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A Protein–Glucan Intermediate during Paramylon Synthesis

By A. DERI TOMOS* and D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

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A sodium deoxycholate extract containing glucosyltransferase activity was obtained from a particulate preparation from *Euglena gracilis*. It transferred glucose from UDP-[^{14}C]glucose into material that was precipitated by trichloroacetic acid. This material released β-(1→3)-glucan oligosaccharides into solution on incubation with weak acid, weak alkali and β-(1→3)-glucosidase. The products of the incubation of the deoxycholate extract with UDP-[^{14}C]glucose were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Radioactive bands were obtained that had the properties of β-(1→3)-glucan covalently linked to protein by a bond labile to weak acid. High-molecular-weight material containing a β-(1→3)-glucan was also shown to be present by gel filtration. The bond linking glucan to aglycone is possibly a pyrophosphate linkage. It is proposed that in *Euglena gracilis* β-(1→3)-glucan (paramylon) is synthesized on a protein primer.

The role of a protein as a primer for the initiation of polysaccharide synthesis has been described for several tissues. When enzyme preparations were incubated *in vitro* with radioactive UDP-glucose or glucose 1-phosphate, glucose was incorporated into material that was insoluble in trichloroacetic acid, but that could be released as soluble polysaccharide by the action of proteinases. This was demonstrated for glucose incorporation from UDP-glucose into potato starch (Lavintman & Cardini, 1973; Lavintman *et al.*, 1974) and into glycogen from rat liver (Krisman, 1972, 1973; Krisman & Barengo, 1975; Berthiller *et al.*, 1975) and *Escherichia coli* (Barengo *et al.*, 1975), and for potato starch incorporation from glucose 1-phosphate was also possible (Tandecarz *et al.*, 1975). Analysis of the product after incorporation of radioactive glucose from UDP-glucose by an extract of *Aerobacter aerogens* suggested that *Aerobacter* glycogen was also synthesized on a protein primer (Gahan & Conrad, 1968). It has been shown that a slime polysaccharide secreted by the outer root-cap cells of maize was also assembled on a protein. A glycoprotein intermediate of the polysaccharide was found in the endomembrane system of the cell, where it was formed before secretion (Green & Northcote, 1978).

Paramylon synthesis in *Euglena gracilis* is catalysed by enzymes that use UDP-glucose as substrate. The properties of a membrane-bound UDP-glucose–β-(1→3)-glucan glucosyltransferase have been described (Marechal & Goldemberg, 1964). The enzyme was shown to catalyse the formation of UDP from UDP-glucose even in the presence of a β-(1→3)-glucosidase. This suggested that a primer other than a β-(1→3)-glucan participated in the synthesis of paramylon.

In the present paper we report the incorporation of glucose from UDP-glucose into a trichloroacetic acid-insoluble fraction by a solubilized *Euglena* membrane enzyme that is active in β-(1→3)-glucan (paramylon) synthesis.

Materials and Methods

Preparation of transglucosylase activity

Euglena gracilis (Klebs 1224/52. Pringsheim 1950), supplied by the Culture Centre of Algae and Protozoa, Storey's Way, Cambridge, U.K., was grown in the medium described by Marechal & Goldemberg (1964). This consisted of 5g of Difco Bacto-Peptone (Difco Labs., East Molesey, U.K.), 2g of Difco yeast extract, 15g of glucose and 10μg of cyanocobalamin (vitamin B_{12}) dissolved in 1 litre. The solution was sterilized by autoclaving at 120°C at 103kPa for 30min and growth of the culture was in the dark at 28°C.

Glucosyltransferase activity was obtained by a modification of the method of Marechal & Goldemberg (1964). After 6–10 days the cells (from 2 litres of medium) were harvested by centrifugation at 4200*g* for 10min. The pellets of cells were washed twice in fresh ice-cold water and once in ice-cold 2.2% (w/v) $(NH_4)_2HPO_4$ (pH7.9) by centrifugation at 5000*g* for 5min for each wash. The washed cells (yield 10g wet wt. of cells/litre) were re-suspended in 25ml of 2.2% $(NH_4)_2HPO_4$ solution.

The cells (4°C) were homogenized for 1–2min with a Braun cell homogenizer (model MSK) in the

* Present address: Department of Biochemistry and Soil Science, University College of North Wales, Memorial Buildings, Deiniol Road, Bangor LL57 2UW, Wales, U.K.

presence of 50g of 0.45–0.50mm-diam. beads (Glasperlen; B. Braun, Melsunger AG, from F.T. Scientific Instruments, Bredon, U.K.) while being cooled in a stream of liquid CO_2. The homogenate was passed through a very coarse sintered-glass filter funnel and the retarded material was washed with 100ml of ice-cold 2.2% $(NH_4)_2HPO_4$.

