Biochem. J. (1974) **142**, 153–159
Printed in Great Britain

Cyclitol Glucosides and their Role in the Synthesis of a Glucan from Uridine Diphosphate Glucose in *Phaseolus aureus*

CHARACTERIZATION OF SOME CYCLITOL GLUCOSIDES AND THEIR SYNTHESIS

JENNIFER KEMP* and BRIAN C. LOUGHMAN
Department of Agricultural Science, University of Oxford, Oxford OX1 3PF, U.K.

(*Received* 18 *March* 1974)

Short-chained sugar compounds, thought to be involved in the synthesis of callose, were formed in small amounts from UDP-glucose by soluble extracts from hypocotyls of seedlings of *Phaseolus aureus*. The properties of the glycosides were investigated by treatment with various chemicals and analysis by paper chromatography, g.l.c. and mass spectrometry. The data obtained support the characterization of these compounds as *myo*inositol-β-glucoside and diglucosyl*myo*inositol. The cyclitol moiety was provided by the enzyme extract. Free *myo*inositol was not the immediate substrate but a compound containing *myo*inositol, isolated from the enzyme extract, may be involved. The method of synthesis of these glucosides is compared with that of other cyclitol glycosides.

Several naturally occurring glycosides of *myo*inositol have been found in higher plants. The first report of the synthesis of a compound of this type was by Frydman & Neufeld (1963), who described the transfer of galactose from UDP-galactose to *myo*inositol to form galactinol in the presence of an enzyme extracted from peas. Senser & Kandler (1967) underlined the importance of galactinol as a galactose donor in the synthesis of the raffinose series of oligosaccharides.

In the present work a connexion has also been established between the formation of glycosidic cyclitols and the synthesis of sugar polymers. A study has been made of the initiation of chains of callose, a β-(1→3)-linked glucan. The subsequent synthesis of this glucan has been described by Feingold *et al.* (1958), Flowers *et al.* (1968) and Elbein & Chambers (1970).

Since callose could be synthesized from UDP-glucose in the presence of a soluble enzyme any substance needed to initiate a polysaccharide chain must be present in solution in the enzyme extract and the present paper describes the results of the search for these compounds and their characterization.

Experimental

Materials

UDP-[^{14}C]glucose was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-glucose and anilinediphenylamine spray were obtained from Sigma, Norbiton Station Yard, Kingston-upon-Thames, U.K. Cab-O-Sil (colloidal silica), laminaribiose (Glcβ1→3Glc) and laminaritriose (Glcβ1→3Glcβ1→3Glc) were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Glucosylmannitol was kindly provided by Mr. John A. Farrar, Department of Agricultural Science, University of Oxford, Oxford, U.K.

Methods

Growth of etiolated seedlings of Phaseolus aureus (mung bean). Seeds were obtained from Palms, Oxford, U.K., and before being planted they were soaked in distilled water for several hours. A mixture containing 1.5 litres of sterilized sand and 0.5 litre of distilled water was prepared and the seeds were shallowly planted in this mixture in enamel trays. They were kept in the dark at 25°C and after the first 2½ days were watered daily with about 100ml of distilled water. Seedlings between 3 and 7 days old were used to prepare the enzyme.

Preparation of enzyme. Mung-bean shoots were separated from their roots, cotyledons and young leaves, and were chilled. The enzyme was prepared essentially as described by Flowers *et al.* (1968). Chilled shoots were ground in a pestle and mortar with an equal volume of 0.1M-Tris–HCl buffer, pH7.5. The suspension was filtered through two layers of cheese cloth and the filtrate centrifuged at 1000*g* for 5min. The supernatant was centrifuged at 15000*g* for 30min. All centrifugation was carried out in the cold. The pellet was resuspended in 0.1M-Tris–HCl buffer, pH7.5, by using 20μl of the buffer for every gram of fresh bean shoots, the resulting suspension containing about 20mg of protein/ml.

* Present address: Department of Biochemistry, University of Dundee, Dundee DD1 4HN, U.K.

