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The Incorporation of D-Glucosamine-^{14}C into Root Tissues of Higher Plants

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ABSTRACT

D-Glucosamine-1-^{14}C was rapidly taken up from aqueous solution by both excised bean (*Phaseolus vulgaris*) and corn (*Zea mays*) root tips. The labeled glucosamine did not accumulate in the tissues, however, but was metabolized to *N*-acetyl-D-glucosamine, *N*-acetyl-D-glucosamine phosphates, and uridine diphosphate *N*-acetyl-D-glucosamine. Little or no label was detected in respiratory CO_2, glycolytic intermediates, or D-glucosamine 6-phosphate. Between 5 and 10% of the ^{14}C was recovered in high molecular weight ethanol-insoluble materials which could be solubilized readily with alkali or by treatment with proteases, and which yielded labeled glucosamine upon complete hydrolysis with HCl. Milder hydrolytic conditions released quantities of *N*-acetylglucosamine-^{14}C plus labeled fragments of higher molecular weight. It is concluded that D-glucosamine-^{14}C may be used to label specifically the amino sugar residues of plant as well as animal macromolecules. *N*-Acetyl-D-glucosamine acts similarly as a precursor, except that it is taken up at only about 1/10 the rate of glucosamine and hence is utilized less efficiently.

When labeled D-glucosamine is supplied to animal cells (4, 7, 20), it is usually incorporated specifically into *N*-acetylhexosamine and *N*-acetylneuraminic acid residues of glycoproteins and mucopolysaccharides. This specificity of labeling has been employed in order to study precursor-product relationships in the biosynthesis of macromolecules containing amino sugars (4). UDP-*N*-acetylglucosamine is considered to be the activated form to which GlcN[2] must be converted prior to its incorporation into glycoprotein.

Recently, there have been many reports of GlcN appearing in hydrolysis products of plant glycoproteins and glycolipids (2, 6, 11, 19), and the enzymes involved in a pathway for the formation of UDP-GlcNAc from fructose-6-P have been isolated from germinating mung beans (11). Sugar nucleotide derivatives containing GlcNAc have also been characterized from a number of tissues (3, 21). This raised the question as to whether GlcN could be used to label specifically the amino sugar residues of plant macromolecules and thereby serve as a sensitive tool in their detection and in studies on their structure, biosynthesis, and intracellular transport. In an attempt to answer this question, GlcN has been fed to roots of young seedlings, and some of the products of metabolism have been isolated and characterized.

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[2] Abbreviations: GlcN: glucosamine; GlcNAc: *N*-acetylglucosamine.

MATERIALS AND METHODS

Materials. Grain of *Zea mays* var. Kelvedon 33 (Dunn's Farm Seeds Ltd., Salisbury) was germinated under sterile conditions on 1% (w/v) agar at 25 C in darkness (16). Bean seeds (*Phaseolus vulgaris* var. Red Emperor) were germinated similarly in trays of moist vermiculite. Primary root tips (1 cm) were used within 30 min of excision.

D-Glucosamine-1-^{14}C HCl (3.1 and 57 mc/mmole) and *N*-acetyl-D-glucosamine-1-^{14}C (57 mc/mmole) were products of the Radiochemical Centre, Amersham, England. Radiochemical purity was >98% for each compound as determined by reverse isotopic dilution analysis and paper chromatography prior to these experiments. UDP-*N*-acetyl-D-glucosamine was purchased from Sigma Chemical Company. *N*-acetyl-D-glucosamine 1-P was prepared from the sugar nucleotide by action of snake venom phosphodiesterase (British Drug Houses Ltd.). Alkaline phosphatase from *Escherichia coli*, fungal pectinase, and bacterial pronase were also obtained from Sigma. Snail (*Helix pomatia*) digestive juice enzyme was a product of Industrie Biologique Francaise, Gennevilliers, France. Ariel detergent (Proctor and Gamble Ltd.) was purchased locally.