The combined filtrate and washing were kept at 4°C and were centrifuged at 2000*g* for 10min to remove dense cell debris and unbroken cells. The pellets were washed twice in a minimum volume of $(NH_4)_2HPO_4$, the supernatant and washings were filtered through glass-wool to remove most of the waxy material that formed as a skin on the surface of each tube and the material was centrifuged at 25000*g* for 30min.

The supernatant was cleared of remaining waxy material by filtration through glass-wool and centrifuged at 100000*g* for 1h at 4°C. The pellets were transferred to a 15ml centrifuge tube that had been ground to accommodate a Dounce-type plunger, and suspended by homogenization in a 5ml detergent suspension containing 1.5M-KCl, 1mM-$MgCl_2$ and 0.8% sodium deoxycholate. This mixture was homogenized continuously at 0°C for 30min before centrifugation at 25000*g* for 30min at 4°C. A clear supernatant was produced, which became turbid on decantation owing to a floating layer of lipid. This supernatant was dialysed for 40h (with two changes of buffer) against 10mM-sodium phosphate (pH7.4)/1mM-EDTA at 4°C, during which time a precipitate formed.

The resulting suspension was either used directly or stored at −20°C. More than 50% activity was retained after 20 days. Protein determination (Lowry *et al.*, 1951) gave values of about 3mg/ml. Bovine serum albumin was used as standard.

Incubation of membrane extract with UDP-[^{14}C]-glucose

The enzyme preparation was incubated with UDP-[^{14}C]glucose under the conditions described by Marechal & Goldemberg (1964). The material was incubated for various periods at 23°C in a buffer consisting of 4mM-$MgCl_2$, 150mM-glycylglycine, 5mM-sodium phosphate and 0.5mM-EDTA (pH7.4) in the presence of 2μCi of UDP-[^{14}C]glucose (6.4μM) (The Radiochemical Centre, Amersham, Bucks., U.K.)/ml. Protein concentration in each incubation was about 1.5mg/ml. The preparation gave an insoluble radioactive product that was soluble in hot 2% (w/v) KOH and that was precipitated by 80% (v/v) ethanol from this solution. Analysis of the precipitate by periodate oxidation indicated that it was paramylon (β-1→3-glucan), with a minimum chain length of 30 glucose residues (Hay *et al.*, 1965). This was identical with the results obtained by Marechal & Goldemberg (1964).

The incubations were terminated in a number of ways, each of which is specified, and which depended on the subsequent treatment of the preparation. If the material was not to be analysed immediately the incubation was rapidly frozen in a mixture of acetone and solid CO_2. On thawing (60s) these samples were treated as freshly incubated material.

Preparation of radioactive material insoluble in trichloroacetic acid solutions after incubation of membrane extract with UDP-[^{14}C]glucose

A trichloroacetic acid-insoluble fraction of the incubation was prepared by a modification of the method of Lavintman *et al.* (1974). Samples of the incubation mixture were mixed with excess ice-cold 6% (w/v) trichloroacetic acid in butan-1-ol. Sufficient butanol to dissolve the water from the sample was always used, for example, 0.1ml of the incubation mixture in 10ml of butanolic trichloroacetic acid. This was thoroughly mixed before filtration through a Millipore filter (2.5cm diam.) that had been washed in both butanolic and aqueous (6%, w/v) trichloroacetic acid. Each tube was thoroughly washed twice with butanolic trichloroacetic acid and the washings were filtered through the Millipore filter with the original sample. Retarded material was then washed with 2×15ml of butanolic trichloroacetic acid followed by 2×15ml of aqueous trichloroacetic acid. Finally a further 5ml of butanolic trichloroacetic acid was used to speed up the subsequent drying of the filter. Butanolic solvent was used in addition to the aqueous solvent to remove the large amount of radioactive lipids (mainly steryl glucoside and glucosyl diglycerides) formed during the incubation.

Each filter was dried in a desiccator and assayed for radioactivity by the method of Harris & Northcote (1970). These samples were very stable and showed no release of radioactivity into water-soluble material if stored at −20°C once they were dried, or even if stored at room temperature under toluene scintillant. The Millipore filter with its adsorbed material was easy to analyse and handle. For example, the dried sample could be assayed for radioactivity with scintillation fluid. Subsequently the scintillation fluid could be readily removed with toluene, which affected neither the radioactive material nor the Millipore filter itself.

