# Transient *N*-Acetylglucosamine in the Biosynthesis of Phytohemagglutinin: Attachment in the Golgi Apparatus and Removal in Protein Bodies

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ABSTRACT Cotyledons of the common bean (*Phaseolus vulgaris* L.) synthesize large amounts of the lectin phytohemagglutinin (PHA) during seed development. The polypeptides of PHA are synthesized by endoplasmic reticulum-bound polysomes and co-translationally glycosylated, pass through the Golgi complex, and accumulate in protein bodies, which constitute the lysosomal compartment in these cells. Some of the high-mannose sidechains of PHA are modified in the Golgi complex, and in mature PHA they contain *N*-acetylglucosamine, mannose, fucose, and xylose in the molar ratios 2, 3.8, 0.6, and 0.5. The results reported here show that the Golgi complex is also the site of additional *N*-acetylglucosamine incorporation into the modified sidechains. When developing cotyledons are labeled with [$^3$H]glucosamine and glycopeptides of PHA present in the Golgi complex isolated, the radioactivity can be released as [$^3$H]*N*-acetylglucosamine by digestion of the glycopeptides with $\beta$-*N*-acetylglucosaminidase, indicating that the residues are in a terminal position. Arrival of PHA in the protein bodies is followed by the slow removal of these terminal *N*-acetylglucosamine residues, resulting in a decrease in the $M_r$ of the modified sidechains. The biosynthetic intermediates of the glycoproteins destined for the lysosomal compartments of animal cells contain high-mannose sidechains modified by phosphate groups covered by *N*-acetylglucosamine that is labile to mild acid treatment. When cotyledons are labeled with [$^{32}$P]orthophosphate, there is no radioactivity in PHA obtained from any of the subcellular fractions. There is also no release of radioactivity when [$^3$H]glucosamine-labeled glycopeptides obtained from PHA in the Golgi complex are subjected to mild acid hydrolysis. These results indicate that the sorting-signals and posttranslational processing steps for proteins that are transported to the lysosomal compartment are different in plant cells and animal cells.

In the storage parenchyma cells of developing legume cotyledons the lysosomal compartment consists of numerous protein bodies or protein storage vacuoles with a dual function: they contain the acid hydrolases of the cell (1–3), and store reserve proteins until these proteins are hydrolyzed during seed germination (4–6). Since half the protein in these cells is contained in the protein bodies, developing cotyledons constitute an excellent system to study the transport of proteins to the lysosomal compartment of plant cells. Protein bodies are organelles measuring 2–5 $\mu$m in diameter that have an electron-dense protein matrix surrounded by a limiting membrane. They originate from the subdivision of the central vacuole as storage protein accumulates during the development of the cotyledons. Besides the acid hydrolases and the storage protein, the protein bodies also contain the lectins that occur abundantly in many legume seeds (7–9). In the common bean, *Phaseolus vulgaris*, the protein bodies contain two abundant proteins: the storage protein phaseolin, which is a trimeric protein with subunits $M_r$ ~50,000; and the lectin phytohemagglutinin, which is a tetrameric protein with subunits $M_r$ ~35,000 (5). We are studying the biosynthesis, posttranslational modifications and transport of these proteins, to understand how plant cells regulate protein transport to the lysosomal compartment.

Phytohemagglutinin (PHA)[1] is a glycoprotein, and in the

[1] *Abbreviations used in this paper:* endo H, endo-$\beta$-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; Fuc, fucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; PHA, phytohemagglutinin.

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cultivar, Greensleeves, its carbohydrate moiety consists of two different classes of oligosaccharide sidechains: one is of the high-mannose type containing two *N*-acetylglucosamine residues and eight or nine mannose residues and one is of the modified type with *N*-acetylglucosamine (GlcNAc), mannose (Man), fucose (Fuc), and xylose in the molar ratios of 2.0:3.8:0.6:0.5 (10). As with other lysosomal proteins (11), the biosynthesis of PHA occurs on membrane-bound polysomes, and two high-mannose chains are co-translationally attached in the endoplasmic reticulum (ER) to each polypeptide. Transport of the glycosylated polypeptides to the protein bodies is mediated by the Golgi complex where one of the high-mannose sidechains on each polypeptide is modified and incorporates fucose (12). Fucosylated PHA is first associated with the Golgi cisternae and moves from there to small, dense vesicles before it is transported to the protein bodies (13).

The best understood recognition signal for transport of proteins to the lysosomes of animal cells involves mannose-6-phosphate groups on the oligosaccharide sidechains of the lysosomal enzymes (14). Such groups are synthesized in the Golgi complex by the modification of high-mannose oligosaccharide sidechains. An intermediate in the synthesis consists of a GlcNAc residue linked to a mannosyl residue via an acid labile phosphodiester linkage (15). In this paper we present evidence that the fucose-containing sidechains of PHA are modified in the Golgi complex by the incorporation of terminal GlcNAc residues. These residues are not attached via acid labile phosphodiester linkages and can be removed in vitro by digestion with β-*N*-acetylglucosaminidase. In situ they are slowly removed after PHA arrives in the protein bodies. This processing takes 12 to 24 h to complete.

## MATERIALS AND METHODS

*Materials:* Plants of *P. vulgaris* L. cv. Greensleeves (Burpee Seed Co., Riverside, CA) were grown in a greenhouse. Experiments were carried out with cotyledons weighing 140–200 mg, when the accumulation of PHA is rapid. Organic chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise, and radiochemicals from Amersham Co. (Arlington Heights, IL).

*Radioactive Labeling:* Radioactive labeling was carried out with excised cotyledons as previously described (16). The following precursors were used: L-[5,6-$^3$H]fucose (25.6 Ci/mmol), D-[6-$^3$H]glucosamine hydrochloride (24.8 Ci/mmol), D-[2-$^3$H]mannose (15.8 Ci/mmol), $^{14}$C-amino acids, and carrier free [$^{32}$P]orthophosphate. Cotyledons were labeled at 20°C, with 10 μCi of precursor each, or 1 mCi when [$^{32}$P]orthophosphate was used. The precursor was added to a nutrient medium containing sucrose, asparagine, and the major mineral nutrients (17).