Labeling of Root Tips. A selected number of roots were added to solutions of labeled compounds in distilled water in small Erlenmeyer flasks (10 or 25 ml). These were sealed with rubber serum closures (Kontes Glass Company) equipped with polypropylene cups containing 0.2 ml of *N*-KOH to trap respiratory CO_2. The flasks were shaken (100 oscillations/min) in a water bath at 30 C in subdued light. At intervals, small aliquots were removed from the medium to determine the disappearance of ^{14}C.

Fractionation of the Roots. Root tips were removed from the solution, washed several times with water, and then killed and ground in boiling 70% (v/v) ethanol using a motor-driven, all glass homogenizer. Ethanol-insoluble residues were washed repeatedly with fresh portions of 70% ethanol to remove all traces of soluble ^{14}C, then extracted successively with 5 ml of 100% ethanol (twice) and 5 ml of ether (twice), and finally air-dried.

Paper Chromatography. Compounds were subjected to descending paper chromatography on Whatman No. 1 paper. The following solvents were used for separation of amino sugars and various monosaccharides: A: *n*-butanol–acetic acid–water (37:25:9); B: ethyl acetate–pyridine–water (8:4:1); C: pyridine–ethyl acetate–acetic acid–water (5:5:3:1); D: *n*-butanol–pyridine–water (6:4:3); E: phenol saturated with water.

Solvents F to J were employed for phosphates and nucleotides: F: ethanol–M ammonium acetate, pH 3.8 (5:2); G: ethanol–M ammonium acetate, pH 7.5 (5:2); H: isobutyric acid–*N* ammonia–2 M EDTA (50:30:1); I: *n*-butanol–acetone–formic acid–5% (w/v) ammonium formate (7:5:3:5); J: ethanol-concentrated ammonia solution–water (20:1:4).

Paper Electrophoresis. Charged compounds were subjected to electrophoresis in 0.2 M ammonium formate buffer at pH 3.7

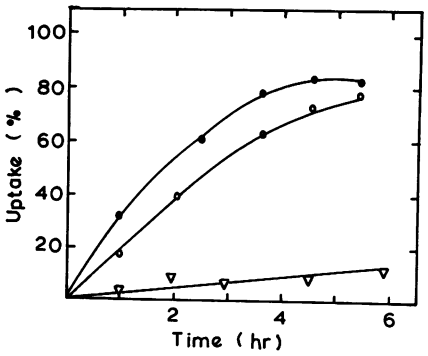

FIG. 1. Uptake of D-glucosamine-^{14}C and *N*-acetyl-D-glucosamine-^{14}C by root tips. For conditions see Table I, experiment 1. ●: *Zea* roots plus GlcN; ▽: *Zea* roots plus GlcNAc; ○: *Phaseolus* roots plus GlcN.

(50 v/cm; 1 hr) using a Mini Pherograph equipped with platinum block electrodes and cooled to 0 C with methanol-water. Picric acid was always run as a colored marker.

GlcNAc, amino sugars, and monosaccharide standards were detected on paper by means of alkaline silver nitrate. Alternatively, amino sugars were located by spraying with ninhydrin in butanol and heating the papers at 105 C for a few minutes. Sugar nucleotides were detected by viewing under an ultraviolet lamp and sugar phosphates with ammonium molybdate spray reagent (5).

Determination of Radioactivity. Soluble radioactive samples diluted to 1 ml with water were counted in 10 ml of toluene–Triton X-100 scintillant (2:1, v/v) (22). The toluene contained 0.4% w/v PPO and 0.01% POPOP. All measurements were carried out using a Nuclear-Chicago, Mark 1 liquid scintillation spectrometer. Counting efficiency was determined for each sample by the channels ratio method and was usually in the region 73 to 76%. Insoluble samples were counted suspended in Cab-O-Sil (Packard Instrument Co., Downer's Grove, Illinois). Radioactivity on paper chromatograms was detected by either scanning using a windowless gas flow counter or by exposure to Kodirex x-ray film.