Gel electrophoresis of components soluble in sodium dodecyl sulphate after incubation of membrane extract with UDP-[^{14}C]glucose

Samples (100μl) from the incubation were mixed with 100μl of 20% (w/v) sodium dodecyl sulphate and were treated by the method of Fairbanks *et al.* (1971),

except that a final sample concentration of 4% sodium dodecyl sulphate was used to increase the amount of material that could be loaded on each gel and run in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis: 5mm-diam. gels were sufficient to detect radioactivity, but 10mm-diam. gels were required to show the pattern of protein by visible staining.

Gels were stained for protein with Coomassie Brilliant Blue by the method described by Fairbanks *et al.* (1971). However, for 10mm-diam. gels at least twice the time stated was allowed, to facilitate diffusion of fixative, stain and destainer into the gel.

To assay 5mm-diam. gels for radioactivity they were frozen on a sheet of aluminium, cooled on solid CO_2 and then sliced into 1mm segments. An array of 120 razor blades bolted together, separated by washers, was used for this procedure. The slices, taken in pairs, were transferred to 50mm×13mm tubes and dried at 60°C for 16h. To each pair of slices was added about 200μl of H_2O_2 (100-volume). The tubes were stoppered and incubated at 60°C for 24h, which usually proved sufficient for the destruction of the cross-linked gel and released all the components and contents of the gel into aqueous solution. The radioactivity of each pair of solubilized slices was then measured by the method of Patterson & Greene (1965). The radioactivity counting fluor tT 21 (Koch-Light, Colnbrook, Bucks., U.K.) was used with 2.67g of PPO (2,5-diphenyloxazole)/litre and 33.3mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre.

Preparation by gel filtration of high-molecular-weight material synthesized from UDP-[^{14}C]glucose by the membrane extract

Gel filtration was performed on columns of various sizes (1–2cm diam.×15–20cm long) containing Bio-Gel P-4, P-200 and P-300 (Bio-Rad Laboratories, Watford, U.K.). After incubation the material was dissolved in a minimum volume of 20% sodium dodecyl sulphate and left for 1–16h. This was diluted with sodium phosphate buffer to a final concentration of 2% sodium dodecyl sulphate/20mM-phosphate (pH7.4). This mixture was run on a gel column previously equilibrated with 2% sodium dodecyl sulphate/20mM-phosphate (pH7.4). The eluate was assayed for radioactivity by the modified method of Patterson & Greene (1965). The A_{280} was measured with an LKB Uvicord detector.

Analysis of radioactively labelled fractions of the membrane extract

Sugars were chromatographed on Whatman no. 1 paper with ethyl acetate/pyridine/water (8:2:1, by vol.) as solvent (Harris & Northcote, 1970). The chromatograms were cut into strips (40mm×10mm) and their radioactivity was measured (Harris & Northcote, 1970). Sugars were detected by the aniline phthalate method of Wilson (1959) or by the alkaline-$AgNO_3$ method (Trevelyan *et al.*, 1950).

Sugars were electrophoresed on Whatman no. 1 paper in sodium tetraborate buffer (19g/litre; pH9.4) at 4kV for 45min (80V/cm). The radioactivity was detected by the same method as that used for the chromatograms. Electrophoresis at pH2 was carried out on Whatman no. 1 paper in 8% (v/v) acetic acid/2% (v/v) formic acid at 5kV for 30min (100V/cm). Sugar phosphates were detected with the molybdenum stain of Bandurski & Axelrod (1951).

Material collected on Millipore filters was hydrolysed in 0.07M-NaOH (pH12.5) at room temperature (approx. 20°C) for 5min (mild alkaline hydrolysis). The reaction was terminated by transferring the Millipore filter from the alkali into ice-cold water, followed by 6% trichloroacetic acid in butanol. Soluble material was hydrolysed in 0.1M-HCl at 100°C for 10min (mild acid hydrolysis). The reaction was terminated by cooling the mixture in ice, followed by addition of Amberlite IR-4B (HCO_3^- form) anion-exchange resin. Mild acid hydrolysis of material on Millipore filters was terminated by rapidly transferring the Millipore to cold water and thence to butanolic trichloroacetic acid. The soluble products were neutralized by passage through columns (10cm×1cm) of Amberlite IR-4B (HCO_3^- form) anion-exchange resin.