*Homogenization and Isolation of Organelles:* The labeled tissue was collected by cutting a thin slice from the cotyledon with a razor blade. The remainder of the cotyledon was discarded. The tissue was homogenized in 100 mM Tris-Cl, pH 7.8, containing either 1 mM EDTA (medium A) or 2 mM $MgCl_2$ (medium B) and 12% (wt/wt) sucrose. This homogenization procedure disrupts the large fragile protein bodies and protein body proteins are admixed with cytosolic proteins. The cell walls and debris were removed by centrifugation at 1,000 *g* for 5 min and the supernatants used for the isolation of subcellular fractions. The supernatants were loaded on linear 16–54% (wt/wt) sucrose gradients prepared either in medium A or B, and the gradients centrifuged at 150,000 *g* for 2 h. The gradients containing EDTA were used for the isolation of the protein body contents (soluble fraction) and those dense vesicles that banded at 1.22 g·cm$^{-3}$. The gradients containing $MgCl_2$ were used for the isolation of Golgi complex that banded at 1.13 g·cm$^{-3}$ and of ER that banded at 1.18 to 1.19 g·cm$^{-3}$ (14). The bands were recovered by hand with a Pasteur pipette.

For the experiment described in Fig. 5, the cleared homogenate was first passed through a Sepharose 4B column to separate the organelles from the soluble fraction (18).

*Extraction of PHA:* The affinity procedure of Felsted et al. (19) was used to isolate PHA. Porcine thyroglobulin was linked to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and the affinity gel used in 0.5-ml portions in small plastic columns. The gel was washed extensively with PBS (0.15 M NaCl in 10 mM K-phosphate, pH 7.4), then with PBS containing 1 M NaCl. PHA was eluted with 3 ml of 100 mM glycine-HCl, pH 3.0, containing 0.5 M NaCl. The solution was dialyzed for 3 h at 4°C against water and lyophilized. SDS PAGE was performed as described (5); fluorographs were made using XAR-5 film (Eastman Kodak, Rochester, NY).

*Isolation of PHA Glycopeptides and Gel Filtration Chromatography:* Affinity-purified lyophylized PHA was resuspended in 500 μl of 50 mM Tris-Cl, pH 8.5, and 1 mg of proteinase K (Merck Chemical Div., Merck & Co., Rathway, NJ) was added; 1 drop of toluene was added and the solution incubated at 37°C. After 24 h, 1 mg of pronase (CB grade [Calbiochem-Behring Corp., San Diego, CA]) was added and incubation carried out for an additional 24 h. The reaction was stopped by adding 15 μl of glacial acetic acid and the solution was loaded on a column (1.5 × 20 cm) of Bio-Gel P-4 (minus 400 mesh) (Bio-Rad Laboratories, Richmond, CA) equilibrated with 0.1 N acetic acid, pH 3.5, to separate the glycopeptides from salt, amino acids, and small peptides. Chromatography was performed at room temperature. Fractions (1.0 ml) were collected and 100 μl counted for radioactivity. The radioactive peak, containing the mixture of the glycopeptides, was lyophylized, and either used for further enzymatic treatment with glycosidases or analyzed by gel filtration on a column (1.0 × 100 cm) of Bio-Gel P-4 (minus 400 mesh) in the same conditions as the previous one.

*Hydrolysis of the Glycopeptides by Glycosidases:* Endo-β-*N*-acetylglucosaminidase H (endo H) (from *Streptomyces plicatus* [Miles Laboratories Inc. Elkhart, IN] digestion of the glycopeptides was performed by incubation at 37°C for 24 h in 500 μl of 100 mM Na-acetate, pH 5.8, with 10 mU of enzyme or without enzyme as a control. Digestion with α-mannosidase (from jack bean [Sigma Chemical Co., St. Louis, MO] was performed by incubation at 37°C for 24 h in 500 μl of 50 mM Na-citrate, pH 4.6, 5 mM $ZnSO_4$ with 4 U of enzyme or without enzyme as a control. Digestion with β-*N*-acetylglucosaminidase (from jack bean) was performed by incubation at 37°C for 48 h in 500 μl of 50 mM Na-citrate, pH 5.0, with 5 U of enzyme or without enzyme as a control. α-Mannosidase and β-*N*-acetylglucosaminidase were dialyzed overnight against 10 mM phosphate buffer, pH 7.2, at 4°C before use. A drop of toluene was added at the start of the enzymatic digestions. For mild acid hydrolysis the glycopeptides were dissolved in 500 μl of 10 mM HCl and heated to 100°C for 10 min in a sealed vial.

## RESULTS

Our previous work (10) showed that when mature PHA obtained from the protein bodies of the cotyledons is exhaustively digested with proteolytic enzymes, two classes of glycopeptides result, which can be separated on a 100 cm Bio-Gel P-4 column. By thin-layer chromatography and carbohydrate analysis we showed that the slower moving glycopeptide class, termed $GP_1$, has high-mannose oligosaccharide sidechain containing two GlcNAc residues and eight or nine Man residues, while the slightly faster moving glycopeptide class, termed $GP_2$, has modified oligosaccharide sidechains containing GlcNAc, Man, Fuc, and xylose in the molar ratios 2.0:3.8:0.6:0.5. Proteolytic digestion of radioactive PHA present in the protein bodies of developing cotyledons labeled for 24 h with [$^3$H]glucosamine ([$^3$H]GlcN), [$^3$H]Man, or [$^3$H]-Fuc, produced two glycopeptide classes with the same characteristics of $GP_1$ and $GP_2$. The elution pattern of these glycopeptides, when [$^3$H]GlcN is used as a label, is shown at the bottom of Fig. 1 and is labeled "soluble, 24 h." Our previous work (20) had shown that the oligosaccharides of PHA undergo a slow processing step that results in a slight reduction of the $M_r$ of the polypeptides. This processing step takes 12 to 24 h to come to completion. To understand the processing steps which the oligosaccharide sidechains undergo in the course of the transport of PHA, we labeled cotyledons of developing beans for 3 h with [$^3$H]GlcN and the homogenates fractionated by means by isopycnic sucrose gradients into ER, Golgi complex, dense vesicles, and soluble fraction. The dense vesicles are the organelles that mediate the transport of PHA from the Golgi complex to the protein bodies