This particulate fraction was treated with an equal volume of a 1.6% (w/v) solution of digitonin. In a few early experiments the digitonin was dissolved in 0.05M-sucrose. The mixture was kept on ice for 15min with occasional shaking and centrifuged at 15000*g* for 20min. The supernatant thus prepared catalysed the formation of glucan and oligosaccharides from UDP-glucose and contained about 2mg of protein/ml.

Incubation conditions. Incubations (50μl) were carried out in a low concentration (often 1μM) of UDP-glucose containing about 20000d.p.m. of UDP-[^{14}C]glucose and 2mM-$MgCl_2$ in 80mM-Tris–HCl buffer, pH7.5. Usually 10μl of enzyme was used and incubations were carried out for different periods at room temperature (18°C). The enzyme reactions were stopped by boiling.

Thin-layer and paper chromatography. Double development of cellulose t.l.c. plates in propan-1-ol–ethyl acetate–water (7:1:2, by vol.) was found to be a fairly quick and satisfactory way of separating oligosaccharides of different sizes. Paper chromatography was found to be superior for separating similar oligosaccharides, especially when large amounts of material were involved. Separation of oligosaccharides on paper was carried out by descending chromatography on Whatman no. 1 paper. Usually the solvent was propan-1-ol–ethyl acetate–water (7:1:2, by vol.). Development was carried out either for 48h at room temperature or for 36h at 30°C.

Radioactive paper chromatograms were cut up into half-centimetre strips and counted in a scintillation spectrometer. T.l.c. plates were divided into half-centimetre strips and the plate material was scraped from the glass base with a razor blade before being counted in the scintillation spectrometer. The cohesive properties of the cellulose enabled accurate recovery of the plate material. Standard sugars were detected with either aniline–diphenylamine spray from Sigma or alkaline silver nitrate spray (Block *et al.*, 1958). Nucleotides were located by their u.v. absorption.

Large-scale preparation of glucosides. Glucosides were prepared from incubations of low concentrations of UDP-glucose with digitonin-solubilized enzyme by using the normal concentrations of Tris–HCl buffer and $MgCl_2$ but scaling up to several millilitres according to the yield required. For preparations for mass spectrometry and gas chromatography the concentration of UDP-glucose was usually 50μM and the radioactivity was decreased to the minimum amount required for detection of the product on a paper chromatogram.

After incubation for 10min the preparation was boiled to kill the enzyme and then freeze-dried before being spotted on to paper and chromatographed in propan-1-ol–ethyl acetate–water (7:1:2, by vol.) for about 36h at 30°C. The radioactive peak near the laminaribiose (Glcβ1→3Glc) marker was eluted with water and the eluate was evaporated to dryness in a vacuum desiccator over P_2O_5.

Determination of radioactivity. Radioactive samples were counted in a Beckman liquid-scintillation spectrometer, in scintillant containing 7g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l of toluene. Where cellulose-plate material was to be counted for radioactivity 25g of Cab-O-Sil (colloidal silica)/l was added to the scintillant and each sample was shaken to disperse and suspend the plate material. In experiments using compounds labelled only with ^{14}C the counting efficiency was of the order of 70%.

Enzymic hydrolysis. A preparation of β-(1→3)-glucanase, described by Manners & Patterson (1966), was kindly supplied by Professor D. Manners, Herriott-Watt University, Edinburgh, U.K. Approximately 0.75mg of this enzyme was incubated with about 1μg of the glucoside in 0.05M-dithiothreitol in a volume of 200μl for 1h or more.

Mild acid hydrolysis. Mild acid hydrolysis was carried out in 1M-HCl for 15min at 100°C. The acid was evaporated in a vacuum desiccator over KOH and the products were redissolved in water and then evaporated to dryness twice more.

Borohydride reduction. The reaction mixture consisted of 20μl of sugar (15000c.p.m.; 70pmol), 10μl of fresh $NaBH_4$ solution (1mg/ml) and 10μl of boric acid (2mg/ml). Incubation was carried out for 30min at room temperature and the reaction was stopped by the addition of 20μl of 10% (v/v) acetic acid. The mixture was then passed through a small Dowex 50 column (H^+ form) to remove Na^+ ions. A very high proportion of the radioactive material could be recovered from the eluate, which was evaporated to dryness in a vacuum desiccator over P_2O_5 and then treated with methanol and evaporated to dryness three times. This removed the boric acid as the volatile methyl ester. If boric acid was omitted from the initial incubation the product was glucose, presumably formed by hydrolysis. Reduction of laminaribiose by this method gave a high yield of glucosylglucitol.