Periodate oxidation followed by borohydride reduction and hydrolysis was carried out by a modification of the method of Elbein (1969). Samples containing not more than 1mg of polysaccharide were incubated for 96h at 4°C in the dark with 1ml of 0.05M-sodium metaperiodate. Two portions (0.5ml) of 0.1M-$NaBH_4$ [made alkaline by the addition of one pellet (approx. 0.1g) of NaOH to 40ml of solution] were added at 30min intervals. After a further 30min the mixture was made up to 4% (w/w) H_2SO_4 and hydrolysed at 103kPa at 120°C for 1h. The samples were deionized by passage through anion (HCO_3^- form) and cation (H^+ form)-exchange resins. The hydrolysate chromatographed on paper in ethyl acetate/pyridine/water (8:2:1, by vol.) for 16–18h; this gave a good separation of glucose, erythritol and glycerol.

Incubations with enzymes

The samples were treated with the following enzyme preparations. In each case the incubation was carried out under toluene to prevent bacterial hydrolytic activity. (*a*) Salivary amylase (Olaitan & Northcote, 1962); 1ml at 25°C in 0.2M-sodium phosphate (pH7.0) for 15h. (*b*) Pronase (BDH Chemicals, Poole, Dorset, U.K.) (1–2mg/ml) at 25°C in 0.2M-sodium phosphate (pH7.2) or 0.2M-Tris/acetate

(pH7.2) for 15h. (*c*) Subtilisin at 1mg/ml at 25°C in 0.1M-sodium phosphate (pH7.4) for 18h. (*d*) Thermolysin (Calbiochem, San Diego, CA, U.S.A.) at 1mg/ml at 25°C in 0.1M-sodium phosphate (pH7.4) for 18h. (*e*) Phosphodiesterase (*Crotalus terrificus*; Boehringer, London W.5, U.K.) (1–10mg/ml) at 25°C in 0.2M-Tris/acetate (pH8.9) for various periods of time up to 36h. (*f*) Ll *Cytophaga* lytic enzyme (BDH) at 0.5mg/ml at 37°C in 0.2M-sodium phosphate/acetic acid (pH5.0) for 1–18h; this mixture of enzymes degrades β-(1→3)-glucans, but is inactive against β-(1→4)-glucans under these conditions (Marshall, 1973).

Results

Analysis of the radioactive fraction insoluble in aqueous and butanolic trichloroacetic acid

Preliminary experiments had shown that radioactive material could be precipitated from an incubation of solubilized paramylon synthase with UDP-[^{14}C]glucose by the addition of trichloroacetic acid. The method used in this work, where the material was isolated on a Millipore filter, was rapid and allowed the radioactive material to be quantitatively collected and analysed. Incorporation of radioactivity into the precipitate formed on addition of trichloroacetic acid is illustrated in Fig. 1.

Requirement for trichloroacetic acid during preparation. Although deoxycholate solubilizes the transglycosylase activity a precipitate appeared in the preparation on dialysis. To ensure that the material rendered insoluble by trichloroacetic acid was not entirely contained in this precipitate, an experiment was performed by the filtration technique, in the absence of trichloroacetic acid, with water and butanol alone. The results showed that absence of trichloroacetic acid decreased the yield of radioactive material by almost 40% (12500c.p.m. compared with 20000c.p.m.). It is considered that the material that was precipitated during dialysis contained similar components to that precipitated by trichloroacetic acid.

Periodate oxidation. A sample of radioactive material collected on a filter was treated with propylene oxide to dissolve the nitrocellulose of the filter. The labelled material was left in suspension. This was washed with fresh propylene oxide and finally dried. The residue was oxidized by periodate, reduced and hydrolysed, and the deionized hydrolysate was chromatographed. All the detectable radioactivity ran as glucose, indicating the presence of a β-(1→3)-glucan of moderate length. The absence of measurable radioactive glycerol indicated a chain length of at least 25 residues.

Mild acid hydrolysis. The bond linking radioactive glucan to the insoluble fraction was shown to be labile in mild acid (0.1M-HCl at 100°C for 10min). Labelled material (70%) was released on mild acid hydrolysis, compared with 39% released by the control treatment of incubation in sodium phosphate buffer at pH7.0 at 100°C for 10min (Fig. 1). In a separate experiment incubation of the material with 0.2M-sodium phosphate/acetic acid (pH5.0) at 37°C for 18h released 10% of the radioactivity into aqueous solution.