(13). The soluble fraction contains the contents of the protein bodies, most of which break during the homogenization. PHA was isolated from each fraction, digested with proteinase K and pronase, and the labeled glycopeptides analyzed by gel filtration (Fig. 1). The glycopeptides isolated from the PHA present in the ER eluted from the column as a single peak. Glycosylation of PHA occurs cotranslationally in the ER and is a dolichol-mediated process consisting of the addition of two high-mannose sidechains (20). The glycopeptides from the PHA present in the other organelles eluted as two partially overlapping but distinct peaks. In the Golgi complex fraction, the peak with a slower mobility eluted in the same position as the single peak from the ER, while a new peak appeared with a larger apparent molecular mass. The elution profile of the PHA glycopeptides did not change during transport of the protein from the Golgi complex to the protein bodies (compare Golgi, vesicles, and *soluble, 3 h* in Fig. 1); however, after many hours in the protein bodies, the PHA sidechains acquired their definitive size (compare *soluble, 3 h* and *soluble, 24 h* in Fig. 1).

To identify the precursors of $GP_1$ and $GP_2$, we analyzed the glycopeptides of [$^3$H]Fuc-labeled PHA, isolated from the Golgi complex or from the soluble fraction after labeling for 24 h (Fig. 2). The arrows in Fig. 2 indicate the positions of the two peaks obtained when [$^3$H]glucosamine was used as a label (see Fig. 1). The results show that only the larger glycopeptide of the Golgi complex fraction contains [$^3$H]Fuc, and is therefore the precursor of $GP_2$. When [$^3$H]Fuc-labeled PHA was isolated from the dense vesicles or the soluble fraction after labeling for 3 h, the elution profile of its glycopeptides was the same as for the Golgi complex ones (not shown). The results shown in Figs. 1 and 2 indicate that modifications in the Golgi complex, such as the incorporation of Fuc (12), result in the formation of a larger precursor glycopeptide ($pGP_2$) of $GP_2$, which is transformed into $GP_2$ by processing events taking place in the protein bodies. The precursor of $GP_1$ ($pGP_1$) is not substantially modified during the transport of PHA from the ER to the protein bodies but there may be a slight decrease in its molecular weight after it reaches the protein bodies to give rise to the mature $GP_1$.

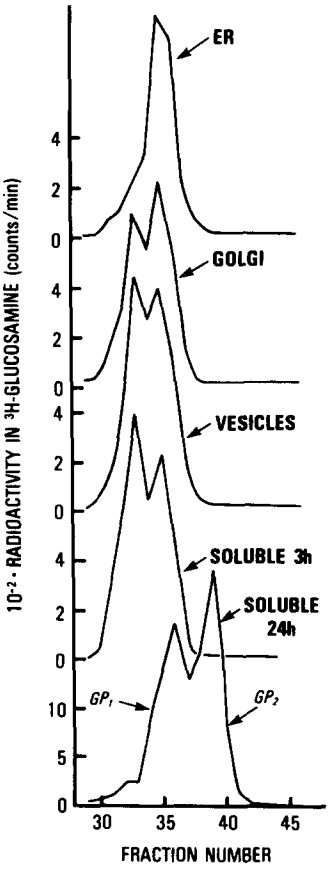


FIGURE 1 Gel filtration profile of [$^3$H]GlcN-labeled glycopeptides obtained by the proteolytic digestion of PHA. Cotyledons were labeled for 2 h (*ER, Golgi,* and *vesicles*), 3 h (*soluble*), or 24 h (*soluble*) and the various subcellular fractions isolated. PHA was purified by affinity chromatography, digested with proteinase K and pronase, and the digest fractionated on a 100 cm Bio-Gel P-4 column. *Soluble* represents the contents of the protein bodies.

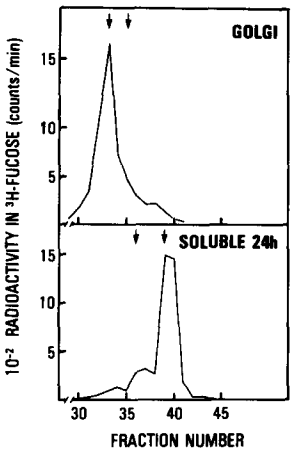


FIGURE 2 Gel filtration profile of [$^3$H]Fuc-labeled glycopeptides obtained by the proteolytic digestion of PHA. As in Fig. 1, except that cotyledons were labeled for 1 h (*Golgi*) or 24 h (*soluble*) with [$^3$H]fucose. Arrows indicate the positions of the glycopeptides of PHA isolated in the same subcellular compartments when [$^3$H]GlcN is used as a label (see Fig. 1). Note that the fucose-containing glycopeptide in the Golgi is the larger of the two, but in the soluble (24 h) it is the smaller of the two.