Oxidation with lead tetra-acetate. A dried preparation of the unknown disaccharide (approx. 7000d.p.m.; 0.15μg) was treated with 20μl of lead tetra-acetate (0.1mg/ml), 75μl of acetic acid and 5μl of water. The mixture was stirred and left for 2h. Oxalic acid (1μl of a solution containing 0.6mg/ml) was added and after 30min the mixture was centrifuged at 20000*g* for 20min to separate the precipitate of lead oxalate from the radioactive solution, which was then examined by paper chromatography.

Silylation of sugar compounds. Sugars were converted into the trimethylsilyl derivatives before gas chromatography or mass spectrometry. The samples were completely dried and then treated with bistrimethylsilylacetamide; 1μl of this reagent was added for each microgram of sugar present. The

reaction vessel was then tightly sealed and left overnight at room temperature. Complete silylation was effected under these conditions.

Gas chromatography. The apparatus used was a Pye model with a flame ionization detector. The column (1.5m×0.6cm) was stainless steel. The support was 1–120 diatomite C and the stationary phase was 3% SE52. The gas-flow rate was 1ml/s. Operation was carried out with a single column and a constant temperature.

Trimethylsilyl derivatives of monosaccharides were separated at 150°C and those of disaccharides were separated at 180°C. Each injection consisted of about 1 μg of sample in 1 μl. Standards for comparison were prepared under identical conditions and were injected at the same time as the experimental samples. Retention times and half-height peak widths were measured where necessary.

Mass spectrometry. Mass spectrometry of the trimethylsilyl derivatives of the unknown biose and some standards were carried out by Dr. Aplin in the Dyson Perrins Laboratory, Oxford, U.K. About 5 μg of the sugar was used in each run.

Results

An experiment was designed to isolate compounds which might be the products of early stages in the synthesis of the glucan. If some component of the soluble enzyme extract was required for chain initiation then soluble short-chain oligosaccharides might be formed under conditions where the proportion of that component to UDP-glucose was high. To investigate this possibility 1 μM-UDP-glucose was incubated with enzyme which had been extracted from seedlings aged 4½–5 days. After incubation for 5min the mixture was boiled, analysed by t.l.c. and the chromatogram sectioned and counted. Fig. 1 shows that about 80% of the UDP-glucose had reacted to form a compound which appeared in the position of laminaribiose (Glcβ1→3Glc). In some experiments using seedlings of different ages a trisaccharide and a tetrasaccharide were also formed.

Chemical analyses

The fastest-moving compound, which we now designate biose, co-chromatographed with laminaribiose in several solvents. However, the radioactive sample was separated from authentic laminaribiose by developing a paper chromatogram in propan-1-ol–ethyl acetate–water (7:1:2, by vol.) for 72h at room temperature. The radioactive trisaccharide (designated triose) could be separated from laminaritriose by using the same solvent and developing the chromatogram for 100h. Under these conditions the unknown biose could be distinguished from some of the commonly occurring disaccharides, namely, cellobiose (Glcβ1→4Glc), maltose (Glcα1→4Glc), lactose (Galβ1→4Glc) and trehalose (Glcα1→α1→Glc).

A straight line was obtained by plotting the mobilities of the biose, triose and tetrasaccharide against the number of sugar units they contained; this, according to French & Wild (1952) is a test of whether the compounds have the same linkage.

When the unknown product was hydrolysed with a β-(1→3)-glucanase about 30% of the radioactivity was converted into a compound with the same chromatographic mobility as glucose. Acid hydrolysis of the biose also gave a radioactive product which had the same chromatographic mobility as glucose. It was probable, therefore, that the unknown biose was the β-glucoside of a non-radioactive moiety.

