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BROWDER, STEVEN KIRK
A STUDY OF SELECTED ASPECTS OF GLYCOPROTEIN STRUCTURE AND BIOSYNTHESIS IN DEVELOPING COTYLEDONS OF PISUM SATIVUM L.

THE UNIVERSITY OF OKLAHOMA, PH.D., 1978

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GRADUATE COLLEGE

A STUDY OF SELECTED ASPECTS OF GLYCOPROTEIN STRUCTURE AND BIOSYNTHESIS IN DEVELOPING COTYLEDONS OF *PISUM SATIVUM* L.

A DISSERTATION

SUBMITTED TO THE GRADUATE COLLEGE

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

By

STEVE K. BROWDER

Norman, Oklahoma

1978

A STUDY OF SELECTED ASPECTS OF GLYCOPROTEIN STRUCTURE AND BIOSYNTHESIS IN DEVELOPING COTYLEDONS OF *PISUM SATIVUM* L.

APPROVED BY

DISSERTATION COMMITTEE

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A STUDY OF SELECTED ASPECTS OF GLYCOPROTEIN STRUCTURE AND BIOSYNTHESIS IN DEVELOPING COTYLEDONS OF *PISUM SATIVUM* L.

BY: STEVE K. BROWDER

MAJOR PROFESSOR: LEONARD BEEVERS

Legumin and vicilin, the major reserve storage proteins in cotyledons of peas, are glycoproteins. A glycopeptide linkage involving N-acetylglucosamine to peptidyl asparagine has been isolated from legumin labeled by exogenously supplied [^{14}C] glucosamine.

The glycoproteins in developing pea cotyledons have been demonstrated to be assembled by lipid intermediate glycosyl carriers *in vitro* particulate preparations. The incorporation of [^{14}C] glucosamine and [^{14}C] mannose *in vivo* into intact pea cotyledons has been observed and the identity of the glycolipids and glycoproteins labeled has been determined. The nature of the acidic lipids formed *in vivo* has been compared to the lipid intermediates formed *in vitro*.

The intracellular location of enzymes responsible for the glycosylation of lipid intermediates and glycoproteins in pea cotyledons has been previously determined. The distribution of these glycosyl transferases, which are differentially responsive to the divalent cations Mg^{2+} and Mn^{2+}, has been determined in developing pea cotyledons 15 and 29 days post anthesis to assess whether the distribution of these enzymes differs from that reported in cotyledons 21 days post anthesis.

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A STUDY OF SELECTED ASPECTS OF GLYCOPROTEIN STRUCTURE AND BIOSYNTHESIS IN DEVELOPING COTYLEDONS OF PISUM SATIVUM L.

CHAPTER I

INTRODUCTION

Developing pea cotyledons contain glycoproteins with glucosamine and mannose as the principal sugar components. The purpose of this investigation was to determine the nature of the glycopeptide bond in the reserve storage glycoprotein legumin, to investigate the nature of glycosyl lipids formed in vivo from [^{14}C] glucosamine and [^{14}C] mannose, and to determine the subcellular distribution of glycosyl transferases during the development of pea cotyledons.

The results of this study are presented in three separate papers. Paper I was prepared according to the instructions for contributors to F.E.B.S. Letters. Papers II and III were prepared according to the instructions for contributors to Plant Physiology.

CHAPTER II

PAPER 1

CHARACTERIZATION OF THE GLYCOPEPTIDE BOND IN LEGUMIN FROM *PISUM SATIVUM* L.

INTRODUCTION

Legumin and vicilin, the reserve storage globulins in pea cotyledons, are glycoproteins containing neutral and amino sugars. The amino sugar has been characterized as glucosamine and the neutral sugars are principally mannose [1]. In native glycoproteins, glucosamine usually occurs as an acetylated derivative (N-acetyl glucosamine) and is frequently involved in glycopeptide linkages [2]. Several workers have demonstrated the involvement of acetylated amino sugars in glycopeptide linkages with the hydroxy amino acids serine or threonine or alternatively, with peptidyl asparagine [3]. However, only a few reports have considered the nature of the glycopeptide linkage in plant glucosamine containing glycoproteins [4-7].

To extend our studies concerning the biosynthesis of glucosamine containing glycoproteins [1,8-10], it is necessary to determine the nature of the glycopeptide linkage. Basha [1] demonstrated that exogenously supplied glucosamine is incorporated into legumin and vicillin in developing pea cotyledons. We now report that some of this exogenously supplied glucosamine may be isolated from legumin as a GlcNAc-Asn glycopeptide linkage.

MATERIALS AND METHODS

[D-U-^{14}C] glucosamine (254 μCi/mmole, 50 μCi/238 μl) was purchased from Amersham Searle Corporation, Arlington Heights, Illinois USA and 1-N-B-L-asparty1-2-deoxy-B-D-glucopyranosylamine (GlcNAc-Asn) was purchased from Bachem Chemicals, Marina Del Ray, Cal. Protease type VI was purchased from Sigma, St. Louis, Missouri USA, and Proteinase K was purchased from Beckman Inst., Co., Palo Alto, Calif.