Radioactive areas were marked, cut out, and placed in counting vials containing 10 ml of toluene/PPO/POPOP without added Triton X-100 and water. Radioactivity in the major spots was expressed as a percentage of the total activity along the solvent track.

Uptake of radioactivity by root tips was estimated by withdrawing aliquots (0.02 ml) and drying these on glass planchets under an infrared lamp. The planchets were then counted at a fixed distance beneath a calibrated Geiger-Müller end window tube.

RESULTS AND DISCUSSION

Uptake of D-Glucosamine and *N*-Acetyl-D-glucosamine. GlcN was rapidly taken up by corn root tips (Fig. 1). The rate observed was comparable to that for D-glucose (15) and D-galactose (14), and at least 10-fold faster than that noted for myoinositol at similar external concentrations (16). GlcNAc, on the other hand, was taken up at a considerably slower rate than GlcN even though it is relatively less polar. Roots killed with ethanol did not incorporate GlcN or GlcNAc.

The production of labeled CO_2 during uptake of either compound was very low (Table I). At no stage in the experiment did it exceed 0.3% of the label accumulated by the tissues. Evidently there was negligible transformation of ^{14}C to hexose sugars or hexose phosphates and subsequent metabolism through normal glycolytic intermediates (17).

At the end of 6 hr, approximately 5 to 10% of the label was taken up as GlcN was recovered in ethanol-insoluble residues of the roots (Table I). These observations have been confirmed in three separate experiments at closely similar external concentrations of GlcN. About 0.1% of the ^{14}C was present in the 100% ethanol and ether extracts, and the remainder in 70% ethanol-soluble materials.

Analysis of the Ethanol-soluble Fraction. Chromatography of portions of the 70% ethanol extracts of both bean and corn (experiment 1) in solvent F, followed by autoradiography revealed seven radioactive spots of varying intensity. Radiochromatogram scans are shown in Figure 2. A major labeled compound (VII) from both tissues ran ahead of GlcN (VI), and further spots were located in the region of GlcN 1-phosphate (IV, V) and UDP-GlcNAc (II).

The whole of the ethanol-soluble extract from experiment 2 (97 μc) was chromatographed on Whatman 3MM paper in solvent F for 16 hours. Bands, II, IV, V, VI, and VII were eluted with water containing 5% ethanol, and the solutions were dried at 25 C on a rotary evaporator.

Bands I and III were not investigated further because their low ^{14}C content made identification difficult.

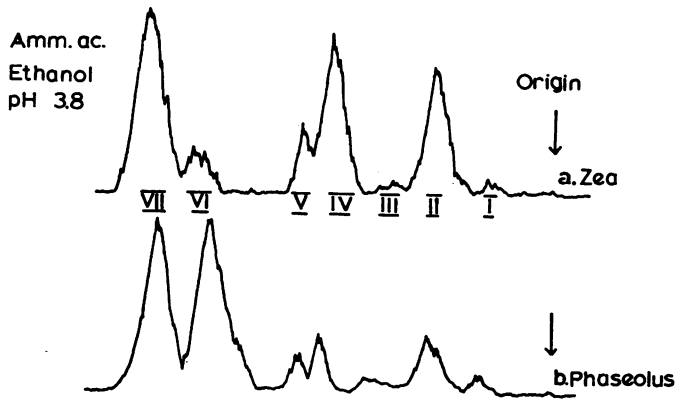

FIG. 2. Radiochromatogram scans of 70% ethanol extracts of roots of *Zea* and *Phaseolus* fed GlcN-^{14}C (experiment 1). Chromatograms were developed in solvent F for 16 hr. Roman numerals refer to radioactive spots detected by x-ray film.

