The Formation of Glycoproteins in Tissues of Higher Plants

SPECIFIC LABELLING WITH D-[1-¹⁴C]GLUCOSAMINE

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1. Radioactivity from D-[1-¹⁴C]glucosamine is incorporated into ethanol-insoluble compounds of high molecular weight in a number of plant tissues, including roots of corn (*Zea mays*), callus cells of sycamore (*Acer pseudoplatanus*), axenic cultures of duckweed (*Lemna minor*) and germinating seedlings of corn, broad bean (*Vicia faba*) and barley (*Hordeum vulgare*). 2. Except in the case of *Lemna*, where some of the radioactivity was recovered in glucose, hydrolysis of these ethanol-insoluble materials with acid released [¹⁴C]glucosamine as the major radioactive product. 3. The labelled compounds isolated from *Zea* roots and the *Acer* cells are believed to be glycoproteins rather than polysaccharides on the basis of their solubility properties, their charge characteristics and their susceptibility to hydrolysis by 0.5M-potassium hydroxide and by the proteases trypsin and Pronase. Further, radioactive peptides were isolated and purified after Pronase treatment and shown to contain glucosamine as well as a number of amino acids. 4. The experiments therefore indicate that D-[¹⁴C]glucosamine can be used as a specific precursor of the amino sugar units of plant as well as animal glycoproteins.

The biosynthesis and structure of glycoproteins from plants have not been widely studied even though some of these compounds are relatively abundant (Montgomery, 1970). Several have received special attention. Thus certain of the phytohaemagglutinins (Jaffé & Hännig, 1965; Kalb, 1968; Lis, Sharon & Katchalski, 1966; Takahashi, Ramachandramurthy & Liener, 1967), some allergens of pollen (Underdown & Goodfriend, 1969; Johnson & Marsh, 1965) and various other antigenic compounds from seeds (Pusztai & Watt, 1970) have been shown to be glycoproteins. These compounds usually contain amino sugars as part of their carbohydrate moiety. Their function is unknown, although some well-characterized plant glycoproteins are enzymes (Scocca & Lee, 1969; Shannon, Kay & Lew, 1966; Stark & Dawson, 1962). Some glycoproteins may also be involved in the maintenance of the primary cell wall of plants where they may form cross-links with the polysaccharide components (Lampert, 1969).

Much of the experimental evidence that has been gained in animals about the structure, biosynthesis and secretion of glycoproteins has involved the use of labelled carbohydrate precursor compounds, in particular D-glucosamine (Marshall & Neuberger, 1968). Clearly, if glucosamine can be used as a specific precursor of the amino sugar components

of plant glycoproteins, a very powerful tool is available for studying these important macromolecules.

Initial experiments have indicated that D-glucosamine is readily converted into UDP-N-acetyl-D-glucosamine by seedling tissues (Roberts, 1970). Further, a proportion (usually 5–10%) of the ¹⁴C was recovered in material that was insoluble in 80% ethanol. This material was of high molecular weight and yielded [¹⁴C]glucosamine on complete hydrolysis with hydrochloric acid; milder treatment released some N-acetyl D-glucosamine plus labelled fragments of high molecular weight. The aim of the present work was to examine whether the macromolecules that became labelled on provision of D-[¹⁴C]glucosamine were glycoproteins and to establish the potential usefulness of this compound in studies with plants.

EXPERIMENTAL

Materials. Corn (*Zea mays* var. Golden Bantam) and beans (*Vicia faba* var. Long Pod) were purchased from Burpee's Seeds, Sanford, Fla., U.S.A. Barley (*Hordeum vulgare* var. Larker) was a gift from Anheuser-Busch Inc., St Louis, Mo., U.S.A. Seeds were surface sterilized with 1.25% (w/v) sodium hypochlorite solution for 15 min and germinated on 1% (w/v) agar at 25°C in darkness.