Analysis of the solubilized material by electrophoresis in sodium borate and by paper chromato-

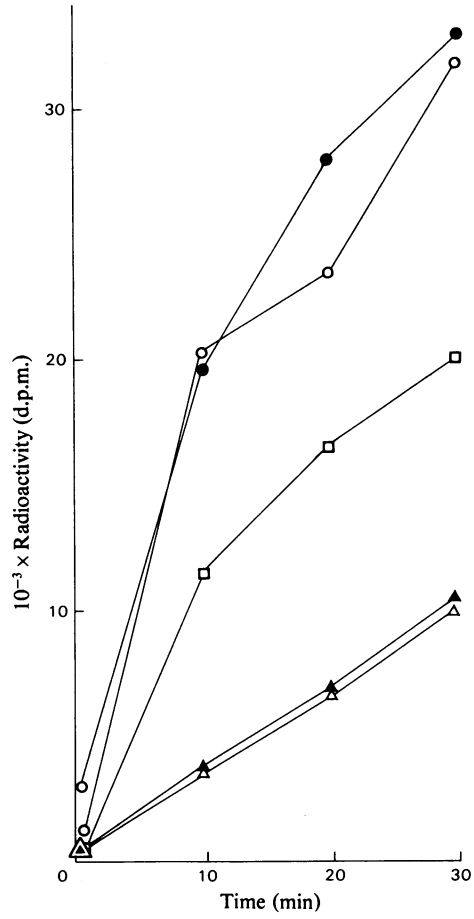

Fig. 1. *Incorporation and release of radioactivity from trichloroacetic acid precipitate*

●, Incorporation of radioactivity from UDP-[^{14}C]-glucose into precipitable fraction. Radioactivity not released from precipitate by the following treatments: ○, 0.2M-sodium phosphate (pH7.0), room temperature for 5min; □, 0.2M-sodium phosphate (pH7.0), 100°C for 10min; ▲, 0.07M-NaOH (pH12.5), room temperature for 5min; △, 0.1M-HCl, 100°C for 10min. For full details see the Materials and Methods section.

graphy showed the presence of oligosaccharides of the laminarin series. Oxidation by periodate and subsequent reduction and hydrolysis of the radioactive products showed that the average chain length of the oligosaccharides released by mild hydrolysis of the material on Millipore filters exceeded 25 glucose units if only one terminal was labelled and 50 if both terminals were labelled. These are minimum values because the amount of radioactive glycerol recovered was too small to measure accurately. Treatment of the solubilized material with Ll *Cytophaga* glucosidase quantitatively converted it into laminaritriose, laminaribiose and glucose. Standard oligosaccharides were produced by partial acetolysis of laminarin (Koch–Light) as described by Clark & Villemez (1972).

Mild alkali hydrolysis. The bond linking radioactive glucan to the insoluble fraction was shown to be very labile to mild alkali. A similar fraction to that released by mild acid was released by the alkali (Fig. 1). Almost no material was released from the control incubation with sodium phosphate buffer at pH7.0 at room temperature (21°C) for 5min. In a separate experiment incubation of the material in 0.2M-Tris/acetate buffer, pH8.9, for 36h at 25°C released less than 5% of the radioactive component into solution.

Analysis of the solubilized material by electrophoresis in sodium borate showed the presence of the laminarin series of oligosaccharides. These oligosaccharides had chain lengths of at least 25 glucose units when they were analysed by periodate oxidation.

An attempt to release the radioactive oligosaccharide by aminolysis by incubation with NH_3 solutions at pH8.9–9.8 released only 5% of the radioactivity into aqueous solution.

Treatment with enzymes. Treatment of the material collected on Millipore filters with Ll *Cytophaga* glucosidase released only 30% of the radioactivity into solution. The solubilized material was shown by electrophoresis in sodium borate to consist of equal amounts of laminaritriose, laminaribiose and glucose.

Digestion of the material with amylase did not release any detectable radioactivity into solution. Incubation with phosphodiesterase, Pronase, subtilisin and thermolysin were equally unsuccessful in releasing radioactive material into solution.

Conclusions. These analyses showed that a fraction of a homogenate of *E. gracilis* known to synthesize paramylon incorporated [^{14}C]glucose from UDP-[^{14}C]glucose into a fraction that was precipitated by trichloroacetic acid. Since this acid does not precipitate oligo- and poly-saccharides the precipitate must consist of sugar attached to an aglycone. The sugar moiety consisted of β-(1→3)-glucan. The bond linking glucan to aglycone was labile to both mild alkali and mild acid, but was resistant to aminolysis. Interpretation of the enzyme hydrolysis was complicated by the observation that, although all the released radioactive glucan was degraded by glucanase, only 30% was degraded *in situ*. The inhibition was presumably due to the insoluble state of the substrate.

Analysis of the radioactive fraction soluble in sodium dodecyl sulphate by polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of material extracted by sodium dodecyl sulphate from the incubation mixture gave six radioactive components (Fig. 2*a*). Two of these corresponded to UDP-[^{14}C]glucose (E) and [^{14}C]glucose 1-phosphate (F) (Fig. 2*b*). No direct comparison of radioactive discs with those stained with Coomassie Blue was possible because of the difficulty of running sufficient material on a 5mm-diam. gel and also the difficulty of measurement of radioactivity on a 10mm-diam. gel. Fig. 2(*c*) shows the position of Coomassie Blue-staining bands positioned according to their mobility relative to the standard proteins (Fig. 2*d*) that were run on both 5mm- and 10mm-diam. gels. Coomassie Blue-staining discs 1, 2 and 3 correspond to the radioactive discs B, C and D.