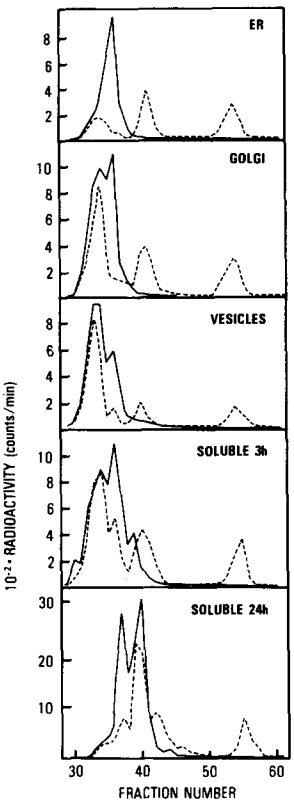


FIGURE 3 Effect of Endo H on the gel filtration profile of [$^3$H]GlcN-labeled glycopeptides of PHA. Cotyledons were labeled for 2 h (*ER, Golgi,* and *vesicles*), 3 or 24 h (*soluble*) and the subcellular fractions isolated. Affinity-purified PHA from each fraction was subjected to exhaustive proteolytic digestion and the glycopeptides purified on a short Bio-Gel P4 column. The glycopeptide mixture was digested with endo H for 24 h and the products fractionated on a long (100 cm) Bio-Gel P4 column. The results show that the endo H resistant peak is first the larger of the two (*Golgi, vesicles,* and *soluble 3 h*) and later the smaller of the two (*soluble 24 h*). (——) Control. (- - -) Treated.

## *Resistance of $GP_2$ to Endo H Is Acquired in the Golgi Complex*

Experiments with endo H showed that $GP_1$ is susceptible to this enzyme while $GP_2$ is resistant (10). The experiment illustrated in Fig. 1 was repeated, but the glycopeptides were digested with endo H to find out at which stage along the transport/modification pathway resistance to endo H was acquired. The results (Fig. 3) clearly show that in the ER the oligosaccharide sidechains are all susceptible to endo H, but

in the Golgi complex, $pGP_2$, the faster moving peak, is resistant. This glycopeptide remains resistant to endo H throughout its further transport to the protein bodies and resistance is maintained in mature $GP_2$ (soluble, 24 h, slower moving peak). Hydrolysis of the endo-H-susceptible glycopeptide (soluble 24 h, faster moving peak in the control) results in the formation of two new labeled peaks containing equal amounts of radioactivity, consistant with the observation that this enzyme splits the *N,N'*diacetylchitobiose units of high-mannose oligosaccharides. $GP_1$ is always susceptible to endo H digestion: in the ER, in the Golgi as $pGP_1$, and in the protein bodies as mature $GP_1$.

Treatment of the glycopeptides with α-mannosidase was used to find out whether both precursors contain terminal mannosyl residues. The glycopeptides were prepared from [$^3$H]GlcN-labeled PHA and analyzed by gel filtration: their change in position after treatment with α-mannosidase was used as a measure of their susceptibility or resistance to the enzyme (Fig. 4). When the glycopeptides were prepared from PHA present in the ER, a change in the position of the peak of radioactivity from fractions 34–35 to fractions 45–46 was observed after treatment with the enzyme (Fig. 4, ER). When the same experiment was performed on [$^3$H]Man-labeled glycopeptides 12% of the total counts eluted after treatment at position 45–46 and 88% at a position co-migrating with free mannose (not shown). The two experiments together show that the glycopeptides of the PHA present in the ER are completely susceptible to α-mannosidase, which removes the eight α-linked Man residues, leaving only the single mannosyl residue β-linked to the *N,N'*-diacetylchitobiose moiety, which is in turn attached to asparagine with flanking amino acids. However, when the glycopeptides of PHA obtained from the Golgi complex were treated with α-mannosidase, $pGP_2$ was found to be totally resistant to the enzyme, while $pGP_1$ was entirely susceptible (Fig. 4, Golgi). A similar result was found when we examined the soluble (protein body) fraction of the homogenate after 3 h of labeling, but the pattern was more complex, with the appearance of a new peak around fraction 37–38 (Fig. 4, soluble 3 h). Treatment of the mature glycopeptides with α-mannosidase (Fig. 4, soluble 24 h) showed that they were both susceptible to α-mannosidase and that two new large peaks appeared: the peak at fractions 45–46 is derived from $GP_1$ and the peak at fraction 39 is derived from $GP_2$ (data not shown, but see ref. 10). Together these results indicate that the modification which $pGP_2$ undergoes in the Golgi complex renders it not only completely resistant to endo H, but also to α-mannosidase. However, the change that $pGP_2$ undergoes in the protein-bodies renders it again susceptible to α-mannosidase, even though only a few mannose residues can be removed. The composition of mature $GP_2$ is GlcNAc:Man:Fuc:xylose in the molar ratios 2.0:3.8:0.6:0.5, and only half of its mannosyl residues are susceptible to α-mannosidase (10).

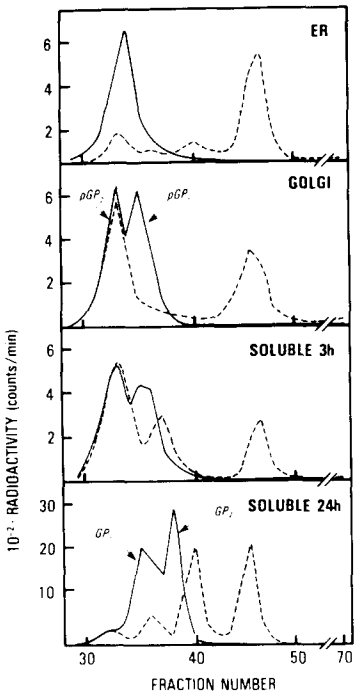


FIGURE 4 Effect of α-mannosidase on the gel filtration profile of [$^3$H]GlcN-labeled glycopeptides. Details as in Fig. 3, except that the glycopeptide mixture was treated with α-mannosidase for 24 h. Note the $pGP_2$ is totally resistant to α-mannosidase degradation. (——) Control. (- - -) Treated.