Preparation of [^{14}C] glucosamine labeled legumin. Seventy cotyledons were collected from developing peas (18-21 days post anthesis) and injected with 1.5 μl of [^{14}C] glucosamine/cotyledon. Following a 4 hr incubation, legumin was isolated from the cotyledons as previously reported [10]. Recovered legumin (71.9 mg protein) was suspended in 20 mls of 0.2 M NaCl 100 mM sodium phosphate buffer pH 7.0 and incubated with 9.0 mg Protease Type VI at 37°C for 48 h. The sample was overlaid with a small amount of toluene to prevent bacterial growth. An additional 9 mg of Protease was added after 48 h and the incubation continued for an additional 48 h. After each protease addition the medium was readjusted to pH 7.0. Protease digestion was terminated by incubation in boiling water for 5 min and the undigested residue was centrifuged out. The supernatant from this initial digestion was evaporated to dryness in vacuo,

and suspended in 50 mM tris-HCl buffer pH 7.5 and redigested as described above. Redigestion, followed each time by fractionation of products on G-15, was repeated a total of 5 times. Digestion 2 was completed in 10 ml buffer, digestions 3 and 4 in 5.0 ml buffer and digestion 5 was carried out in 3.0 ml buffer at 47°C for 72 h with a total of 6.0 mg Proteinase K, half of which was added after 24 h of incubation.

Alkali digestion. [^{14}C] labeled glycopeptides, isolated after 2 proteolytic digestions, were dissolved in either 1 ml of 0.1 N NaOH or H_2O and incubated at 37°C for 72 h. Additional samples were dissolved in 1 ml of 1.0 N NaOH or H_2O and incubated for 6 h at 100°C. After incubation, the samples were adjusted to pH 7.0 with 2.0 N HCl and the products analyzed by paper chromatography in solvent system A.

Acid hydrolysis. [^{14}C] glycopeptides, isolated after 5 proteolytic digestions, were dissolved in 2.0 ml of 2.0 N HCl and placed in boiling water for 20 min. Following incubation, the mixture was then diluted to 8.0 ml with H_2O and passed through a column (1 X 25 cm) of Dowex X-8 (200-400 mesh acetate resin). The glycopeptides were washed from the column with H_2O. The effluent was evaporated *in vacuo* and the resulting residue was dissolved in H_2O and analyzed by paper chromatography in solvent systems A and B.

Chromatographic procedures. Samples were applied to Whatman No. 3 MM paper and chromatographed in solvent mixtures containing A) 1 butanol/pyridine/H_2O (40/30/40) B) 1-Butanol/acetic acid/H_2O (12/3/5).

Radioactive areas of chromatograms were determined by cutting the chromatograms into 1 X 4.5 cm strips and counting each of these strips in 4 ml of scintillation cocktail (8.0 gms PPO, 1 liter Triton X-100, 2 liters toluene) in a Beckman LS-100 scintillation counter. GlcN and GlcNAc-Asn were detected by spraying chromatograms with a solution of 0.2% (w/v) ninhydrin in acetone, followed by heating at 100°C for 5 minutes. GlcNAc was detected using Ehrlichs reagent as described by Basha [1].

Analytical methods. Carbohydrate was determined by the anthrone method [11] using D-mannose as a standard. Amino nitrogen was determined by the ninhydrin method [12] using L-leucine as a standard.

RESULTS AND DISCUSSION

The products of protease digestion of labeled legumin were fractionated by exclusion chromatography on Sephadex G-15. The distribution of radioactivity, carbohydrate, and amino nitrogen are shown in Figure 1. Over 95% of the radioactivity applied to the column was recovered in the pooled glycopeptide fractions. Since anthrone does not react with glucosamine or N-acetylglucosamine, the association of [^{14}C] glucosamine with anthrone positive material indicates that the label was incorporated into carbohydrate containing components. This agrees with the report that exogenously applied [^{14}C] glucosamine is incorporated into reserve glycoprotein [1]. The radioactivity associated with glycopeptides was distinctly separated from α amino nitrogen fractions, indicating that the [^{14}C] glucosamine did not undergo conversion to amino acids during the incubation period.

Paper chromatography of untreated [^{14}C] labeled glycopeptides indicated that radioactivity was associated with components showing little mobility in solvent system A. Mild alkali treatment had no major influence on the chromatographic mobility of the radioactivity. In contrast, strong alkali treatment of labeled glycopeptides released radioactivity which migrated to the center of the chromatogram. The heterodispersion of radioactivity in the chromatogram

ABSTRACT

Legumin and vicilin, the major reserve storage proteins in cotyledons of peas, are glycoproteins. A glycopeptide linkage involving N-acetylglucosamine to peptidyl asparagine has been isolated from legumin labeled by exogenously supplied [^{14}C] glucosamine.

The glycoproteins in developing pea cotyledons have been demonstrated to be assembled by lipid intermediate glycosyl carriers in *in vitro* particulate preparations. The incorporation of [^{14}C] glucosamine and [^{14}C] mannose *in vivo* into intact pea cotyledons has been observed and the identity of the glycolipids and glycoproteins labeled has been determined. The nature of the acidic lipids formed *in vivo* has been compared to the lipid intermediates formed *in vitro*.

The intracellular location of enzymes responsible for the glycosylation of lipid intermediates and glycoproteins in pea cotyledons has been previously determined. The distribution of these glycosyl transferases, which are differentially responsive to the divalent cations Mg^{2+} and Mn^{2+}, has been determined in developing pea cotyledons 15 and 29 days post anthesis to assess whether the distribution of these enzymes differs from that reported in cotyledons 21 days post anthesis.

implies that oligosaccharide components of varying degrees of complexity have been released from the glycopeptides [13].

Resistance of the [^{14}C] glycopeptides to mild alkali hydrolysis tends to preclude the involvement of serine or threonine in the glycopeptide linkage, while the susceptibility of the glycopeptide to strong alkali is consistent with the occurrence of a GlcNAc-Asn linkage [2]. However, the demonstration of a specific type of linkage cannot be based solely on B-elimination studies, but relies on the isolation of the putative linkage compound from the glycopeptide in question [14].