The unknown biose was treated with lead tetraacetate under conditions which would oxidize both

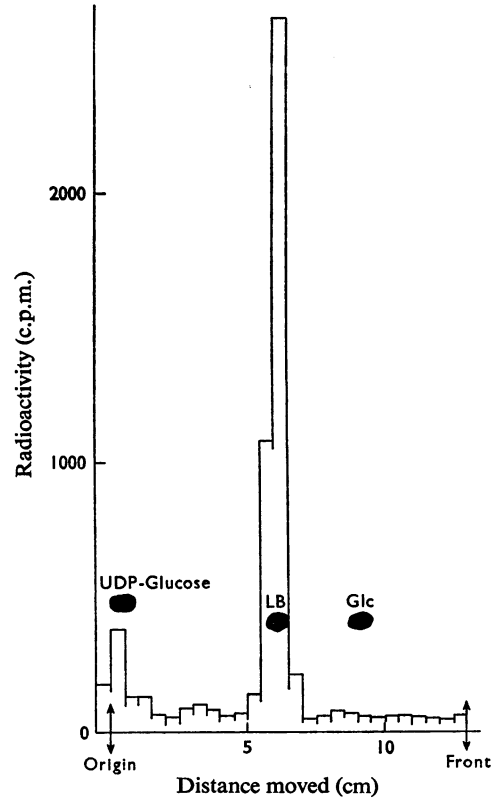

Fig. 1. *Chromatogram showing the products after incubating 1 μM-UDP-[^{14}C]glucose with enzyme extracted from 4½–5-day-old seedlings of Phaseolus aureus*

The incubation with 1 μM-UDP-[^{14}C]glucose and 20 μl of enzyme was for 5min. A portion of the resulting mixture was separated by t.l.c. and the radioactivity measured. All the methods are described in the text. LB, laminaribiose.

laminaribiose and cellobiose. The unknown compound was also treated with $NaBH_4$ under conditions which would reduce both laminaribiose and cellobiose. Neither oxidation products nor reduction products could be isolated, suggesting that the biose itself was non-reducing. Acid hydrolysis of the radioactive compounds left after both these reactions gave a product which had the same mobility as glucose. Clearly there was no free reducing group in the glucose moiety, its C-1 carbon atom being involved in the linkage.

Further chemical identification depended on the isolation of microgram quantities of the biose so that the techniques of g.l.c. and mass spectrometry could be used and thus the identification would no longer depend on the presence of ^{14}C.

The trimethylsilyl derivative of the unknown compound gave a single g.l.c. peak. This was additional evidence for it being a non-reducing compound. The biose derivative was clearly separated by g.l.c. from the derivatives of most of the common disaccharides and could be distinguished from glucosylβ1$\rightarrow$3glucitol, prepared by reduction of laminaribiose and also from glucosylβ1$\rightarrow$3mannitol, with this method. However, the unknown compound could not be separated from sucrose.

Assuming that the detector-response factor for the unknown compound was similar to those for known disaccharides, a calculation of the peak height gave an estimate of the amount of biose present. The amount of glucose incorporated into the biose could be calculated from the specific radioactivity of the UDP-[^{14}C]glucose and the radioactivity in the compound. Comparison of these two values suggested that one sugar moiety was not radioactive, i.e. that the UDP-glucose was the source of only one of the sugar units.

Mass spectroscopy of the trimethylsilyl derivatives of the unknown biose, sucrose and laminaribiose was carried out and the patterns of disintegration were similar but each compound gave peaks that were not present in the other.

The largest fragment produced by the unknown compound has a mass of 903. This was unlikely to be a mass ion but could be a mass ion minus a methyl group. In that case the unknown could have the molecular weight of a dihexose.

Kochetkov *et al.* (1968, 1973) claim that dihexoses break to give certain fragments which are diagnostic of the linkages in those disaccharides. The spectrum from the unknown biose did not contain any of these specific fragments, suggesting that it was not a dihexose but a glycoside with a C_6 aglycone.

Batches of radioactive biose and triose were then isolated and subjected to mild acid hydrolysis. After removal of the acid, trimethylsilyl derivatives were made and the components separated by g.l.c. Each preparation gave three peaks and appeared to contain the same sugar moieties, which were characterized more fully in the triose. A mixture of the trimethylsilylated hydrolysate of the triose with trimethylsilyl glucose showed three peaks, two of which could thus be identified as trimethylsilyl α-glucose and trimethylsilyl β-glucose. A mixture of the trimethylsilyl hydrolysate with trimethylsilyl fructose, however, gave four peaks, showing that fructose was not one of the hydrolysis products.