## *Terminal GlcNAc Residues Are Added in the Golgi Complex*

A number of experiments were carried out to find out which molecular groups that blocked the action of α-mannosidase on $pGP_2$ may have been added to PHA in the Golgi complex. By carrying out double-labeling experiments with [$^3$H]GlcN and $^{14}$C-amino acids we found that additional GlcNAc residues are added to PHA in the Golgi complex. Cotyledons were labeled for 2 h simultaneously with [$^3$H]-GlcN and $^{14}$C-amino acids and the radioactive tissue homogenized in medium A (containing 1 mM EDTA) or medium B (containing 2 mM $MgCl_2$). The organelles were separated from the soluble molecules with Sepharose 4B columns eluted with the same media, and then fractionated on 16–54% (wt/wt) isopycnic sucrose gradients. The ER and Golgi complex were located by their marker enzymes NADH-cytochrome *c* reductase and inosinediphosphatase, respectively (13). The positions of the marker-enzymes are shown as horizontal bars in Fig. 5, with a solid bar for inosine-diphosphatase and a dashed bar for NADH-cytochrome *c* reductase. Actual enzyme distributions have been shown elsewhere (13). The Golgi complex banded at a density of 1.13 $g \cdot cm^{-3}$ in both types of gradients, while the ER banded at 1.14 $g \cdot cm^{-3}$ in the EDTA-containing medium and at 1.18 $g \cdot cm^{-3}$ in the $MgCl_2$-containing medium. This difference in density is due to the removal of ribosomes by the EDTA. PHA was extracted from each gradient fraction with affinity gel, and the radioactivity of $^3$H and $^{14}$C in PHA determined. The results (Fig. 5) show that $^{14}$C-amino-acid-labeled PHA is found primarily in the ER, as shown by the shift of the peak of $^{14}$C-amino acid labeled PHA with the marker enzyme NADH-cytochrome *c* reductase depending on the medium used for homogenization and gradients. [$^3$H]GlcN-labeled PHA does not follow exactly the same pattern. There are two additional peaks: one in the area of the Golgi complex as identified by inosinediphosphatase, and one in the area where the dense vesicles band (1.22 $g \cdot cm^{-3}$) (13). The results also show that PHA in the Golgi complex and in the vesicles has a $^3$H to $^{14}$C ratio two to three times higher than in the ER where the high-mannose oligosaccharides are added. This result indicates that additional

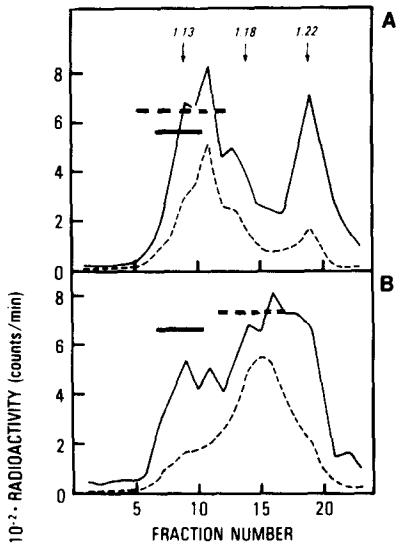


FIGURE 5 Fractionation with isopycnic sucrose gradients of the organelles containing PHA labeled with [$^3$H]GlcN and $^{14}$C-labeled amino acids. Cotyledons were labeled for 2 h with [$^3$H]glucosamine and $^{14}$C-labeled amino acids and homogenized in medium A (with EDTA) or medium B (with $MgCl_2$). The homogenate was fractionated in the same medium on Sepharose 4B to isolate the organelles. The organelles were fractionated on a 16–54% (wt/wt) sucrose gradient in 100 mM Tris, pH 7.8, containing either 1 mM EDTA or 2 mM $MgCl_2$. After centrifugation for 2 h at 150,000 *g*, gradient fractions were collected and analyzed for NADH-cytc reductase, inosine diphosphatase incorporation of $^{14}$C-labeled amino acids and [$^3$H] glucosamine in PHA. PHA was isolated with thyroglobulin-Sepharose from every gradient fraction. The distribution of NADH-cytochrome c reductase in the two gradients is indicated by the dashed horizontal bars and inosine diphosphatase by the solid horizontal bar. (*A*) medium with EDTA; (*B*) medium with $MgCl_2$. Note that at a density of 1.22 g·cm$^{-3}$ (vesicles) in *A* and at a density of 1.13 g·cm$^{-3}$ (Golgi) in *B* there is a high ratio of [$^3$H]glucosamine over $^{14}$C-amino acids. (– – –) Amino acids. (——) Glucosamine.

GlcN residues are being added as the PHA moves from the ER to the Golgi complex, and later into the vesicles.

## *Phosphate-linked GlcNAc?*

Incubation of fibroblasts with [$^{32}$P]orthophosphate results in the incorporation of radioativity in the newly-synthesized lysosomal hydrolases that contain GlcNAc residues linked to Man residues via mild acid labile phosphodiester linkages (21, 22). The synthesis of such groups occurs in the Golgi apparatus and involves a membrane-bound *N*-acetylglucosaminylphosphotransferase (23). We used two approaches to determine if similar linkages were present in PHA which had not yet reached the protein bodies and therefore contained the additional GlcNAc residues added in the Golgi complex. Cotyledons were incubated in the presence of [$^{32}$P]orthophosphate (1 mCi per cotyledon) for 2 h, and the organelle fraction (ER, Golgi complex, and dense vesicles) separated from the soluble fraction on a discontinuous sucrose gradient. The organelle and soluble fractions and PHA purified by affinity chromatography from these fractions were analyzed by SDS PAGE and fluorography. Many proteins were radioactive (i.e., phosphorylated), but there was no incorporation of radioactivity into PHA (Fig. 6, lanes *8* and *9*).