Sycamore (*Acer pseudoplatanus*) callus on agar was

obtained from Dr R. G. Stanley, University of Florida. The cells originated in a clone first isolated by Lamport & Northcote (1960). Cultures used in this study have been maintained on the agar medium described by Murashige & Skoog (1962). During transfer to new medium, pale-yellow or colourless regions (100–200 mg fresh wt.) were selected and placed aseptically on 50 ml of freshly prepared medium in Erlenmeyer flasks. The cultures were kept at 27°C in subdued light for a selected growth period and then the clones were removed, freed of adhering agar and weighed.

Lemna minor was grown axenically in 100 ml of E medium (Cleland & Briggs, 1967) in 250 ml Erlenmeyer flasks at 25°C close to a window. Cultures (usually two or three clones) were transferred to new media every 3–4 weeks.

D-[1-¹⁴C]Glucosamine (specific radioactivity 53 μ Ci/ μ mol) was a product of The Radiochemical Centre, Amersham, Bucks., U.K. Analysis, just before these experiments, indicated that its radiochemical purity was greater than 98%.

Pronase was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., crystalline trypsin was from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and other biochemicals were from Sigma Chemical Co., St Louis, Mo., U.S.A.

Labelling experiments. *Acer* cultures and *Lemna* were provided with D-[1-¹⁴C]glucosamine in their culture media at a radioactive concentration of approx. 1 μ Ci/10 ml. In some experiments seeds of beans, barley or corn were germinated on 1.0% (w/v) agar containing 2–5 μ Ci of the radioactive glucosamine.

In experiments with corn roots non-radioactive seedlings were grown for 3 days in darkness and the corn root-tips excised with a surgical blade. The tissues were blotted dry and shaken (100 rev./min) in water at 30°C containing the desired concentration of [1-¹⁴C]glucosamine. Samples of the media were removed at hourly intervals to follow the uptake of ¹⁴C. After 6 h the roots were washed free of medium and ground in hot 80% (v/v) ethanol in an all glass motor-driven homogenizer. After centrifuging at 1000g for 5 min the residue was re-extracted several times with 80% ethanol to remove all soluble radioactivity, treated successively with 100% ethanol (twice) and ether (twice), and finally air-dried. The final residue is referred to as the ethanol-insoluble residue. This material was then treated in various ways as described in the Results section to extract its incorporated radioactivity. Essentially similar procedures were employed to recover the ethanol-insoluble residues from the other tissues used in these experiments.

Hydrolysis procedures. To release and identify the amino sugars, portions of ethanol-insoluble residue were hydrolysed with 2M-HCl in an autoclave at 121°C for 30 min. After centrifugation the supernatant solution was dried *in vacuo* to remove HCl and analysed by paper chromatography. To hydrolyse peptides, the compounds were heated with 6M-HCl at 105°C overnight (16 h) in sealed glass tubes, dried to remove the acid and redissolved in 0.1M-HCl before amino acid analysis. Results have not been corrected for destruction of amino sugars or amino acids under these hydrolytic conditions.

Paper chromatography. Total hydrolysates were chromatographed in the following solvents: A, butan-1-ol-

pyridine-0.1M-HCl (5:3:2, by vol.); B, pyridine-ethyl acetate-acetic acid-water (5:5:3:1, by vol.) in a tank equilibrated with pyridine-ethyl acetate-water (11:40:6, by vol.) (Fischer & Nebel, 1955); C, ethyl acetate-pyridine-water (8:2:1, by vol.); D, butan-1-ol-acetic acid-water (37:25:9, by vol.).

Detection of radioactivity on paper chromatograms. Radioactive regions were detected either by exposure to Kodak X-ray film or by radiochromatogram scanning (Packard model 7201 scanner). Material was then eluted from the papers with water and samples were taken for measurement of radioactivity by liquid scintillation.