The third peak in the hydrolysate had a longer retention time than the trimethylsilyl hexoses and was similar to that of trimethylsilyl *myo*inositol. When a *myo*inositol standard was mixed with the hydrolysate, no fourth peak appeared and the increase in height of the slow peak did not appear to be accompanied by an increase in peak width. Other cyclitols can be clearly separated from *myo*inositol under similar conditions (Lee & Ballou, 1965) and although it has not been possible to check the retention times of other cyclitols under the precise conditions used in the present work it can be fairly confidently claimed that the third peak was trimethylsilyl *myo*inositol. These results are shown in Fig. 2. It can also be seen that the peaks from glucose and inositol are in the proportions expected if the triose contained 1 molecule of inositol and 2 of glucose. It appeared therefore that the biose was the β-glucoside of *myo*inositol and that the triose was diglucosyl*myo*-inositol. It has already been suggested that the biose, triose and tetraose were possibly from the same family of oligosaccharides. The tetraose might therefore be triglucosylinositol.

Biosynthesis of the glucosides

The particulate enzyme fraction obtained before digitonin treatment also catalysed the formation of a disaccharide which had the same mobility as that shown in Fig. 1. It seemed therefore that the treatment with digitonin was not necessary for the formation of the compound and there was no evidence that its synthesis was unphysiological.

Experiments were designed to investigate the biosynthesis of the disaccharide glucosylinositol. Dual-labelling experiments were carried out to see whether *myo*inositol could be used as a substrate for biose production. Fig. 3 shows a chromatogram obtained after incubation of [^{3}H]inositol and UDP-[^{14}C]glucose with the enzyme. A single biose peak was formed which contained both ^{14}C and ^3H, providing good evidence for the incorporation of ^3H into the biose together with further confirmatory evidence for the chemical identification of the biose as glucosylinositol.

However, only a very small proportion of the [^{3}H]inositol reacted to form biose and the specific radioactivity of the ^3H in the aglycone was clearly much decreased. Table 1 shows the results of an