When the components produced by mild acid hydrolysis of the [^{14}C] labeled legumin glycopeptides were neutralized and subjected to paper chromatography in solvent System A (Fig. 2), the majority of radioactivity was localized in a peak which co-chromatographed with authentic GlcNAc-Asn. A second radioactive component co-chromatographed with glucosamine. A similar chromatographic profile was observed with acid hydrolysates of labeled legumin glycopeptides in solvent system B.

CONCLUSION

[^{14}C] Glucosamine is incorporated into glycoprotein in the developing pea cotyledon, and a portion of this glucosamine is incorporated into a GlcNAc-Asn glycopeptide linkage. The existence of this linkage compound was demonstrated by the results of B-elimination studies and isolation of the [^{14}C] GlcNAc-Asn linkage compound from mild acid hydrolysates of legumin glycopeptides.

ACKNOWLEDGEMENTS

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LEGENDS FOR FIGURES

Figure 1. Analysis of [^{14}C] legumin glycopeptides on Sephadex G-15. Legumin glycopeptides were digested five times and applied to a Sephadex G-15 column (1.5 X 105 cm), equilibrated and eluted with 0.1 M pyridine acetate pH 5.0. 4.0 ml fractions were collected and analyzed for radioactivity ●·●·● , carbohydrate, ■-■-■ and amino nitrogen o·o·o .

Figure 2. Paper chromatography of the products of mild acid hydrolysis of [^{14}C] legumin glycopeptides. Legumin glycopeptides were subjected to acid hydrolysis, as described in Materials and Methods, and the products chromatographed on Whatman 3 MM paper in solvent system A for 24 hrs. Radioactivity ●-●-● was determined and the distribution compared to the standard compounds, 1-N-B-L-asparty1-2-deoxy-B-D-glycopyranosyl-amine (GlcNAc-Asn), glucosamine (GlcN), and N-acetyl glucosamine (GlcNAc).

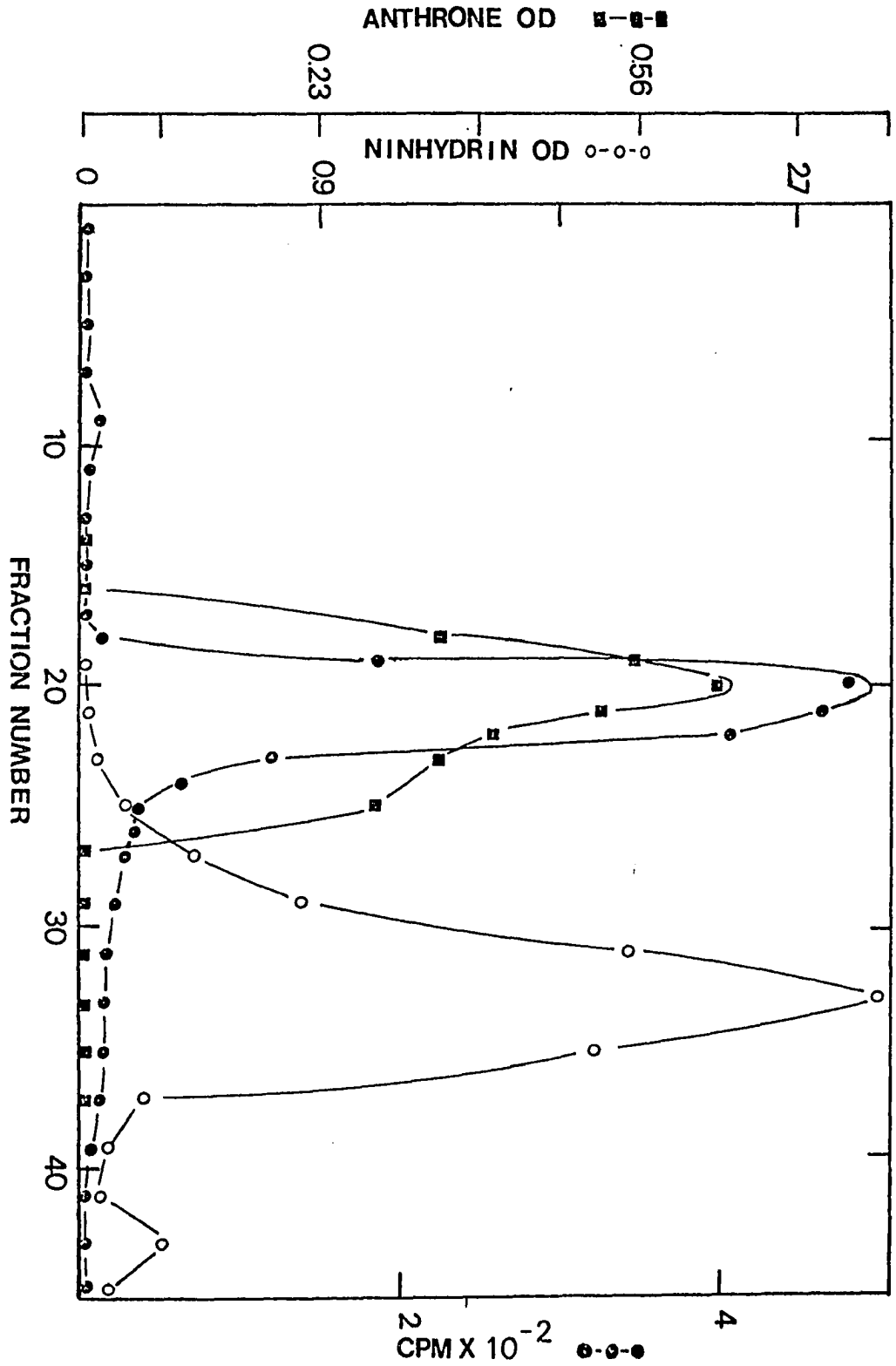
ANTHRONE OD
0.23
0.56
NINHYDRIN OD
0
0.9
27
10
20
30
40
FRACTION NUMBER
2
4
CPM X 10^-2