Column chromatography. Columns of Sephadex G-100, DEAE-Sephadex A-25 (Cl⁻ form) and CM-Sephadex C-25 (Na⁺ form) were prepared according to the manufacturers' recommendations. Linear gradients of NaCl (0.01–0.6M) were employed with the Sephadex ion-exchangers. The mixing flask, which was stirred continuously, contained 250 ml of 0.01M-NaCl in the appropriate equilibrating buffer, and the reservoir 250 ml of buffered 0.5M-NaCl. A peristaltic pump maintained a constant rate of elution. Fractions (10 ml) were collected and samples removed for determination of ¹⁴C. The column of Sephadex G-100 (2.5 cm $\times$ 45 cm) was equilibrated with 0.01M-tris-HCl buffer, pH 8.2, and calibrated for determination of molecular weights by using Blue Dextran and the following purified proteins: bovine serum albumin, jack-bean urease, ovalbumin and α -chymotrypsin. The flow rate was approx. 30 ml/h and fractions between 2.5 and 5 ml were collected. The effluent from the column was scanned continuously for u.v. absorption, and the readings were recorded on a chart that had event markers triggered by the fraction collector.

For ion-exchange chromatography of small peptides we employed a column (1.2 cm $\times$ 15 cm) of Sephadex A-25 (HCO₃⁻ form) and eluted with a linear gradient (0.01–0.5M) of NH₄HCO₃.

Protease digestions. Samples of ethanol-insoluble residue were treated with ice-cold performic acid (Hirs, 1967) for 2 h to oxidize disulphide bonds, freeze-dried to remove the volatile acid and treated with either trypsin or Pronase. A 2 ml portion of the trypsin solution (0.1 mg/ml in 0.2M-NH₄HCO₃) was used for approx. 20 mg of residue. The digest was layered with a drop of toluene to prevent bacterial contamination and kept at 37°C for 20 h. Similar conditions were used for Pronase, but to ensure as complete a hydrolysis as possible the incubation was continued for 5 days and further Pronase (0.1 mg/20 mg of residue) was added every 24 h.

After incubation the Pronase solutions were dried several times under vacuum to remove the volatile NH₄HCO₃ and the residues dissolved in either water or 0.01M-tris-HCl buffer, pH 8.2.

Peptide 'mapping'. Peptide 'maps' were prepared in two ways. (a) Material was spotted 1 cm from the bottom and approximately half way along 20 cm $\times$ 20 cm thin-layer cellulose plates (E. Merck A.-G., Darmstadt, Germany) and run in solvent E [the upper (organic) phase of butan-1-ol-acetic acid-water (4:1:5, by vol.)] until the solvent front had reached the upper edge of the plate. After chromatography the plates were dried, sprayed with buffer F [pyridine-acetic acid-water (1:1:78, by vol.), pH 4.5] and subjected to electrophoresis

in the second dimension (400 V for 1 h). A second method (b) was used to isolate larger amounts of the radioactive peptides. Materials were spotted on Whatman no. 1 or 3MM paper, chromatographed in solvent *E* for 24 h and then subjected to high-voltage electrophoresis in the second direction with buffer *F* (2000 V at 40 V/cm for 1 h). Radioactive regions were detected by using X-ray film.

Determination of radioactivity. The radioactivity of soluble radioactive samples, diluted to 1 ml with water, was counted in 10 ml of toluene-Triton X-100 (2:1, v/v) scintillant (Turner, 1968). All procedures, including estimations of quenching, have been described previously (Roberts & Tovey, 1969; Roberts, 1970).

Synthesis of 2-deoxy-[(2-hydrozynaphthylmethylene)-amino]-D-glucose. The derivative was prepared as described by Stacey & Webber (1962), except that the amounts of the reactants were decreased approximately fourfold. The radioactive product was recrystallized from hot methanol. However, it is sufficiently soluble in water to allow its radioactivity to be counted in the aqueous liquid-scintillation system we employ, although it quenches strongly and so only small samples can be used. The degree of quenching was estimated by the channels-ratio method (see Turner, 1968).