Using a different approach, we subjected GlcN-labeled glycopeptides of PHA to mild acid hydrolysis. Cotyledons were labeled with [$^3$H]glucosamine for 3 h and the homogenate fractionated into an organelle and soluble fraction as described above. Affinity-purified PHA from the organelle fraction was subjected to exhaustive proteolysis, and the glycopeptides isolated on a 20 cm Bio-Gel P4 column. The glycopeptides were subjected to mild acid hydrolysis and the products again chromatographed on a 20 cm Bio-Gel P4 column. The results showed that the mild acid hydrolysis removed <3% of the radioactivity, indicating the absence of phosphodiester-linked GlcNAc residues (data not shown).

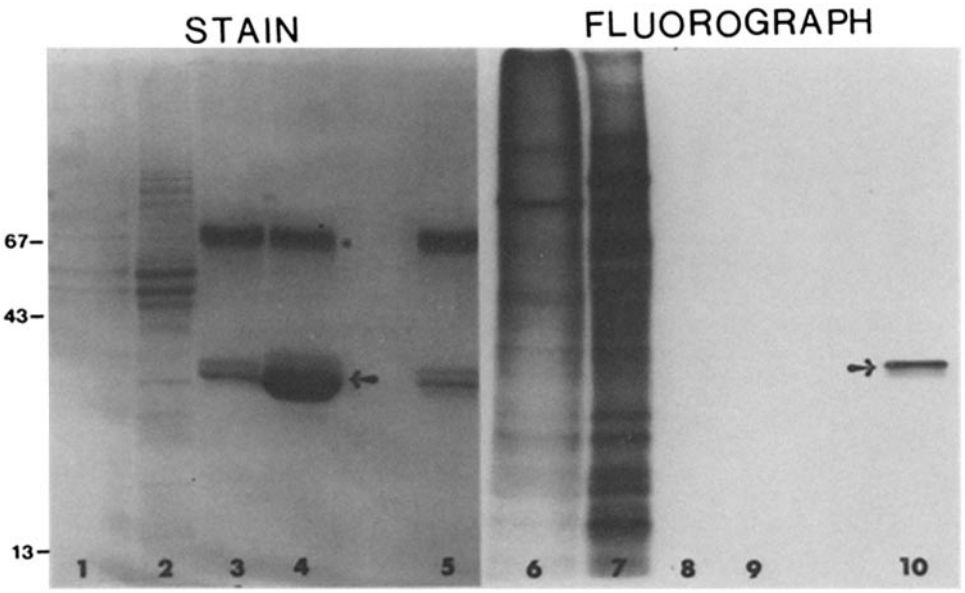


FIGURE 6 Incorporation of [$^{32}$P]orthophosphate. Cotyledons were incubated for 2 h and the membranous organelles separated from the soluble fraction. Total proteins and purified PHA were analyzed by SDS PAGE and fluorograph. Lanes *1* and *6*: membranous organelles, total proteins; lanes *2* and *7*: soluble fraction, total proteins; lanes *3* and *8*: PHA from membranous organelles; lanes *4* and *9*: PHA from soluble fractions; lanes *5* and *10*: $^{14}$C-labeled PHA from membranous organelles. Arrows indicate the position of PHA. The intense band (asterisk) in lanes *3–5* is carrier bovine serum albumin. The numbers on the left indicate the positions of molecular size markers bovine serum albumin (67,000), ovalbumin (43,000), and ribonuclease (13,000).

## $GP_2$ Is Formed by the Removal of the Terminal GlcNAc Residues from $pGP_2$

The finding that PHA receives additional glucosamine residues was confirmed by subjecting [$^3$H]GlcN-labeled glycopeptides to digestion with β-*N*-acetylglucosaminidase. Cotyledons were labeled with [$^3$H]GlcN and the homogenate used to isolate organelles (ER, Golgi complex, and vesicles) or soluble fraction. The glycopeptides were isolated from purified PHA, digested with β-*N*-acetylglucosaminidase, and the products chromatographed on a 100 cm Bio-Gel P4 column (Fig. 7). The results showed that the hydrolase removed very little [$^3$H]GlcN (or GlcNAc) from the glycopeptides obtained from PHA in the ER (Fig. 7, ER) or from mature PHA (Fig. 7, *soluble, 24 h*). However, when glycopeptides obtained from PHA in the Golgi, dense vesicles, or soluble fraction after a 3-h pulse, were digested with β-*N*-acetylglucosaminidase, $pGP_2$ disappeared almost completely, and a new peak appeared in the position of mature $GP_2$, together with a large peak that coeluted with free [$^3$H]GlcN. Analysis by paper chromatography of the radioactive material removed by β-*N*-acetylglucosaminidase showed that it co-chromatographed with GlcNAc, rather than with GlcN. To check if the new peak appearing in the same position of $GP_2$ after digestion of $pGP_2$ with β-*N*-acetylglucosaminidase was indeed the mature glycopeptide, we performed the following experiment. The

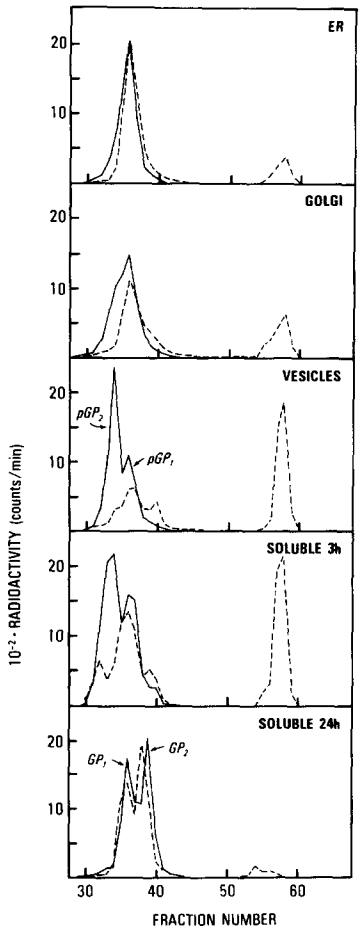


FIGURE 7 Effect of β-*N*-acetylglucosaminidase on the gel filtration profile of [$^3$H]GlcN-labeled glycopeptides. Details as in Fig 3, except that the glycopeptide mixture was treated with β-*N*-acetylglucosaminidase for 48 h. (——) Control. (- - -) Treated.