Table I. *Uptake and Utilization of D-Glucosamine-^{14}C and N-Acetyl-D-Glucosamine-^{14}C by Root Tips*

Experiment	No. of Root Tips	Labeled Compound	Radioactivity	Concn	olume	Uptake	Pecentage of Total ^{14}C Taken up (6 hr)			
							CO_2	Ethanol-soluble	Lipid	Residue
			μc	*M*	ml	%				
1. Bean	20	GlcN	2.8	4.5×10^{-4}	2.0	78	0.3	87.1	0.1	12.5
1. Corn	20	GlcN	2.8	4.5×10^{-4}	2.0	84	0.1	93.7	0.1	6.1
1. Corn	20	GlcNAc	50.0	4.8×10^{-4}	2.0	11	<0.1	88.8	...[1]	11.2
2. Corn	120	GlcN	151.0	5.7×10^{-4}	5.0	88	0.2	91.6	0.1	8.1

[1] Not estimated.

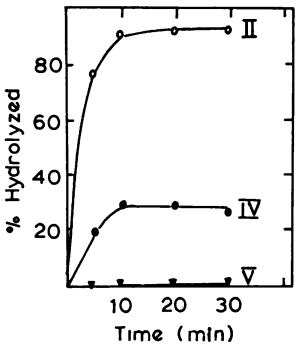

FIG. 3. Time course for hydrolysis of labeled compounds in bands II, IV and V by 0.1 N HCl at 100 C. The product in each case was GlcNAc-^{14}C which was separated from starting material by paper chromatography in solvent D.

Band II. This compound appeared radiochemically homogenous upon chromatography in solvents F to J and upon electrophoresis. In each case the radioactive spot had a mobility identical with that of authentic UDP-GlcNAc. It was not attacked by alkaline phosphatase, but treatment with phosphodiesterase gave GlcNAc-1-P. A combination of the phosphodiesterase and alkaline phosphatase treatments at pH 10.4 released labeled GlcNAc as the sole labeled product. Mild hydrolysis (0.1 N HCl; 100 C) gave almost complete breakdown to GlcNAc in 10 min (Fig. 3). Compound II was stable in the strongly alkaline solvent J, a characteristic feature of nucleotides of GlcNAc (3). Most sugar nucleotides decompose rapidly at pH values above 10 to give the sugar 1,2-cyclic phosphate. It was concluded that the labeled compound in band II was UDP-GlcNAc.

Band IV. This compound became firmly bound to anion exchange resins, was completely hydrolyzed to GlcNAc by alkaline phosphatase, and had a mobility close to GlcNAc-1-P in solvents F, G, H, and I. Hydrolysis with 0.1 N HCl (Fig. 3), however, released only 25% of the ^{14}C as GlcNAc in 10 min. Prolonging the mild hydrolysis for a further 20 min failed to increase the yield of GlcNAc. Band II, therefore, was a mixture of GlcNAc-phosphates. Approximately 25% of the label was in GlcNAc-1-P.

Band V. The radioactivity in this spot comprised about 6% (5.8 μc) of the total in the ethanol-soluble fraction of experiment 2 (Table II). It could not be separated from IV except in solvent F, and alkaline phosphatase treatment gave complete hydrolysis to GlcNAc. No GlcN could be detected in the hydrolysis mixture The compound was resistant to 0.1 N HCl (Fig. 3) and only partially hydrolyzed by 1.0 N HCl at 121 C for 30 min. Although its identity was not confirmed, band V was considered to be a phosphate ester of GlcNAc.

Band VI. In experiment 2, this labeled compound contributed less than 2% (3.0 μc) to the total radioactivity in the ethanol-soluble fraction, though in experiment 1 this value was nearer 8% (0.22 μc). It was identified as GlcN by chromatography in solvents A to E and by its characteristic mobility toward the cathode during electrophoresis. Its identity was further confirmed by reverse isotopic dilution analysis of a sample (5.6 μc) with 113 mg of unlabeled GlcN-HCl. There was no change in specific activity after three successive recrystallizations from water-methanol-acetone. Recoveries were 83.9 mg (1.09 μc/mmole), 61.5 mg (0.98 μc/mmole), and 43.0 mg (1.13 μc/mmole).