Other analytical procedures. Protein was determined by the method described by Campbell & Sargent (1967), total polysaccharide with α -naphthol (Dische, 1962), amino sugars by either the method of Elson & Morgan (Winzler, 1955) or on the amino acid analyser with the use of ninhydrin. All column eluates were scanned for absorption at 280 nm. Monosaccharides were detected on paper chromatograms by alkaline AgNO_3 spray reagent, amino sugars, amino acids and peptides by ninhydrin in butan-1-ol. Hydrolysed peptides were analysed for amino sugar and amino acid content on a Beckman model 120C amino acid analyser.

RESULTS

Incorporation of D-[1- ^{14}C]glucosamine into corn roots

Labelling of tissues. Groups of 200 roots were shaken with D-[1- ^{14}C]glucosamine (50 μCi) for 6 h in 10 ml of water. During this time each lot took up about 80% of the radioactivity (40 μCi), and of this approx. 4 μCi was recovered in the ethanol-insoluble residues of the roots (380 mg of dried material).

Identification of D-glucosamine. As in previous experiments (Roberts, 1970), hydrolysis of small portions of this residue released a radioactive compound with the chromatographic mobility of glucosamine in solvents *A*, *B* and *C*, plus compounds that were retained close to the origin. The latter are presumed to be the products of partial acid hydrolysis. Further hydrolysis of these compounds (4M-hydrochloric acid at 100°C for 4 h) released more [^{14}C]glucosamine. No radioactivity was detected in glucose and in the other major sugars of the cell wall. Galactosamine contained less than 10% of the radioactivity recovered in glucosamine.

To confirm the identity of the D-[^{14}C]glucosamine in hydrolysates, a portion was eluted from a chromatogram, diluted with authentic D-glucosamine (84.3 mg) and converted into 2-deoxy-2[(hydroxynaphthylmethylene)amino]-D-glucose. The specific radioactivity of the derivative (17 600 d.p.m./mmol) was close to that of the starting D-glucosamine (18 800 d.p.m./mmol). Further recrystallization from hot methanol did not alter this value significantly. The yellow derivative had m.p. 195–196°C, somewhat lower than the literature value [m.p. 202–203°C (decomp.) (Stacey & Webber, 1962)].

Chromatography of radioactive macromolecules. When the ethanol-insoluble residue from the roots was treated with 0.5M-sodium chloride, between 75 and 80% of the ^{14}C was extracted. This material was non-diffusible on dialysis, precipitable with 5% (w/v) trichloroacetic acid and 80%-saturated ammonium sulphate and, when it was chromatographed on a column of Sephadex G-100, most of the radioactivity was eluted as a narrow peak, coinciding exactly with the void front. A small amount of carbohydrate and material absorbing at 280 nm was associated with the radioactivity.

Portions of the salt extract were also chromatographed on either DEAE-Sephadex or CM-Sephadex. In the former case 92% of the ^{14}C was retained on the column and was eluted as a series of peaks by a linear salt gradient (Fig. 1*a*). By contrast only 25% became bound to CM-Sephadex at pH 4.0. Most of this was eluted in two overlapping peaks at low concentrations of salt (Fig. 1*b*).

It is clear from these results that radioactivity from D-glucosamine is incorporated into a number of different macromolecules of high molecular weight, most of which bear a net negative charge at pH 8.2, but some of which are positively charged at pH 4.0. This amphoteric characteristic is consistent with these molecules being glycoproteins.

Alkali extraction of corn residue. Treatment with hot dilute alkali is expected to solubilize proteins and to result in at least partial hydrolysis of their peptide chains. It will also extract much of the non-cellulosic polysaccharide from cell-wall material without hydrolysing it appreciably, though there will be some degradation of the exposed reducing end of the chains (Whistler & Richards, 1970). When the corn-root residue was treated with 0.5M-potassium hydroxide (100°C for 1 h) more than 95% of the ^{14}C was extracted. This solution was neutralized with acetic acid, centrifuged to remove precipitated hemicellulose A and chromatographed on the standardized column of Sephadex G-100 (Fig. 2*a*). Although most of the carbohydrate appeared at the void front, the radioactive materials were eluted as a series of overlapping bands that were only partially excluded by the gel. This material contained only small amounts