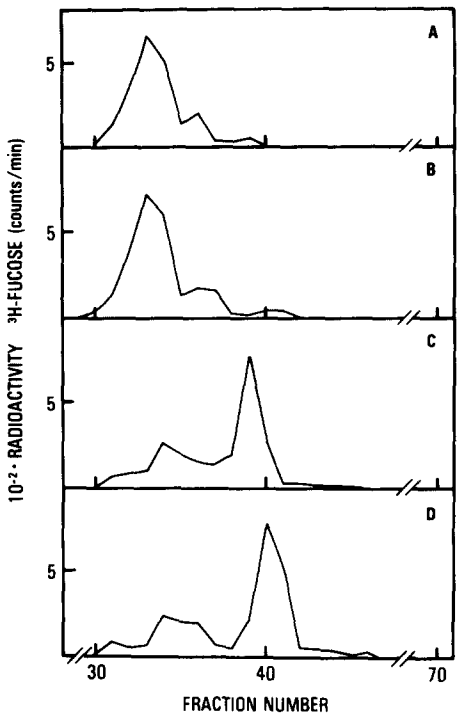


FIGURE 8 Effect of α-mannosidase and β-*N*-acetylglucosaminidase on the elution profile of [$^3$H]fucose-labeled glycopeptides. Cotyledons were labeled for 1 h with [$^3$H]fucose, and PHA isolated from the membranous organelles fraction. Exhaustive proteolytic digestion of PHA was followed by purification of the glycopeptides. Glycopeptides were then digested with β-*N*-acetylglucosaminidase and/or α-mannosidase and analyzed through a 1 m Bio-Gel P4 column. (*A*) Control, no digestion; (*B*) α-mannosidase; (*C*) β-*N*-acetylglucosaminidase; (*D*) β-*N*-acetylglucosaminidase followed by α-mannosidase.

membranous organelles were isolated from cotyledons labeled with [$^3$H]Fuc (in this way $pGP_1$ remains unlabeled) for 2 h, and PHA was purified. The glycopeptides were isolated and treated with α-mannosidase (Fig. 8*b*), with β-*N*-acetylglucosaminidase (Fig. 8*c*), or with β-*N*-acetylglucosaminidase followed by α-mannosidase (Fig. 8*d*), and analyzed by gel filtration. Treatment with α-mannosidase alone did not affect $pGP_2$, as expected, while the glycopeptide moved to a position corresponding to that of $GP_2$ when digestion was performed with β-*N*-acetylglucosaminidase. When the action of the two enzymes was combined the resulting glycopeptide appeared to be smaller than $GP_2$. The decrease in molecular weight is slight but the elution position corresponds exactly with that of mature $GP_2$ treated with α-mannosidase (see Fig. 4, *soluble 24 h*).

## DISCUSSION

PHA is a glycoprotein whose carbohydrate moiety consists of two different classes of oligosaccharide sidechains: one, termed $GP_1$, is of the high-mannose type, and a second one, termed $GP_2$, is of the modified type with GLcNAc, Man, Fuc, and xylose, in the molar ratios of 2.0:3.8:0.6:0.5 (10). Here we present evidence that the transport and accumulation of PHA in the protein bodies is accompanied by the transient attachment of terminal GlcNAc residues to the modified oligosaccharide chain. We have shown elsewhere (12, 13) that half the high-mannose sidechains of PHA are modified in the Golgi complex by the incorporation of fucose, and that the

arrival in the protein bodies is accompanied by a slow processing step resulting in a reduction in size of the fucose-containing oligosaccharides (20). This processing step takes 12–24 h to complete and for this reason we chose to compare the size and structure of glycopeptides after a 3-h pulse and a 24-h chase. Pulse-chase experiments with [$^3$H]fucose showed that all the PHA that accumulates in the protein bodies passes through the Golgi complex (13). The results presented here show that $GP_2$ that is partially susceptible to digestion by α-mannosidase (10) is present in the Golgi complex as a precursor ($pGP_2$) which is larger than $GP_2$ and resistant to α-mannosidase. Treatment of $pGP_2$ with β-*N*-acetylglucosamidase renders it susceptible to α-mannosidase digestion (see Fig. 8) indicating the presence of terminal GlcNAc residues. Treatment with α-mannosidase brings about but a small change in the elution position on Bio-Gel P4, similar to that obtained when mature $GP_2$ is treated with this enzyme. Since mature $GP_2$ contains only four mannosyl residues, half of which can be removed by α-mannosidase (10), we conclude that part of the high-mannose oligosaccharides that were attached to PHA in the ER, specifically the ones destined to become $GP_2$, were trimmed in the Golgi apparatus by the removal of mannosyl residues. The presence in the Golgi complex of specific α-mannosidases that trim mannosyl residues from high-mannose oligosaccharides has been demonstrated in animal cells (for a review, see reference 14), but not yet in plant cells. The fact that $pGP_2$ can be converted in vitro to a glycopeptide with the same elution position as mature $GP_2$ by the removal of terminal GlcNAc residues, indicates that the Golgi complex is probably also the site of xylose incorporation into the processed sidechains of PHA. The processing steps that the modified oligosaccharides of PHA undergo are summarized in Fig. 9.