FIGURE 1

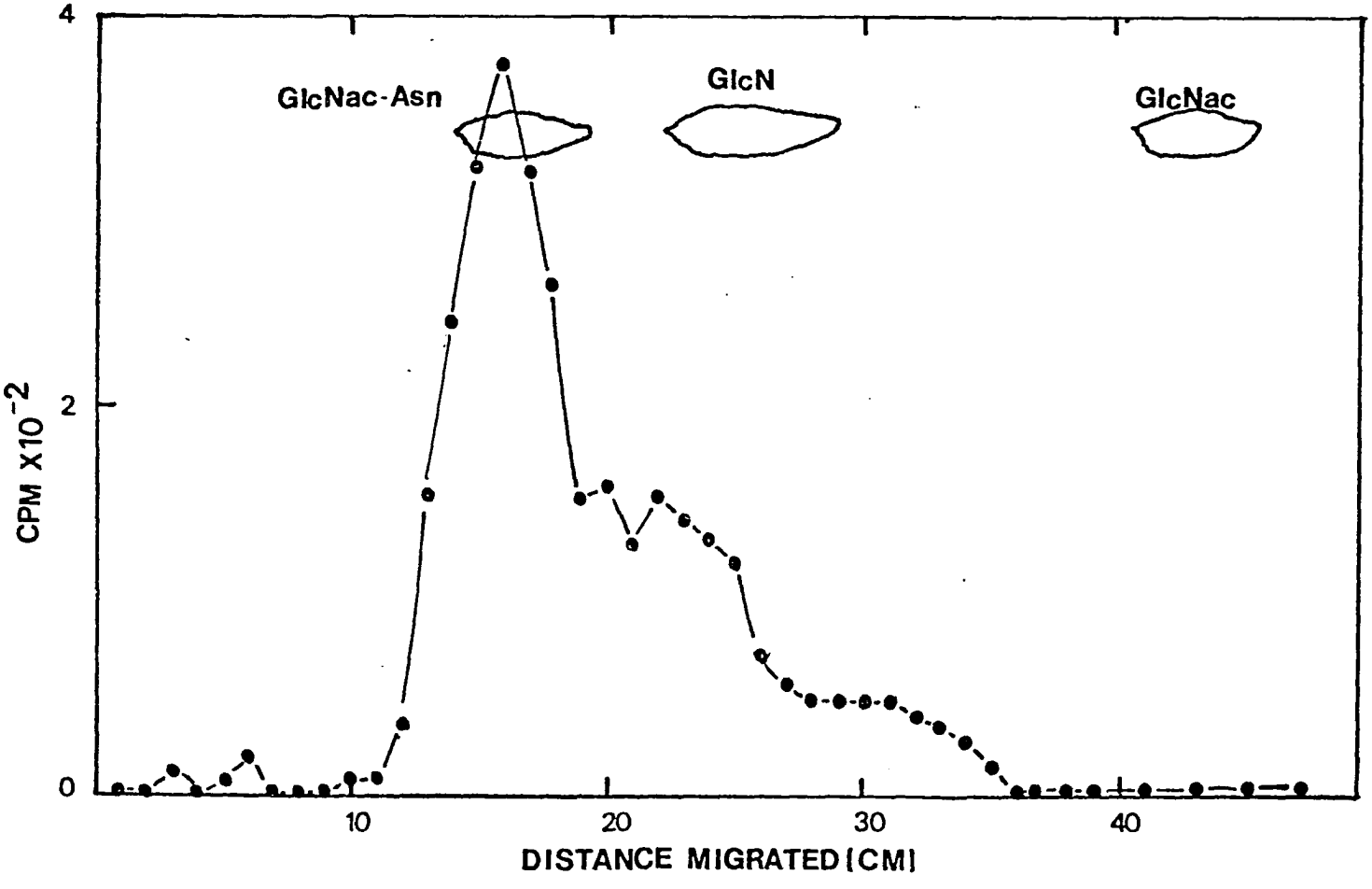
GlcNac-Asn
GlcN
GlcNac
CPM X10^{-2}
4
2
0
10
20
30
40
DISTANCE MIGRATED (CM)

FIGURE 2

CHAPTER III

PAPER II

THE *IN VIVO* INCORPORATION OF [^{14}C] GLUCOSAMINE AND [^{14}C] MANNOSE INTO GLYCOLIPIDS AND GLYCOPROTEINS IN COTYLEDONS OF *PISUM SATIVUM* L.