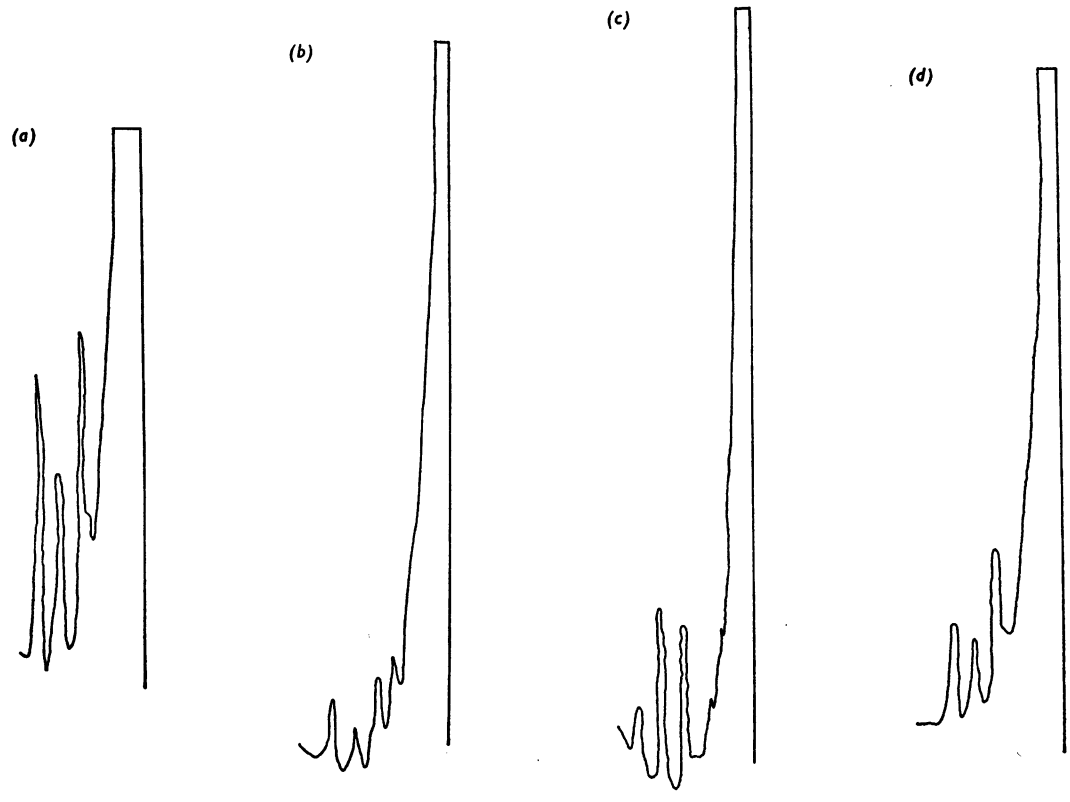

Fig. 2. *Separation and analysis by g.l.c. of the trimethylsilylated products after hydrolysis of the unknown trisaccharide*

The unknown trisaccharide was hydrolysed with HCl, treated with bistrimethylsilylacetamide and the products were separated by g.l.c. Details of these methods are given in the text. (*a*) Hydrolysate+*myo*inositol; (*b*) hydrolysate+fructose; (*c*) hydrolysate+glucose; (*d*) hydrolysate.

experiment to test the effect of the concentrations of UDP-[^{14}C]glucose and [^{3}H]inositol on their incorporation into biose. It is clear that more ^{3}H is incorporated at higher concentrations but that the added cyclitol is the source of only a minor part of the inositol in the biose. It seemed unlikely therefore that free inositol was the immediate substrate required to make biose.

Moreover, in all these experiments a small proportion of the ^{3}H accumulated at the origin of the chromatogram. No way has yet been found of increasing the amount of this product so accurate measurements cannot yet be made to assess whether this unknown product was the source of the inositol in the synthesis of the biose.

Plants aged 4½–5 days were used to show the effect of the concentration of UDP-glucose on the synthesis of the biose. The enzyme preparation was used as the source of the inositol, which may have been limiting the reaction at higher concentrations of UDP-glucose. The results are shown in Fig. 4. Under the conditions used in the experiment the glucoside was neither converted into higher oligosaccharides nor broken down to glucose and the biose was the major product, although about 5% of the UDP-glucose was incorporated into an unidentified product.

It is clear from this experiment that the cyclitol moiety must be present in the extract at a concentration of at least 11 μM. This corresponds to a concentration of about 600 pmol/g of fresh bean tissue. The kinetic data were also used to obtain rough estimates of the apparent K_m (1.8×10^{-5} M) and $V_{max.}$ (2.4 nmol/min per mg) of the reaction forming the biose with respect to the UDP-glucose.

The addition of 1 mM-UDP or 1 mM-UMP to the incubation mixture completely inhibited the synthesis of the glucoside and under similar conditions 1 mM-laminaribiose, -cellobiose or -glucose decreased

the yield of cyclitol glucoside to less than half. In all these experiments the inhibition was accompanied by the accumulation of two unknown compounds containing glucose but there were no callose oligosaccharides or polymer, although 40% or more of the UDP-glucose remained unchanged.

