.
11. Matheson, N. K. (1971) *Phytochemistry* **10,** 3213.
12. Geddes, R., Greenwood, C. T. and MacKenzie, S. (1965) *Carbohydr. Res.* **1,** 71.
13. Matheson, N. K. and Wheatley, J. (1962) *Aust. J. Biol. Sci.* **15,** 445.
14. Greenwood, C. T. and Thomson, J. (1962) *Biochem. J.* **82,** 156.
15. Duffus, C. M. and Murdock, S. M. (1979) *Cereal Chem.* **56,** 427.
16. Lavintman, N., Tandecarz, J., Carceller, M., Mendiara, S. and Cardini, C. E. (1974) *Eur. J. Biochem.* **50,** 145.
17. Itzhaki, R. F. and Gill, D. M. (1964) *Analyt. Biochem.* **9,** 401.
18. Trinder, P. (1969) *Ann. Clin. Biochem.* **6,** 24.
19. Loewus, F. A. (1952) *Analyt. Chem.* **24,** 219.
20. Huber, C. N., Scobell, H. D., Tai,, H. and Fisher, E. E. (1968) *Analyt. Chem.* **40,** 207.