The nature of radioactive discs A, B, C, E and F was further investigated (disc D contained too little radioactivity to be analysed further). Gel slices corresponding to these components were collected in pairs and desiccated at room temperature in a vacuum desiccator over NaOH. The pairs of dried gel discs were rehydrated with 0.5ml of 0.1M-HCl and incubated at 100°C for 10min before being cooled in ice. Water (2ml) was added to each pair, and they were left at 4°C for 15h. This allowed diffusion of soluble material from the gel. The solution was decanted from the discs and stored at −20°C while the gel slices were allowed to stand for a further 24h at 4°C in fresh water. These washings were added to the stored solutions.

Each fraction was deionized with anion (HCO_3^- form)- and cation-exchange resin before being analysed by paper chromatography and electrophoresis in sodium borate. All radioactive material released from fractions E and F was shown to be glucose. Glucose, laminaribiose and laminaritriose were identified by both electrophoresis and paper chromatography in the hydrolysates of components A, B and C. Electrophoresis showed the presence of radioactive material with a mobility corresponding to laminaridextrans of greater degree of polymerization than the trisaccharide. No qualitative difference could be detected between the hydrolysates of fractions B and C. The electrophoretic pattern of fraction A was more complex, but was not further analysed.

Conclusions. It is most improbable that fractions B and C contained free oligosaccharide phosphates, since (*a*) monomeric glucose was among the products of mild acid hydrolysis of the fractions, but fractions

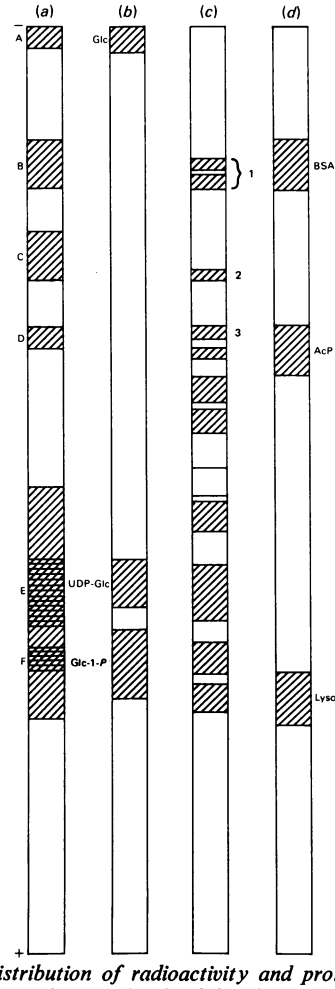

Fig. 2. *Distribution of radioactivity and protein-staining material on sodium dodecyl sulphate/polyacrylamide gels of [^{14}C]glucose-labelled material from incubation of membrane extract with UDP-[^{14}C]glucose*

(*a*) Radioactive material from incubation. (*b*) Radioactive standards: [^{14}C]glucose (Glc); UDP-[^{14}C]-glucose (UDP-Glc); [^{14}C]glucose 1-phosphate (Glc-1-*P*). (*c*) Protein-staining pattern of material from incubation. (*d*) Protein standards: bovine serum albumin (BSA); acid phosphatase (AcP); lysozyme (Lyso). Details of the procedures used are given in the Materials and Methods section.

B and C did not have the mobility of glucose 1-phosphate (F), (*b*) it is unlikely that the mobilities of different oligosaccharide phosphates of the same series would be so similar to each other and yet so different from that of glucose 1-phosphate. Thus the oligosaccharide must be linked to aglycone moieties that are not just phosphate groups. However, the aglycone may include phosphate attached to another compound.

The components therefore had a charged anionic group in sodium dodecyl sulphate solution, and this may be carried on the aglycone or the bond linking the sugar to the aglycone. C. Brett (Department of Biochemistry, University of Cambridge; unpublished work) has shown that dolichyl phosphate glucose formed by rat liver enzyme travels with the low-molecular-weight marker on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with a 5%-cross-linked stacking gel on a 10%-cross-linked gel. This suggests that the aglycone of fractions B and C was not a polyprenyl phosphate. The radioactive material could not be stained directly with Coomassie Blue, but fractions containing protein had a similar mobility to the radioactive fractions B, C and D. It seemed probable, therefore, that the aglycone moiety was a protein. The mobility of the fractions appeared not to be altered by different lengths of glucose oligosaccharide present in them, which suggests that the aglycone was relatively much larger than the linked oligosaccharide. It is possible that three different proteins were involved as carriers to give the bands B, C and D. However, it is more reasonable to suggest that there is only one carrier protein which holds one, two or more chains of the same type of oligosaccharide and that these compounds had different mobilities.