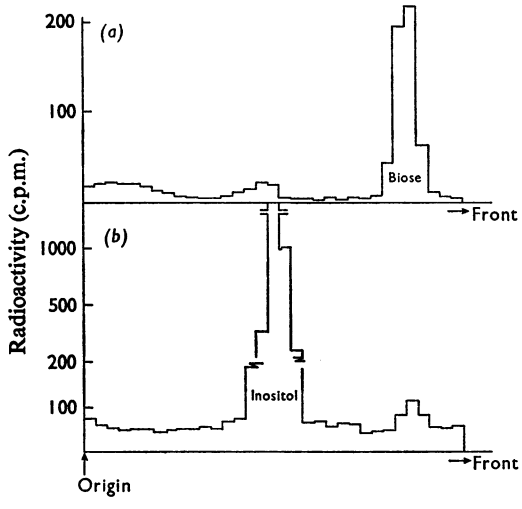

Fig. 3. *Incorporation of ^3H from* myo*inositol into glucosyl*myo*inositol*

A single chromatogram showing the radiation from UDP-[^{14}C]glucose (1 μM) (*a*) and from [^{3}H]*myo*inositol (0.1 μM) (*b*) after incubation for 10 min with enzyme (10 μl) in 90 mM-Tris–HCl, pH 7.5, and 2 mM-$MgCl_2$ in a total volume of 50 μl. The separation by paper chromatography is described in the text. The counting efficiencies were not estimated in this experiment but they differ markedly from all experiments where a single isotope was used.

Discussion

Elbein & Chambers (1970) and Clark & Villemez (1972) have noted the production of a disaccharide by a preparation from mung beans which catalysed the formation of β-(1→3)-linkages in glucans. In both cases this compound was presumed to be laminaribiose from its mobility on a paper chromatogram. Since glucosyl*myo*inositol and laminaribiose have very similar mobilities on paper it is possible that the product noted by these two groups of workers is identical with the disaccharide described here.

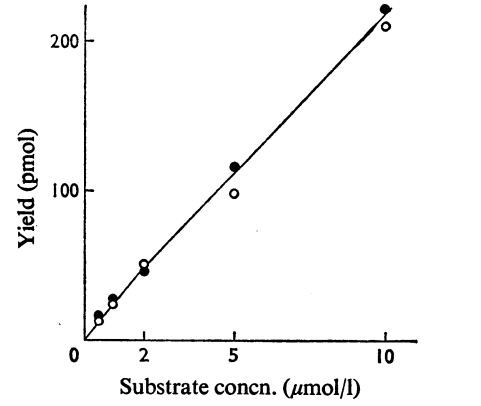

Fig. 4. *Effect of the concentration of UDP-glucose on the yield of glucosyl*myo*inositol with the enzyme extract as the sole source of the cyclitol*

Enzyme was extracted from 4½-day-old beans. Incubations were carried out by the method described in the text, with 20 μl of enzyme. Portions of the resulting mixtures were separated either by paper chromatography for 24 h by the method described in the text (○) or by chromatography in 0.3 M-sodium formate, pH 3, for 40 min on paper impregnated with polyethyleneimine (●).

Table 1. *Effect of concentration of UDP-[^{14}C]glucose and* [^{3}H]myo*inositol on the proportion of each incorporated into glucosylinositol*

The enzyme was extracted from 4-day-old beans. The experimental details are described in the legend to Fig. 3. This Table does not necessarily show the total yield of disaccharide, since, in some cases, there were indications of its conversion into higher oligosaccharides.

Substrate concn. (μM)		^{14}C or ^{3}H incorporated into glucoside (pmol)		^{3}H at origin of chromatogram (pmol)
UDP-[^{14}C]glucose	[^{3}H]*myo*Inositol	^{14}C	^{3}H	
1	0.1	40	0.02	0.005
10	10	344	2.5	0.85
10	100	366	6.5	7.5
100	100	1900	27.5	10.5
100	1000	2460	130.0	110.0

A major problem was that of obtaining sufficient product for analysis. This partly arose from the fact that one substrate was being supplied by the enzyme system and appeared to limit the synthesis at higher concentrations of UDP-glucose. Difficulty was also caused in some cases by the enzymic removal of the synthesized disaccharide for the formation of glucan. This reaction varied with the age of the seedling and increased with higher concentrations of UDP-glucose (Kemp & Loughman, 1973). However, considerable progress with the chemical identification has been achieved. All the evidence supports the conclusion that the disaccharide is *myo*inositol-β-glucoside and the trisaccharide is diglucosyl*myo*-inositol.

The occurrence of glucopyranosyl*myo*inositol in potatoes has been shown by Urbas (1968) and the synthesis of two forms of glucosyl*myo*inositol by *Sporobolomyces* has been described by Gorin *et al.* (1965). It is believed that this is the first description of diglucosyl*myo*inositol, although the occurrence of digalactosyl*myo*inositol (Petek *et al.*, 1966) and dimannosyl*myo*inositol (Tanner, 1967) has been noted.