ABSTRACT

Developing pea cotyledons incorporate radioactivity *in vivo* from [^{14}C] glucosamine and [^{14}C] mannose into glycolipids and glycoproteins. Several different lipid components are labeled including neutral, ionic-nonacidic, and acidic lipids. The acidic lipids labeled *in vivo*, with the possible exception of the glucosamine lipid soluble in chloroform-methanol (2:1), appear to be identical to the polyisoprenoid lipid intermediates formed *in vitro* in pea cotyledons (Beevers, L., R. M. Mense, 1977, Plant Physiol. 60: 703-708). Each of the types of lipids present in the total cotyledonary extract can also be isolated from defined subcellular membrane fractions. Radioactivity from [^{14}C] glucosamine and [^{14}C] mannose is incorporated into glycoproteins whose molecular weight has been determined. Considerable redistribution of [^{14}C] mannose into other glycosyl components found in endogenous glycoproteins is observed. An N-acetyl glucosamine to asparagine glycopeptide linkage has been isolated from [^{14}C] glucosamine labeled glycoproteins.

INTRODUCTION

The major reserve storage proteins in pea cotyledons, legumin and vicilin, are glycoproteins containing glucosamine and mannose [4]. These sugars are both components of the "core" oligosaccharide characteristic of many animal, plant, and fungal glycoproteins [22,32]. These core oligosaccharides have the general structure $(\alpha\text{-man})_N$-B-man(1-4)-B-GlcNAc(1-4)-B-GlcNAc-Asparagine.

Studies with animal [22,32], plant [1,6,7,9,14-17,20], and fungal [32] particulate preparations have indicated that these core oligosaccharides are assembled via polyisoprenoid lipid intermediates, which function as glycosyl carriers in the synthesis of the carbohydrate moiety of glycoproteins. The synthesis of this core region by lipid intermediates appears to proceed in the following manner [32]. The most internal sugar in the core, N-acetylglucosamine (GlcNAc), is transferred from its nucleotide derivative, UDP-GlcNAc, to a lipid, forming a GlcNAc-lipid. Another GlcNAc residue is transferred directly to this GlcNAc-lipid, from UDP-GlcNAc, to form a GlcNAc(1-4)B-GlcNAc-lipid (chitibiosyl lipid). Finally, mannose, from GDP-mannose, is transferred directly to the disaccharide chitibiosyl-lipid to form a B-man(1-4)-B-GlcNAc-(1-4)-B-GlcNAc-lipid (trisaccharide lipid). The last phase of synthesis of the core involves yet another lipid

intermediate. Mannose is transferred from GDP-mannose to a lipid carrier to form a mannosyl-lipid. This lipid monosaccharide serves as a mannose donor for the outer α-linked mannose residues, which are ultimately transferred to the trisaccharide lipid. The resulting lipid oligosaccharide, which now contains the completed core oligosaccharide, is then transferred to the asparagine residue of a polypeptide, by forming an N-glycosidic linkage between the innermost GlcNAc of the core and asparagine. The result is a glycosylated protein. Additional sugar residues can possibly be added to the core as side branches directly from nucleotide sugars, without the involvement of lipid intermediates. The elucidation of the lipid intermediate scheme was greatly aided by the property [32] that mono, di, and trisaccharide lipids are soluble only in mixtures of chloroform-methanol (2:1), while lipid oligosaccharides can only be extracted in chloroform-methanol-water (1:1:0.3). The lipid carrier in animals, fungi [32] and some plants [11] has been identified as the α-unsaturated polyisoprenol, dolichol.

Although in vitro studies have demonstrated the synthesis of lipid intermediates implicated in the biosynthesis of glycoprotein using particulate preparations, little information is available on the in vivo occurrence of these compounds. Roberts [26] has demonstrated the incorporation of [^{14}C] glucosamine into lipids and glycoproteins of Phaseolus aureus, but the nature of the lipids was not

investigated. A few reports concerning the nature of glycosylated lipids have been reported in animal systems [3,28-30].

As part of our continuing study of protein synthesis in the developing pea cotyledon, we report the partial characterization of the lipids and glycoproteins labeled *in vivo* by exogenously supplied [^{14}C] glucosamine and [^{14}C] mannose. The characteristics of the labeled glycolipids and glycoproteins are compared to the glycosylated lipids formed *in vitro* [6], which have been implicated as lipid intermediates in the assembly of pea cotyledon glycoproteins.

MATERIALS AND METHODS

Preparation of [^{14}C] mannose and [^{14}C] glucosamine labeled lipids and lipid free residue. Developing pea cotyledons (Pisum sativum L. cv. Burpeeana) were grown as previously described [4]. Thirty cotyledons, 21 days post anthesis, were harvested, the testae and embryo removed, and each cotyledon was injected with either 3 µl of [D-U-^{14}C] glucosamine (254 mCi/mmol, 200 µCi/ml - Amersham Searle) or [D-1-^{14}C] mannose (59 mCi/mmol, 200 µCi/ml - Amersham Searle). After injection, the cotyledons were incubated on moist filter paper in a glass petri dish for 4½ hours in the light. Following incubation, the cotyledons were homogenized in (4:1 v/wt) 50 mM potassium phosphate buffer (pH 7.0) in a Polytron for 1 minute.

The resulting suspension was filtered through cheesecloth and aliquots taken for determination of radioactivity. The remaining suspension was centrifuged at 250 g in an SS-34 rotor in a Sorvall RC-2B centrifuge. After the radioactivity in the pellet was determined, this fraction was discarded and the remaining supernatant was sequentially extracted by lipid solvents as previously described [6] to obtain the lipids soluble in chloroform-methanol (2:1 v/v), chloroform-methanol-water (1:1:0.3 v/v/v), and a lipid free residue. Aliquots of each of the lipid extracted fractions (c.f. Table I)

were placed in scintillation vials, evaporated to dryness, and, after the addition of scintillation fluid to the vials (8.0 gms PPO, 2 liters toluene, 1 liter Triton-X-100), the samples were counted in a Beckman LS-100 liquid scintillation counter. The lipid-free residue, remaining after the final lipid extraction of the original homogenate, was suspended in 5% (w/v) trichloroacetic acid, the precipitated pellet was washed with water and reprecipitated, and aliquots were taken to determine radioactivity as described above.