Fig. 1. (a) Ion-exchange chromatography of dialysed salt extract from *Zea* residue on a column of DEAE-Sephadex. About 10 mg of ethanol-insoluble residue was treated with 5 ml of 0.5M-NaCl, and the extract dialysed for 16 h to remove salt and then chromatographed on the column. The conditions of elution are described in the text. ^{14}C (●) was measured for each fraction. The concentration of salt in the gradient is indicated (----). (b) Ion-exchange chromatography of dialysed salt extract from *Zea* residue on a column of CM-Sephadex. About 10 mg of residue was treated with 5 ml of 0.5M-NaCl and the dialysed extract chromatographed on the column. The conditions of elution are described in the text. ^{14}C (●) was measured for each fraction. The concentration of salt in the gradient is indicated (----).

of carbohydrate but much of the u.v.-absorbing material. The fractions collected between 70 and 125 ml were combined, dried *in vacuo* at 40°C and analysed by high-voltage electrophoresis at pH 4.5 (40 V/cm for 1 h). Several major and many minor cationic and anionic bands of radioactivity could be detected by radioautography. The darkest band, which showed a slight movement (3 cm) towards the cathode, was eluted and analysed on the amino acid analyser. It contained glucosamine, a trace of galactosamine and all of the common amino acids typically found in protein hydrolysates. The amino sugar content, however, was less than 0.5%, and it seems likely that the band contained a mixture of unlabelled as well as labelled compounds.

Treatment with proteases. Corn-root residue was oxidized with performic acid and then treated with either trypsin or Pronase. When the trypsin digest was 'fingerprinted' on thin-layer cellulose plates by successive chromatography and electrophoresis at least 15 well-defined radioactive spots could be detected by radioautography. These did not correspond closely to any of the regions that stained most deeply with ninhydrin.

Prolonged digestion with Pronase released about 85% of the radioactivity from corn-root residue into solution, scarcely more than that solubilized with sodium chloride. Gel chromatography of the result-

ing brown solution, however, revealed that most of this ^{14}C was no longer associated with the polysaccharide peak at the void front, but was eluted as a slightly asymmetric band between 90 and 135 ml, ahead of the salt volume and the major portion of the materials absorbing at 280 nm (Fig. 2b). The salt peak also contained the brown material noted in the original digest.

The calculated molecular weight of compounds emerging at 110 ml in the column eluate is 10000–15000. Pronase clearly causes a partial breakdown of the macromolecules labelled with D- ^{14}C glucosamine into smaller components.

Analysis of peptides. The asymmetric nature of the radioactive peak eluted from the Sephadex G-100 column suggested that it consisted of two or more compounds differing in molecular size. Fractions between 95 and 130 ml were therefore pooled and dialysed for 18 h against 3 litres of water. More than 90% of the ^{14}C was non-diffusible. This material was concentrated and subjected to ion-exchange chromatography on a column of DEAE-Sephadex (HCO_3^- form) (Fig. 3). Four major peaks of radioactivity (CA, CC, CD and CE) were detected, confirming the heterogeneity of the sample. The fractions within each peak were combined and dried, and the salt was removed by repeated evaporation. Each sample

Fig. 2. (a) Chromatography of the neutralized alkali extract from *Zea* residue on a column of Sephadex G-100. The starting material was 200 mg of ethanol-insoluble residue. This was treated with 5 ml of 0.5 M-KOH at 100°C for 1 h, and the extract neutralized with acetic acid and concentrated to 3 ml. This solution was applied to the column. Fractions (each about 5 ml) were collected and assayed for radioactivity (●) and total carbohydrate (▲). Extinction at 280 nm (····) was measured throughout the elution. (b) Chromatography of the Pronase digest from *Zea* residue on a column of Sephadex G-100. The starting material was 200 mg of ethanol-insoluble residue, which was treated for 5 days with Pronase solution as described in the Experimental section. The digest plus washes were then concentrated to 2.0 ml and chromatographed on the standardized column. Fractions were assayed in a manner similar to (a).