It is clear that the synthesis of glucosylinositol in mung beans differs markedly from that in *Sporobolomyces* (Gorin *et al.*, 1965) since in the latter the glucose moiety is supplied from either cellobiose or lactose to free inositol. It also appears that the synthesis of glucosylinositol in the mung bean differs from that of galactosylinositol (galactinol) in the pea (Frydman & Neufeld, 1963) where UDP-galactose reacts with free inositol.

It is fairly clear that free *myo*inositol does not play an important role in glucosylinositol synthesis since it is not incorporated with equimolar amounts of glucose. If free inositol cannot act as the direct substrate then the source of the cyclitol, available in the enzyme extract, must be formed in small amounts from free *myo*inositol. The substance isolated at the origin of the paper chromatograms in the experiments using [^{3}H]inositol appears to be the only compound which conforms to these requirements and it is possible that the compound is an intermediate in the synthesis of the disaccharide rather than in competition with it.

Recent work by Tanner (1967) has shown the formation of mannosylinositol in yeast and in this system the inositol donor has been shown to be a lipid component. It cannot be ruled out that the system in mung beans parallels that in yeast, since the inositol donor in the former, originally isolated in a particulate enzyme fraction, has been brought into solution by treatment with a detergent, digitonin. It has been found that the enzyme extracted in this way does contain lipids (Flowers *et al.*, 1968).

Most of the experiments described in this paper were carried out under conditions where the disaccharide was the final product of the reaction. However, there is evidence that these cyclitol glucosides are involved in the synthesis of a glucan which is probably callose (Kemp & Loughman, 1973).

We are grateful to Professor D. J. Manners for supplying the β-(1→3)-glucanase, to Dr. R. Aplin for help with the mass spectrometry and to Mr. J. A. Farrar for help with g.l.c. We thank Mr. M. G. Beesley and Mr. K. Mutch for their able technical assistance. We gratefully acknowledge financial support from the Agricultural Research Council.

References

Block, R. J., Durrum, E. L. & Zweig, G. (1958) *A Manual of Paper Chromatography and Paper Electrophoresis* p. 170, Academic Press, New York

Clark, A. F. & Villemez, C. L. (1972) *Plant Physiol.* **50**, 371–374

Elbein, A. D. & Chambers, J. (1970) *Arch. Biochem. Biophys.* **138**, 620–631

Feingold, D. S., Neufeld, E. F. & Hassid, W. Z. (1958) *J. Biol. Chem.* **233**, 783–787

Flowers, H. M., Batra, K. K., Kemp, J. & Hassid, W. Z. (1968) *Plant Physiol.* **43**, 1703–1709

French, D. & Wild, G. M. (1952) *J. Amer. Chem. Soc.* **75**, 2612–2616

Frydman, R. B. & Neufeld, E. F. (1963) *Biochem. Biophys. Res. Commun.* **12**, 121–125

Gorin, P. A. J., Horitsch, K. & Spencer, J. F. T. (1965) *Can. J. Chem.* **43**, 2259–2264

Kemp, J. & Loughman, B. C. (1973) *Biochem. Soc. Trans.* **1**, 446–448

Kochetkov, N. K., Chizkov, O. S. & Molodtsov, N. V. (1968) *Tetrahedron* **24**, 5587–5593

Kochetkov, N. K., Chizhov, O. S. & Bochkov, A. F. (1973) in *MTP International Review of Science* (Aspinall, G. O. & Hey, D. H., eds.), Ser. 1, vol. 7, pp. 148–190, Butterworth University Park Press, London & Baltimore

Lee, Y. C. & Ballou, C. E. (1965) *J. Chromatogr.* **18**, 147–149

Manners, D. J. & Patterson, J. C. (1966) *Biochem. J.* **98**, 19c

Petek, F., Villarroya, E. & Courtois, J. E. (1966) *C. R. Acad. Sci. Ser. D* **263**, 195–197

Senser, M. & Kandler, O. (1967) *Phytochemistry* **6**, 1533–1540

Tanner, W. (1967) *Ber. Deut. Bot. Ges.* **80**, 592–607

Urbas, B. (1968) *Can. J. Chem.* **46**, 49–53