Chromatographic methods. [^{14}C] Glucosamine and mannose labeled lipids, extracted with chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3), were fractionated by column chromatography on DEAE cellulose (acetate), prepared by a modification of the procedure of Rouser [27] as described previously [6]. Chloroform-methanol (2:1) soluble mannose and glucosamine labeled lipids were applied to DEAE columns (2.5 X 35 cm) and eluted sequentially with 250 ml of chloroform-methanol (2:1), 200 ml 99% methanol, followed by a 500 ml linear gradient of 0-0.2 M ammonium acetate in methanol. Fractions of 10 ml were collected and 2 ml aliquots were placed in scintillation vials, evaporated to dryness, and the radioactivity determined. The fractions eluted from the column with chloroform-methanol (2:1) (hereafter referred to as Peak I) were pooled, as were those fractions eluted by the ammonium acetate gradient (Peak II), and evaporated to dryness in vacuo at 30°C. The Peak II pooled

fractions were extracted with chloroform-methanol (2:1) and water, as described by Chambers [9], to remove ammonium acetate from this fraction. [^{14}C] Mannose and glucosamine labeled lipids, soluble in chloroform-methanol-water (1:1:0.3), were applied to DEAE columns (2.5 X 35 cm), eluted with 250 ml of chloroform-methanol-water (1:1:0.3), followed by a 500 ml linear gradient of 0-0.2 M ammonium formate in chloroform-methanol-water (1:1:0.3). Fractions of 10 ml were collected and 2 ml aliquots were assayed for radioactivity. The radioactive fractions eluted with the starting solvent (Peak I) were pooled, as were the fractions eluted with the salt gradient (Peak II), and evaporated to dryness in vacuo.

Thin layer chromatography of chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids was performed on precoated Silica Gel G-25 plates (Brinkman Ins., Des Plains, Ill.) in solvent systems A (chloroform-methanol-acetic acid-water, 50:30:8:4), B (chloroform-methanol-NH_4OH, 75:25:4), and on Eastman 13254 cellulose plates in solvent system C (isobutyric acid-1 N NH_4OH, 5:3). After development, the plates were dried, divided into 1 cm segments, scraped into scintillation vials, and counted directly.

Descending paper chromatography was performed on Whatman 3 MM paper in solvent systems D (ethylacetate-acetic acid-formic acid-water, 18:3:1:4), E (butanol-pyridine-water, 4:3:4), and F (butanol-acetic acid-water, 12:3:5). The chromatograms were dried, and cut into 1 cm sections for

determination of radioactivity. Sugars were detected on chromatograms by the alkaline silver nitrate method [2]. GlcNAc-Asn, glucosamine, and N-acetyl glucosamine were detected on chromatograms as described previously [8].

Mild acid and base treatment of [^{14}C] glucosamine and [^{14}C] mannose labeled chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids. Mild acid treatment of lipids was performed in .01 N HCl in 50% isopropanol and partitioned into aqueous and organic phases as described previously [6]. Radioactivity in each phase was determined.

Mild base treatment of lipids was carried out in chloroform-methanol (1:4) containing 0.1 N NaOH. Samples were heated at 37°C for 15 min and, after neutralization with HCl, the samples were separated into aqueous and organic phases as described by Chambers [9]. The radioactivity in each phase was determined.

Characterization of the [^{14}C] glucosamine and mannose labeled lipid free residues. Lipid free residue, prepared as described in Materials and Methods, was incubated with protease and the percentage of radioactivity released from the residue by proteolytic digestion was determined as described previously [6].

Strong acid hydrolysis of residue fraction was carried out in 4 N HCl for 2 hr at 100°C. After treatment, the HCl

was removed by repeated evaporation and dissolution of the sample in water. The sugars liberated from the residue by acid treatment were identified by paper chromatography in solvent system D for 24 hr.

SDS polyacrylamide gel electrophoresis of the residue fraction was performed as described by Studier [31], modified for use in cylindrical gel tubes. The samples were dissociated in buffer containing SDS [31] and applied to 9.0 cm 12.5% acrylamide gels with a 1.5 cm 5% stacking gel. Samples of 50-150 μg of protein, determined by the method of Lowry [21], were applied to each gel and run at 8 milliamp/tube for 2-3 hr. The gels were then sliced into 1 mm sections and the radioactivity determined as previously described [4]. SDS polyacrylamide gel electrophoresis was also carried out with the following proteins of known subunit molecular weight; Cytochrome-c (12,384), trypsin (23,800), horseradish peroxidase (40,000), and pyruvate kinase (57,000).

Identification of the GlcNAc-Asn glycopeptide linkage in [^{14}C] glucosamine labeled lipid free residue. Strong alkali (1.0 N NaOH at 100°C for 6 hr) and mild alkali treatments (0.1 N NaOH at 37°C for 72 hr) were performed with [^{14}C] lipid free residues as described previously for legumin [8]. The radioactive products of alkali digestion were analyzed by paper chromatography in solvent system E for 24 hr. Repeated proteolytic digestion of the residue, followed each time by fractionation of the products by gel filtration, was

described previously [8]. [^{14}C] Lipid free residue, digested 5 times with protease, was subjected to mild acid hydrolysis in 2 N HCl for 20 min at 100°C. After the hydrolysate was neutralized, the radioactive products were analyzed by paper chromatography in solvent systems E for 24 hr and F for 96 hr.