Band VII. Chromatography in solvents F, G, I, and J revealed a fast moving band which ran ahead of GlcN with a mobility identical with that of GlcNAc. Further chromatography in solvents A to E and reverse isotopic dilution analysis of a sample (1.45 μc) with 109 mg of authentic GlcNAc confirmed this identification. Recoveries after recrystallization were 72.1 mg (2.84 μc/mmole), 52.3 mg (2.71 μc/mmole), and 39.7 mg (2.73 μc/mmole). The ^{14}C in band VII accounted for more than 40% of the ^{14}C in the ethanol-soluble fraction and for over 35% of the total label taken up by the root-tips (Table II).

Most of the label supplied as GlcN accumulated, therefore, as UDP-GlcNAc, GlcNAc phosphates, and GlcNAc. In both animals and plants, UDP-GlcNAc competitively inhibits the enzyme L-glutamine-D-fructose 6-phosphate amidotransferase (EC 2.6.1.16) (8, 11). This enzyme catalyzes the first reaction in a multistep pathway leading to the formation of UDP-GlcNAc. An accumulation of the sugar nucleotide, therefore, will thus inhibit its own synthesis from fructose-6-P. However, when GlcN was fed to animal tissues, the control step was by-passed and UDP-GlcNAc accumulated (8). Similarly, in the experiments described here with roots, the metabolism of GlcN is presumably unaffected by the feedback inhibition, and the incorporation into the sugar nucleotide continues unimpeded. One explanation for the growth inhibition observed when GlcN was supplied to roots (10) is that the balance within the sugar nucleotide pool was disturbed as UDP-GlcNAc accumulated and as UTP became depleted. The initial step in the metabolism of GlcN in animals and microorganisms is usually considered to be a conversion to GlcN-6-P, a reaction which is catalyzed by a nonspecific hexokinase (17). Although Saltman has characterized a similar enzyme in plants (18), no GlcN-6-P was detected in the experiments described here. Indeed, a similar accumulation of GlcNAc-phosphates and UDP-GlcNAc has been described in rat liver after feeding GlcN (12), and it was suggested that the metabolism of GlcN proceeded via *N*-acetylation followed by phosphorylation rather than vice versa.

Ethanol-insoluble Residue. Approximately 12 μc of the ^{14}C in experiment 2 was recovered in the ethanol-insoluble residues (130 mg) of the tissue. Treatment of portions (5 mg) of this residue with 2 ml of 1.0 N HCl (121 C; 30 min) released more than 90% of the incorporated ^{14}C into solution. Chromatography of this hydrolysate in solvents A to E revealed that most of the label (>80%) was associated with a spot coincident with GlcN while the rest was located over the origin. Sugars and amino acids were not radioactive. Milder acid hydrolysis with 1.0 N HCl (1 hr; 90 C) released mainly GlcNAc with smaller amounts of GlcN. Radioactivity was high at the origin, presumably owing to products of incomplete hydrolysis. It was concluded that GlcN was in the acetylated form when incorporated into the ethanol-insoluble fraction of the roots.

Most of the ^{14}C in these residues could be solubilized using *N*-alkali at 30 C (Fig. 4). Pronase and Ariel detergent (a detergent mixed with a bacterial protease) was similarly effective. Crude glycosidase preparations (cellulase and pectinase) also caused a

Table II. *Radiochemical Analysis of Ethanol-soluble Fraction of Zea Roots Fed* D-*Glucosamine-*^{14}*C (Experiment 2)*

Compound[1]	Percentage of Total ^{14}C Recovered in Ethanol-soluble Fraction		
	Solvent F	Solvent H	Solvent I
UDP-GlcNAc, II	24.3	24.4	21.0
GlcNAc-phosphates, IV, V	26.4[2]	27.6[2]	25.7[2]
GlcN, VI	2.2	1.2	1.7
GlcNAc, VII	42.3	43.7	49.0
Others	4.8	3.1	2.6

[1] Roman numerals refer to the band number of the compound upon chromatography in solvent F.