was 'fingerprinted' on Whatman no. 1 paper to purify the peptides and the radioactivity was detected by radioautography. Each peptide 'map' was clearly complex, consisting of up to four or five radioactive spots and often 20 or more areas that reacted positively with ninhydrin. Peak CE from the DEAE-cellulose column chromatography, for example, contained three major and three minor areas of radioactivity (Fig. 4). Spots CE1, CE2 and CE3 were well separated from the major ninhydrin-positive areas on the 'map'. The major

spot (CE2) had the composition listed in Table 1. The total sample contained only 1.4 nmol of glucosamine (specific radioactivity $6.5 \mu\text{Ci}/\mu\text{mol}$) but no other amino sugars. A wide spectrum of the more common amino acids was detected, however.

Peak CC was less complex than the others and only two radioactive regions (CC1 and CC2) were detected by radioautography (Fig. 4). One of these, spot CC1, had a slight (4 cm) mobility towards the cathode, but showed little movement on chromatography. Ninhydrin spraying of the

Fig. 3. Ion-exchange chromatography of radioactive peptides released from *Zea* residue by treatment with Pronase. Material, after Sephadex G-100 chromatography, was concentrated, dialysed and loaded on to the column. Unbound materials were washed through with 2 bed volumes of 0.01 M- NH_4HCO_3 . The conditions for elution of bound compounds are described in the Experimental section. Radioactivity (●) and E_{280} (·····) were followed throughout the elution. The concentration of salt in the gradient (----) is indicated.

surrounding region of the paper indicated no appreciable overlap by non-radioactive peptides. Spot CC1 contained, as well as glucosamine, a number of amino acids. However, the amino sugar content was relatively high. The specific radioactivity of the recovered glucosamine was $5.6 \mu\text{Ci}/\mu\text{mol}$, about one-tenth that of the glucosamine supplied to the roots.

All other such purified compounds that have been isolated so far from the Pronase digests have contained glucosamine and a number of amino acids. They are clearly peptides that contain covalently bound glucosamine and that have resisted complete hydrolysis by Pronase. In each case they have been recovered in such low amounts that a detailed investigation of their complete carbohydrate composition has been precluded.

Incorporation of D-[1- ^{14}C]glucosamine into sycamore-cell cultures

Essentially similar results to those found with corn roots were obtained with *Acer*-cell cultures grown on a 1% (w/v) agar medium containing D-[1- ^{14}C]glucosamine. The distribution of ^{14}C in the various extracts and in the final insoluble residue is summarized in Table 2. Hydrolysis of the ethanol-insoluble fraction with 2 M-hydrochloric acid released [^{14}C]glucosamine, some products of partial acid hydrolysis, but no trace of galactosamine or mannosamine. Exhaustive extraction of the 80%-ethanol-insoluble residue with 0.5 M-sodium chloride released only about 60% of the incorporated ^{14}C . Most of the remainder could be extracted with 0.5 M-potassium hydroxide. The sodium chloride

Fig. 4. Peptide 'maps' of peaks CC and CE. Peaks CC and CE, isolated by ion-exchange chromatography (Fig. 3), were spotted on separate sheets of Whatman 3MM paper and 'fingerprinted' as described in the Experimental section. This composite diagram shows the distribution of radioactive compounds on the papers. Radioactive peptides from peak CC are shown with a broken line, those from peak CE with a continuous line.

extract was of high molecular weight and was eluted as two peaks on the standardized column of Sephadex G-100, the first coinciding with the void front, the second only partially excluded by the gel (Fig. 5a).