Analysis of high-molecular-weight material bound to β-(1→3)-glucose oligosaccharide

Analysis of the eluates of gel filtration showed that all the radioactivity was associated with either low-molecular-weight material (UDP-glucose and glucose 1-phosphate) or with fully excluded material that absorbed u.v. (280nm). The distribution was quantitatively the same for each grade of gel used (Table 1). Bio-Gel P4 columns were used for the isolation of the high-molecular-weight component. Bio-Gel P4 has an exclusion limit of mol.wt. 4000 and would be expected not to exclude free oligosaccharides under 20 residues in length (mol.wt. 3600).

A sample of the high-molecular-weight material was made up to 0.1M-HCl and incubated at 100°C for 10min, cooled in ice and deionized with anion (HCO_3^- form)- and cation-exchange resin. The hydrolysate was analysed by electrophoresis in sodium borate and paper chromatography. The presence of glucose, laminaribiose and laminaritriose was demonstrated by both techniques. Radioactive material having the mobility of laminaridextrans of higher degree of polymerization than laminaritriose was detected on the electrophoretograms.

Conclusions. Since free oligosaccharides of the size found in the high-molecular-weight fractions would not be excluded by any of the filtration gels used, they must be attached to high-molecular-weight aglycones. Exclusion from Bio-Gel P300 indicated that the

Table 1. *Gel filtration of incubation mixture*
Samples (100μl), from an incubation of membrane extract with UDP-[^{14}C]glucose for 100min, were mixed with 100μl of 20% sodium dodecyl sulphate and left for 1h at room temperature before being diluted with sodium phosphate buffer (20mM; pH7.4) to 2% sodium dodecyl sulphate; final concn. of sodium phosphate (pH7.4) was 20mM. The clear solutions were passed through gel-filtration columns (approx. 12cm×2.5cm) of various exclusion limits. The radioactivity in the fully excluded fraction was measured for each column.

Grade of gel used in column	Exclusion limit (mol.wt.)	Fully excluded radioactivity (c.p.m.)
Bio-Gel P-4	4000	15200
Bio-Gel P-200	200000	16250
Bio-Gel P-300	300000	16000

glucan–aglycone compound had a mol.wt. exceeding 300000. Sodium dodecyl sulphate–glycoprotein complexes of this size would contain glycoprotein components with mol.wts. of at least 180000, allowing for a 40% increase caused by the bound sodium dodecyl sulphate (Putnam & Neurath, 1945).

The incorporation of radioactivity from UDP-[^{14}C]glucose into the high-molecular-weight material completely excluded by the Bio-Gels was quantitatively the same as that incorporated into the material isolated by precipitation with trichloroacetic acid and collected on Millipore filters. This indicated that the two fractions represented the same material.

Evidence for the nature of the aglycone-glucan bond

The product of mild alkali hydrolysis of material collected on Millipore filters after an incubation of the membrane fraction for 1min with the radioactive UDP-glucose was analysed by electrophoresis at pH2.0. Fig. 3 illustrates the distribution of radioactivity and showed that most radioactivity had a mobility greater than that of glucose but less than that of glucose 1-phosphate. An anion group charged at pH2.0 must be attached to the material, and such a group is likely to be phosphate. Alkaline hydrolysis of pyrophosphate bonds attached to glucose produces a cyclic diester. This is shown on alkaline hydrolysis of UDP-glucose, which gives UMP and glucose 1:2-cyclic phosphate (Leloir & Paladini, 1957).

In a separate experiment direct analysis by electrophoresis at pH2.0 of material synthesized by the enzyme extract separated a mobile fraction that released laminaribiose and trehalose after prolonged storage at 4°C. Trehalose phosphate is known to occur in *Euglena* extracts (Belocapitow & Marechal, 1972), and our experiment indicated the presence of laminaribiose phosphate also.