A glycosyltransferase that transfers GlcNAc residues from UDP GlcNAc to mannosyl residues of PHA and phaseolin has been shown to be associated with the membranous organelles of *P. vulgaris* cotyledons (24). When membranous organelle fractions were incubated with labeled UDP-GlcNAc it was found that most of the GlcNAc that became incorporated into phaseolin and PHA was terminal GlcNAc. This observation was hard to explain since mature PHA and phaseolin, the two major glycoproteins present in the membranous fractions, do not contain terminal GlcNAc in their mature form. Our finding that the terminal GlcNAc residues added to PHA in the Golgi complex are removed again in the protein bodies, helps to explain these previous results. Recent experiments indicate that terminal GlcNAc residues may be present on another protein body protein, jack bean α-mannosidase. Denatured α-mannosidase binds to concanavalin A indicating that it has mannosylated oligosaccharides. This binding is abolished if the denatured protein is incubated with α-mannosidase (native) and β-*N*-acetylglucosaminidase, but not when it is incubated with α-mannosidase alone (25).

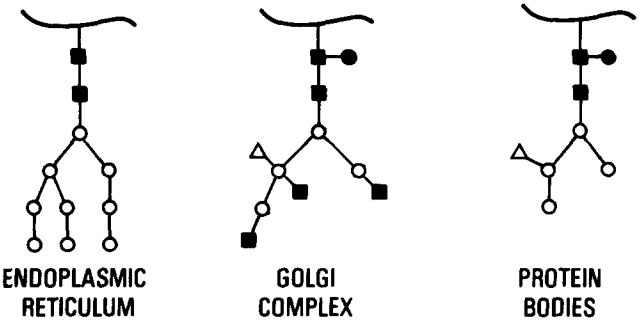


FIGURE 9 Proposed scheme for the processing of the modified asparagine-linked oligosaccharides of PHA. There is as yet no evidence concerning the exact site of attachment of the fucose and xylose residues. (■) GlcNAc; (○) mannose; (●) fucose; (△) xylose.

Calculations based on the results shown in Fig. 5 show that the [$^3$H]GlcNAc to $^{14}$C-amino acid ratio of PHA was 1.83 in the ER, 2.80 in the Golgi complex (contaminated with ER) and 5.09 in the dense vesicles. Assuming that the high-mannose and the modified oligosaccharide sidechain are present in nearly equal amounts in the mature protein (10) there are seven to eight residues of terminal GlcNAc for every two residues of GlcNAc in the *N,N'*-diacetylchitobiose stem of $pGP_2$. This surprisingly high ratio was confirmed by the experiments in which β-*N*-acetylglucosaminidase was used to digest the precursors in the vesicles (Fig. 6*c*). Of the total radioactivity, 55% was released by the enzyme treatment, but the elution pattern indicates that the digestion probably did not go to completion. If we assume that 60–65% release would have been the end of the reaction, there were eight terminal GlcNAc residues for each *N,N'*-diacetylchitobiose unit in $pGP_2$. Thus $pGP_2$, with respect to the high-mannose chain from which it originates, appears to have lost four to five mannosyl residues and to have acquired seven to eight new GlcNAc residues as well as a fucose and probably a xylose residue (However, see *Note Added in Proof.*)

Protein bodies constitute the lysosomal compartment of the storage parenchyma cells of the cotyledons. They contain numerous acid hydrolases, and participate in the autophagic digestion of cytoplasmic components (3, 26). The protein bodies in mung bean cotyledons contain β-*N*-acetylglucosaminidase (27), and this enzyme has also been found in protein bodies of *P. vulgaris* cotyledons (W. Van der Wilden and M. J. Chrispeels, unpublished results). We therefore assume that the enzyme in the protein bodies is responsible for the removal of the terminal GlcNAc residues in vivo, in the same way that they are removed by the jack bean enzyme in vitro. We observed that the arrival of PHA in the protein bodies is followed by a gradual decrease in the $M_r$ of the glycopolypeptides, and that this is due to a slow decrease in the size of the oligosaccharide sidechains. The data presented here indicate that the decrease in size is caused by the gradual removal of GlcNAc residues from the modified sidechains.

In animal cells the high-mannose oligosaccharide chains of the glycoproteins destined for the lysosomal compartment are modified in the Golgi complex by the incorporation of phosphoGlcNAc followed by the removal of the GlcNAc residue, leaving a mannose-6-phosphate group. This particular modification does not seem to occur in PHA. Cotyledons were incubated for 1 h with 1 mCi of [$^{32}$P]orthophosphate each, and newly-synthesized PHA purified from the membranous organelles (ER plus Golgi complex). However, there was no incorporation of radioactivity in PHA. Treatment of [$^3$H]-GlcNAc-labeled $pGP_2$ with 0.1 N HCl for 15 min at 100°C also did not remove any [$^3$H]GlcNAc indicating that the GlcNAc was not linked via an acid-labile phosphodiester linkage involving the first carbon of the aminosugar. There is as yet no information on the sorting signals of plant proteins and, except for the existence of signal peptides on secreted proteins and protein body proteins, no sorting signals have been identified. The proteins that accumulate in protein bodies include some which have no covalently attached car-

bohydrate such as legumin in *Pisum sativum* (28), some which have only high-mannose oligosaccharide sidechains such as phaseolin (29), and some which have modified carbohydrate sidechains such as PHA and other lectins. The experiments reported here indicate that the sorting signals for the glycoproteins whose oligosaccharide sidechains are modified in the Golgi complex are different from the mannose-6-phosphate signal recently identified for the lysosomal hydrolases of animal cells. Whether the transient attachment of GlcNAc residues plays a role in the transport of the protein or is a recognition signal remains to be determined.

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*Note Added in Proof:* During discussion of these results, Dr. Stuart Kornfeld pointed out that the specific radioactivity of the terminal GlcNAc is likely to be much higher than that of the GlcNAc in the chitobiose portion of the oligosaccharides. When the cotyledons are labeled for only 2–3 h, unlabeled PHA from the ER keeps feeding into the Golgi, where it picks up radioactive GlcNAc. This will lead to an overestimation of the amount of peripheral GlcNAc. Since $pGP_2$ is totally resistant to $\alpha$-mannosidase, the number of peripheral GlcNAc residues may be only two, or perhaps only one, depending on the structure of $GP_2$.

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