[2] In solvent F these compounds (IV and V) were partially resolved into two spots. Band IV contributed 20.3% and band V 6.1% to the total radioactivity in the ethanol-soluble fraction. In solvents H and I, IV, and V ran together giving a single spot.

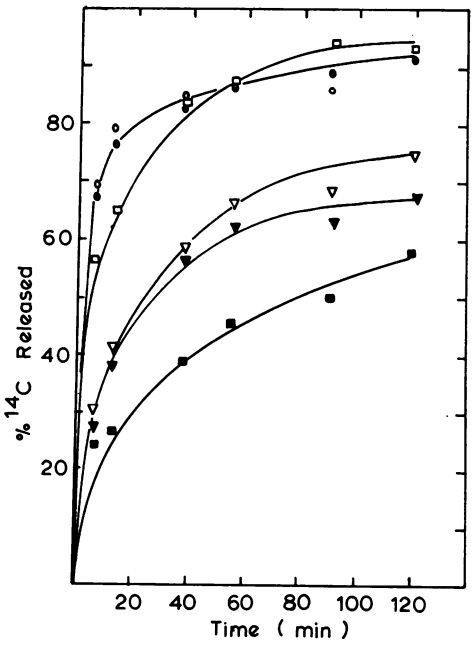

FIG. 4. Release of soluble label from ethanol-insoluble material by treatment with water (■); N KOH (□); 1% (w/v) pronase in 0.02 M tris, pH 8.3 (●); 2% (w/v) Ariel detergent in H_2O (○); 10% (v/v) snail digestive juice in H_2O (▼); and 1% (w/v) pectinase in H_2O (▽). Five milligrams of residue containing 4.6 μc of ^{14}C were treated with 1 ml of solution at 30 C. At selected time intervals, the suspensions were centrifuged and 20-μl aliquots of supernatant were removed for estimation of soluble ^{14}C.

fairly rapid release of ^{14}C. Distilled water at 30 C caused a somewhat slower, but still fairly effective solubilization of ^{14}C over a 2-hr period. The labeled material extracted by water was nondiffusible when dialyzed for 3 hr against 2l buffer (10^{-3} M tris-HCl, pH 8.2) and is probably, therefore, of relatively high molecular weight. Similarly, when the protease and cellulase digests were chromatographed on a column of Sephadex G-25 (75 × 2 cm) with 10^{-3} M tris-HCl as eluant, a radioactive peak appeared at the void front, again suggesting that the ^{14}C was incorporated in macromolecular form (of mol wt >5000). Although this material has not been characterized thoroughly, its properties are not unlike those of glycoprotein.

Pulse Labeling with D-Glucosamine-^{14}C. In an attempt to follow the movement of label from the precursor to the various labeled products noted in experiments 1 and 2, a group of 70 roots were fed 51 μc of GlcN-1-^{14}C (1.8 ml; 5.7 × 10^{-4} M) for 1 hr and then transferred to 2 ml of a cold "chase" (10^{-3} M) for periods of up to 5 hr. Higher concentrations of GlcN were avoided in this chase because of its reported toxicity under such conditions (10). Groups of 10 roots were analyzed immediately after the "pulse" and subsequently after a further 60, 120, 210, and 300 min. The roots were killed in 70% (v/v) ethanol, ground up, and separated into ethanol-soluble and -insoluble fractions. Label in ethanol-insoluble materials was determined after digesting the residue in 1.0 N KOH at 85 C for 1 hr to solubilize incorporated ^{14}C. The ethanol-soluble fraction was assayed for ^{14}C, reduced in volume, and chromatographed in solvents F and H to separate the GlcN, GlcNAc-phosphates, and UDP-GlcNAc (Fig. 5). Some variation in the total amount of ^{14}C was noticed in the separate batches of roots analyzed, even though there was little exchange of ^{14}C (<5%) with the medium during the chase. Thus, the group of 10 roots analyzed at the end of the pulse contained 2.68 μc of ^{14}C, while those after a further 60, 120, 210, and 300 min contained 2.76, 2.46, 2.30, and 2.86 μc, respectively. Because of this variation, results in Figure 5 are presented as a percentage of total ^{14}C in the roots rather than as measured radioactivity. Label in the insoluble fraction did not increase but remained relatively constant at about 3.3% of the total during the chase, even though the amount of ^{14}C in UDP-GlcNAc, the presumed precursor of the macromolecular material stayed at a high level throughout. However, no estimate has been made of the rate of turnover of the ethanol-insoluble fraction, and it may be that the apparent stability of label is an artifact resulting from a balance being achieved between breakdown and synthesis.