Preparation of [^{14}C] glucosamine and mannose labeled cellular membrane fractions. Developing pea cotyledons, 21 days post anthesis, were injected with [^{14}C] glucosamine or mannose for 1½ hr as described in Materials and Methods. Following incubation, the cotyledons were disrupted and the cellular constituents separated on linear 15-60% sucrose gradients as described previously [24,25]. The fractions obtained from microsomal gradients at densities of 1.165 g/cc and 1.12 g/cc were pooled and corresponded respectively to rough endoplasmic reticulum and possibly golgi apparatus membranes [24]. In a similar manner, fractions obtained from mitochondrial gradients at a density of 1.185 g/cc were pooled and corresponded to mitochondrial membranes [24]. Each of these three isolated membrane fractions were extracted with chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3), and the radioactivity in each of these fractions determined [24]. The [^{14}C] glucosamine or mannose labeled lipids extracted in these solvents were analyzed by thin layer chromatography in solvent system A.

RESULTS AND DISCUSSION

Incorporation of radioactivity from [^{14}C] glucosamine and [^{14}C] mannose into lipid and residue fractions. When developing pea cotyledons are incubated in vivo with either [^{14}C] glucosamine or mannose, radioactivity is incorporated into several different components (Table I). When glucosamine is utilized, a low percentage (8.3%) of counts becomes associated with material sedimenting at 250 g. This fraction contains starch, cell wall fragments, intact chloroplasts and nuclei [24]. The majority of the radioactivity is recovered from the methanol extractions and probably represents unicorporated glucosamine, and its low molecular weight soluble derivatives; glucosamine phosphates, N-acetyl glucosamine, and UDP-N-acetyl glucosamine. Roberts [26] has demonstrated that plants have the ability to form UDP-GlcNAc from exogenously supplied glucosamine by way of these glucosamine derivatives. Of specific interest to the current study is the observation that 1% and 5.4% of the radioactivity from [^{14}C] glucosamine is transferred respectively to lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:3). These solvents have been demonstrated to extract lipid intermediates involved in glycoprotein synthesis in in vitro systems [32]. Greater than 8% of the radioactivity is associated with the lipid free residue. This relative

distribution of radioactivity into the glycolipids and lipid free residue is the exact reverse of the pattern observed when *in vitro* studies were performed utilizing particulate fractions from pea cotyledons which were incubated with [^{14}C] UDP-GlcNAc [6]. The distribution is, however, similar to that reported *in vivo* by Speake [28] and Spiro [29] for incorporation of [^{14}C] glucosamine into animal preparations.

In contrast to incorporation of glucosamine, a much greater amount of radioactivity is incorporated into the 250 g pellet when [^{14}C] mannose is utilized (Table I). A large proportion (40%) of the radioactivity is soluble in the methanol solvents. Radioactivity is incorporated into fractions soluble in chloroform-methanol (2:1) (17.4%), chloroform-methanol-water (1:1:0.3) (2.1%), and into a delipidated residue (8.8%). This distribution is in contrast to the *in vitro* incorporation of radioactivity of mannose from [^{14}C] GDP-mannose by particulate preparations of pea cotyledons [6]. In this study the lipid free residue was the fraction most extensively labeled.

Analysis of the [^{14}C] labeled chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids. Thin layer chromatography in 3 solvent systems of the [^{14}C] labeled chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids indicates that radioactivity from [^{14}C] glucosamine and mannose becomes associated with several different components (Table II). This is in contrast to *in*

vitro reports in which only a limited number of radioactive lipid components were produced after incubation of particulate preparations with [^{14}C] GDP-mannose [1,6,7,12,14,17,20] or [^{14}C]-N-acetyl glucosamine [6,7,17]. It therefore appears that additional labeled lipids, not produced in *in vitro* systems, become labeled *in vivo*. This study has attempted to investigate the nature of the lipids formed *in vivo* to determine which, if any, correspond to the lipids that are produced *in vitro* and have been implicated as lipid intermediates in glycoprotein synthesis [6].

Rouser [27] has reported that mixtures of lipids can be separated by DEAE cellulose (acetate) column chromatography. Lipids eluted from this ion exchange resin with chloroform-methanol are non-ionic (neutral) in nature, ionic-nonacidic lipids are eluted with various mixtures of chloroform-methanol-water, and acidic lipids are retained on the column and require the addition of acid, base, or salt gradients for elution.

When the chloroform-methanol (2:1) soluble lipids labeled by [^{14}C] glucosamine were chromatographed on DEAE, two peaks of radioactivity are resolved (Figure 1-A). A large percentage (69%) of radioactivity eluted from the column with the starting solvent, chloroform-methanol (2:1), and would therefore appear to be associated with non-ionic lipids [27] (Peak I). Thirty one percent of the radioactivity was retained by the resin and was eluted from the column with

an ammonium acetate gradient (Peak II). This material thus appears to be acidic lipid components [27]. The chloroform-methanol (2:1) soluble lipids labeled in vivo by [^{14}C] mannose were also fractionated into a neutral lipid (83%) fraction (Peak I) and acidic lipid (17%) fraction (Peak II) by chromatography on DEAE cellulose (Figure 1-B).