In one experiment the fractions within each peak (1, 42–54 ml, and 2, 57–72ml) were concentrated to a small volume and treated with Pronase. In this case the hydrolyses at 37°C were continued for only 24h and not 6 days. The digests were then rechromatographed separately on Sephadex G-100, and the products of hydrolysis were collected as low-molecular-weight radioactive peaks (1P and 2P) between 115 and 130ml (Fig. 5a). The fractions were concentrated and the peptides purified further by high-voltage electrophoresis (2000V for 1h). Only a single radioactive peptide was isolated from each of peaks 1P and 2P. Radioactive peptide 1P moved 4cm, and radioactive peptide 2P 2.6cm, towards the cathode. Each of these was eluted, hydrolysed and analysed for its amino acid content. Their compositions are listed in Table 1. Each contained glucosamine and a number of amino acids. Like the peptides isolated from the corn roots, they were clearly complex, but their later elution from the column of Sephadex G-100 indicated that they were of slightly lower molecular weight.

To complement the experiments with corn, Fig. 5(b) illustrates the chromatographic pattern observed when the entire Pronase digest of a portion of ethanol-insoluble residue from *Acer* was passed through a column of Sephadex G-100. In this experiment no attempt was made to purify the macromolecules in any way before proteolysis, and

Table 1. Composition of radioactive peptides from *Zea* and *Acer* cells given D-[1-¹⁴C]glucosamine

The values listed are the total amounts of each compound recovered after analysis of each purified peptide. CC1 and CE2 represent material eluted from the peptide 'maps' shown in Fig. 4. 1P and 2P are the radioactive peptides isolated from Pronase digests of *Acer* extract (Fig. 5a). Tryptophan was not estimated. Nor were corrections made for destruction of glucosamine during hydrolysis. This could be as high as 50% under the conditions employed here (Neuberger & Marshall, 1966). The specific radioactivity of the glucosamine is determined from the known amount of ¹⁴C in the hydrolysed sample and the amount of glucosamine measured on the analyser. Values are therefore probably high because of glucosamine destruction.

Amino acid	Peptide ...	Amount (nmol)			
		<i>Zea</i>		<i>Acer</i>	
		CC1	CE2	1P	2P
Lysine		—	3.8	—	—
Histidine		—	1.6	—	—
Arginine		—	2.8	—	—
Hydroxyproline		10.7	—	—	—
Aspartic acid		25.5	13.3	32.6	12.1
Threonine		15.3	4.0	49.2	7.7
Serine		24.2	18.8	52.2	11.9
Glutamic acid		11.2	17.8	41.8	7.0
Proline		—	—	12.8	4.7
Glycine		27.6	26.9	56.4	14.5
Alanine		15.6	10.7	61.5	11.4
Half cystine		—	—	—	—
Valine		—	5.7	83.8	8.2
Methionine		—	1.6	—	—
Isoleucine		—	1.8	52.6	—
Leucine		—	2.4	96.2	—
Tyrosine		—	—	18.8	—
Phenylalanine		52.8	—	23.8	—
Glucosamine		49.1	1.4	18.0	4.4
Sp. radioactivity of glucosamine (μCi/μmol)		5.6	6.5	1.4	3

Table 2. Recovery of ¹⁴C from *Acer* pseudoplatanus callus after growth of the tissue on medium containing D-[1-¹⁴C]glucosamine

The weight of tissues at day 0 was 0.50g and that of tissues at day 43 was 7.87g. The radioactivity provided was 5.30 μCi and that incorporated was 2.73 μCi (51.5%).

	Radioactivity	
	(μCi)	(% of total incorporated)
In ethanol-soluble fraction	1.55	56.7
Extracted by NaCl	0.65	23.8
Extracted by KOH	0.40	14.5
In alkali-resistant residue	0.13	5.0