At the end of the pulse (time 0), about 34% of the label in ethanol extracts was isolated as GlcN. This contrasted with experiment 2, in which the content of GlcN-^{14}C was very low. However, in that run, net uptake of label from the medium had been completed at least 1 hr prior to analysis. In the present experiment, the roots were still taking up GlcN at a linear rate when killed. During the chase, label in GlcN fell rapidly, and there was a marked increase in GlcNAc-^{14}C. UDP-GlcNAc and the labeled phosphates showed an early rise in ^{14}C content, followed by a gradual fall. At first sight, the increase in GlcNAc-^{14}C might be interpreted as relating directly to the drop in GlcN-^{14}C. However, label in GlcNAc continued to increase beyond the point at which the GlcN pool became depleted to a negligible level. This continued increase would appear to occur at the expense of the nucleotide sugar and the phosphates, but other explanations are not ruled out.

Incorporation of ^{14}C from *N*-Acetyl-D-glucosamine-1-^{14}C into Root Tips. When GlcNAc-1-^{14}C was fed to excised corn roots, uptake was very low (Fig. 1, Table I). However 0.56 μc of ^{14}C was recovered in the ethanol-insoluble fraction of the roots, and hydrolysis of this material with 1.0 N HCl yielded GlcN-^{14}C as sole radioactive product. The ethanol extracts of the roots contained labeled GlcNAc (3.7 μc), GlcNAc-phosphate (0.59 μc), and UDP-GlcNAc (0.51 μc), but no GlcN. It is concluded, therefore, that root tissues utilize GlcNAc in very much the same way as GlcN, and that this compound may also be employed as a precursor to label amino sugar residues in macromolecules. It is less useful in this respect, however, because it is taken up more slowly and hence utilized less efficiently by the tissues.

Although internal pools of GlcN or GlcNAc are unlikely to be found in most plant tissues, there are a limited number of ways in which one could arise other than from experimentally administered compounds. One possibility is from dephosphorylation of GlcN-6-P formed from fructose-6-P. A second source of free amino sugars might result from breakdown or turnover of existing glycoproteins. Indeed, specific enzymes capable of releasing GlcNAc from glycoproteins have been isolated from germinating seed of several species of higher plants (9) and highly purified from beans (1). Moreover, cotyledonary and endosperm reserves

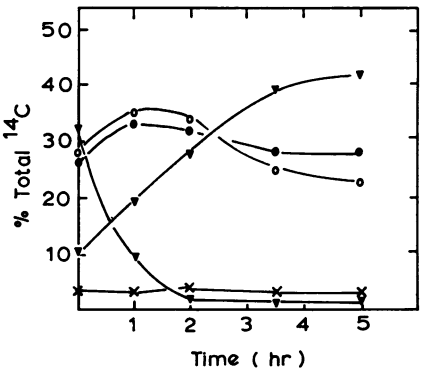

FIG. 5. Distribution of ^{14}C in glucosamine (▼), *N*-acetylglucosamine (▽), *N*-acetylglucosamine phosphates (○), UDP-*N*-acetylglucosamine (●), and insoluble residue (×) after a pulse of D-glucosamine-1-^{14}C and subsequent chase in unlabeled solution.

of seeds are recognized sources of amino sugars (13). It is unreasonable, therefore, to regard pathways capable of utilizing free GlcN or GlcNAc in seedling tissue as either spurious or unnatural.

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