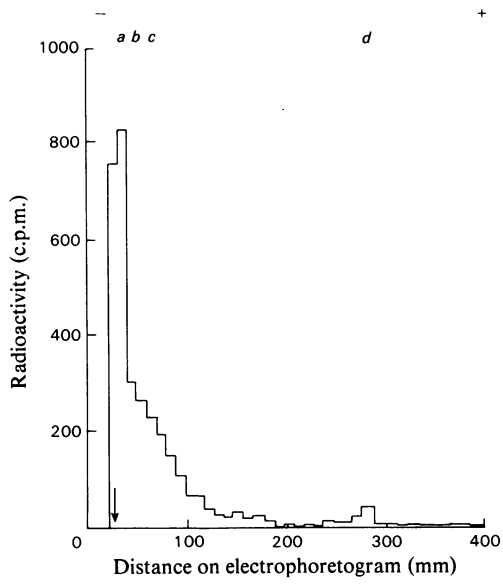

Fig. 3. *Electrophoresis of material released from trichloroacetic acid precipitate of incubation of membrane extract*
An acid precipitate was prepared from an incubation of 1min with radioactive UDP-glucose. This was treated with 0.07M-NaOH (pH12.5) for 5min at room temperature. The solubilized radioactivity was run on electrophoresis at pH2.0 at 5kV for 30min. *a*, *b*, *c* and *d* indicate the positions on the electrophoretogram of the markers glucose, glucuronic acid, galacturonic acid and glucose 1-phosphate respectively. The arrow indicates the starting line of the electrophoretogram.

Discussion

The transglycosylase preparation described by Marechal & Goldemberg (1964) and used in the work described here provides a useful system for the analysis of the initial stages of polysaccharide synthesis. In this work radioactive fractions were obtained after incubation of a system known to be synthesizing paramylon from UDP-[^{14}C]glucose. These fractions released identifiable β-(1→3)-glucan polymers after mild acid hydrolysis and, in some cases, after mild alkali hydrolysis. Mild alkali treatment was not applied to all the fractions isolated, owing to the difficulties of removing interfering anions from the resulting hydrolysate before analysis. This was especially true for hydrolysates containing sodium dodecyl sulphate. Removal of anions with exchange resin was not possible, since this would have also removed any oligosaccharide phosphate that was present. Nevertheless it appeared from the analysis of material collected on Millipore filters by trichloroacetic acid precipitation that the bond

linking sugar to aglycone was labile to both mild acid and mild alkali.

The occurrence of short β-(1→3)-glucan oligosaccharides in high-molecular-weight material indicated that they were covalently attached to a large aglycone.

Nature of the aglycone

Short laminaridextrans were shown to be bound to a high-molecular-weight aglycone that was precipitated by trichloroacetic acid and had the gel-electrophoretic properties of a protein. Coomassie Blue-staining material was observed that corresponded to the radioactive fractions containing oligosaccharide separated by gel electrophoresis. The material precipitated by trichloroacetic acid did not release oligosaccharide after treatment with proteinase. However, only 30% of the radioactive glucan was hydrolysed by incubation of the intact material with glucosidase, whereas it was all susceptible to hydrolysis after release of the oligosaccharides into aqueous solution by acid hydrolysis. It was possible therefore that proteinase failed to release oligosaccharides because of the insoluble nature of the material.

We suggest that the radioactive fractions contained oligosaccharide bound to protein, and since the oligosaccharide moiety of a proportion of the material was shown to consist of short β-(1→3)-glucan polymers we suggest that the glucoprotein represented the initiation stages of the synthesis of paramylon. The occurrence of such a primer explains the ability of Marechal & Goldemberg (1964) to demonstrate transglycosylase activity in the absence of a β-(1→3)-glucan acceptor.

Nature of aglycone–glucan linkage

The extreme alkali-lability of the bond linking aglycone to glucan limits the number of linkages that are possible. The conditions are milder than those required to achieve β-elimination of glycosidic linkages to serine or threonine (Gottschalk, 1972). Pyrophosphate is, however, extremely alkali-labile and these groups would also show the observed sensitivity to acid. The resistance of the bond to aminolysis showed that a pseudoester bond between the glycosidic carbon atom of the sugar and an acidic group was not involved. In contrast with the compound formed during paramylon synthesis, Krisman (1972) has shown that the bond between the oligosaccharide and protein formed during glycogen synthesis is resistant to alkali hydrolysis.

Alkali hydrolysis of the trichloroacetic acid precipitate gave material corresponding to radioactive glucose 1-phosphate when this was analysed by electrophoresis. Polymeric material obtained after alkali hydrolysis was also shown to move with a negative charge on electrophoresis at pH2, and since carboxylic acids would be protonated at this pH value the charge on the oligosaccharides was probably due to a mineral-acid ester such as phosphate. Laminaribiose carrying a strongly ionized anionic group was also detected in an extract from an enzyme incubation, and this also suggested that the growing polysaccharide chain carried a phosphate bond.

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