Fig. 5. (a) Chromatography of the salt extract from *Acer* residue on a column of Sephadex G-100. The starting material was 200 mg of ethanol-insoluble residue. This was treated with 0.5 M NaCl and chromatographed as described for *Zea* extract in Fig. 1(a). Radioactivity (●), total carbohydrate (▲) and E_{280} (····) were measured throughout the elution. The inset in (a) shows the elution of ^{14}C from the same column after peak 1 (○) and peak 2 (△) had been treated with Pronase. These low-molecular-weight peaks are referred to as 1P and 2P respectively. (b) Chromatography of the Pronase digest of unextracted *Acer* residue on a column of Sephadex G-100. The starting material was 187 mg of ethanol-insoluble residue, which was treated for 5 days with Pronase solution as described in the Experimental section. The digest plus washes were then concentrated to 2.0 ml and chromatographed on the standardized column. Fractions were assayed as in (a).

so the broad asymmetric band eluted between 100 and 145 ml presumably contains the radioactive peptides 1P and 2P, as well as other labelled fragments. The Pronase extracted 87% of the

total radioactivity in the residue, considerably more than that removed by salt. Larger amounts of polysaccharide were also present, but again this material was eluted close to the void front.

*Incorporation of D-[1-¹⁴C]glucosamine into
other tissues*

D-[1-¹⁴C]Glucosamine was provided in the growth medium of axenic cultures of *Lemna minor* and to seeds of beans (*Vicia faba*), corn (*Zea mays*) and barley (*Hordeum vulgare*) germinating sterily on 1.0% agar. In each case the tissues incorporated radioactivity into compounds of high molecular weight that resembled proteins in their physical properties. Further, except with *Lemna*, the radioactivity was located specifically in amino sugar residues. In the case of *Lemna*, however, some radioactive glucose (about 20% of the total ¹⁴C) was detected in hydrolysates.

DISCUSSION

Glycoproteins may be defined as naturally occurring compounds of high molecular weight in which carbohydrate and protein are covalently linked (Gottschalk, 1966; Montgomery, 1970). They consist of one or more polypeptide chains to which are attached one or more carbohydrate groups. The chemical composition, structure and properties of mammalian glycoproteins are very varied, but most of them contain hexosamines as well as other carbohydrate constituents. Radioactive D-glucosamine has been widely used in studies on glycoprotein biosynthesis in animals because it acts as a specific precursor of N-acetylhexosamine and neuraminic acid residues (Marshall & Neuberger, 1968).

Glycoproteins containing amino sugars have been identified in a large number of plants. Our experiments show that D-[¹⁴C]glucosamine can usually be used as a precursor of the amino sugar components of such glycoproteins. This could provide a useful tool in future studies on their biosynthesis, structure and cellular location. As in animal tissues, however, care must be taken to establish the specificity of the labelling pattern for each experiment. With *Lemna*, a photosynthetic tissue, for example, some of the ¹⁴C from the glucosamine we provided was recovered, not in amino sugars, but in glucose units, possibly in the form of starch. This suggests that glucosamine can, under some circumstances, be deaminated and be converted into hexose phosphates. Similar results have been obtained with mammalian erythrocytes (Kornfeld & Gregory, 1968).

The evidence that the labelled macromolecules formed in corn are glycoproteins and not polysaccharides or lipids is convincing. The compounds are fairly soluble in water, yet largely insoluble in ethanol, 5% (w/v) trichloroacetic acid, and concentrated solutions of ammonium sulphate. Some at least are amphoteric and most are negative-

ly charged at pH 8.2. They are readily degraded by alkali and by solutions of proteases, and the radioactive fragments that were released were clearly peptides containing glucosamine.

It was possible that Pronase did not degrade the peptides as far as their protein-carbohydrate linkage region. The peptides were large and contained several amino acids, and it is not uncommon for a carbohydrate group to interfere with the normal pattern of action of some proteolytic enzymes (Lee & Montgomery, 1962; Fletcher, Marks, Marshall & Neuberger, 1963). Therefore a more complete hydrolysis of the peptide position will be necessary before the nature of the linkage region can be identified. It was also evident that in corn roots the radioactive glycoproteins, though numerous, were present in relatively small amounts. Because of this they were difficult to purify and analyse. In *Acer* cells, however, the total amino sugar content seemed relatively higher and the labelled macromolecules less diverse than in corn.

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