The majority (93%) of radioactivity associated with [^{14}C] glucosamine labeled lipids soluble in chloroform-methanol-water (1:1:0.3) was retained by DEAE cellulose (acetate) and eluted with an ammonium formate gradient (acidic lipids, Peak II). A small percentage (7%) of the radioactivity was not retained by the column (Peak I) (Figure 2-A). In contrast, when the [^{14}C] mannose labeled lipids soluble in chloroform-methanol-water (1:1:0.3) were applied to DEAE cellulose (acetate) columns (Figure 2-B), three peaks of radioactivity are detected. A portion (44%) had no affinity for the resin and was eluted with the starting solvent (Peak I). This material would appear to be ionic-nonacidic lipids [27]. The radioactivity retained on the column appeared to be acidic in nature [27] and eluted as two separate fractions (Peak IIa and Peak IIb - Figure 2-B) with the ammonium formate gradient. The peak of smaller magnitude (IIa) was eluted at a lower salt concentration than the larger peak (IIb).

The chromatographic behavior of the [^{14}C] mannose and glucosamine in vivo labeled acidic lipids, soluble in chloroform-methanol (2:1) and chloroform-methanol-water

(1:1:0.3), on DEAE cellulose (acetate) was similar to that reported for acidic lipids extracted in the same solvents, which were labeled in vitro by [^{14}C] UDP-N-acetyl glucosamine and GDP-mannose [6,9,17,19,32]. However, there are no reports in these in vitro systems of the production of the neutral and ionic-non acidic lipids that we have observed being formed in vivo.

Partial characterization of [^{14}C] Peak I lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3). Thin layer chromatography of the [^{14}C] mannose labeled Peak I (neutral) lipid fraction in solvent system A indicated the presence of a radioactive component with an R_f of .44 (Table III). Therefore, the component of R_f .44 in solvent A in the unfractionated mixture of [^{14}C] mannose labeled lipids soluble in chloroform-methanol (2:1) (Table II) can be attributed to a neutral lipid component. The neutral lipid components (PKI) soluble in chloroform methanol (2:1), which were labeled by [^{14}C] glucosamine, were fractionated into three components with R_f's of .27, .53, and .88 in solvent system A (Table III). These same three components are detected in solvent A in the mixture of [^{14}C] glucosamine labeled lipids soluble in chloroform-methanol (2:1), prior to DEAE fractionation (Table II). Thus, these three components can also be assigned to lipids of neutral nature.

The contention that the radioactivity in Peak I (Figure 1-A and B) is associated with neutral and not polyisoprenoid acidic lipids, was further confirmed by the observation that Peak I lipids were stable to treatment by mild acid hydrolysis, but labile to treatment with mild alkali. These properties are inconsistent with a polyisoprenoid nature but are consistent with the lipids being neutral [18]. Neutral lipids labeled in vivo by [^{14}C] mannose have been reported in animals by Speake [28] and were identified as being triglycerides.

The ionic non acidic lipids, soluble in chloroform-methanol-water (1:1:0.3), labeled by [^{14}C] mannose (Peak I, Figure 2-A) gave two peaks with R_f's of .43 and .94 in solvent system A (Table III). These same two components appeared in the crude mixture of lipids before DEAE fractionation (Table II) and can thus be assigned the designation of ionic-nonacidic lipids. In a similar manner, the Peak I lipids labeled by glucosamine, soluble in chloroform-methanol-water (1:1:0.3), gave a single peak with an R_f of .29 (Table III). This lipid is also present in the crude mixture (Table II) and this component can also be designated as an ionic-nonacidic lipid. The nonacidic nature of each of these labeled lipids was confirmed by their resistance to mild acid and susceptibility to mild alkali treatment [18].

Partial characterization of acidic Peak II lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) labeled by [^{14}C] glucosamine and [^{14}C] mannose. Thin layer chromatography of the [^{14}C] mannose labeled Peak II lipid soluble in chloroform-methanol (2:1) (Figure 1-B) produced a single radioactive component which migrated with an R_f of .84 in solvent system A (Table III). Since this same component was present in the crude lipid mixture (Table II), this component of the mixture can be assigned the designation of an acidic lipid. The migration of this mannose labeled acidic lipid during thin layer chromatography is similar to that observed for the mannosyl lipid formed *in vitro* when particulate preparations are incubated with [^{14}C]-GDP-mannose [6,9,12,14-17,19]. These mannosyl lipids formed *in vitro* have been tentatively identified as polyisoprenoid derivatives [6,9,12,14-17,19] which are characteristically stable to mild alkali treatment but hydrolyzed by treatment with mild acid [18,32]. The acidic Peak II lipid, labeled *in vivo* by [^{14}C] mannose, is stable to mild alkalai but is hydrolyzed by mild acid treatment. This observation, in conjunction with the chromatographic behavior of the lipid, indicates that the [^{14}C] mannose labeled lipid soluble in chloroform-methanol (2:1) (Peak II - Figure 1-B) is a mannosyl polyisoprenol derivative.

The acidic [^{14}C] glucosamine labeled lipid soluble in chloroform-methanol (2:1) (Peak II - Figure 1-A) demonstrates little mobility during thin layer chromatography in solvent system A (Table III). A component of the same mobility is also detected in the crude lipid fraction (Table II) and thus can be assigned the designation of an acidic lipid. The observation that the [^{14}C] glucosamine labeled Peak II acidic lipid was more strongly bound by DEAE cellulose (acetate) than the acidic Peak II lipid labeled by [^{14}C] mannose (Figures 1-A and 1-B) indicates that the former compound has a greater negative charge [27]. This observation is consistent with reports that the glucosaminyl lipid involves a pyrophosphate linkage while the mannosyl lipid has a phosphate linkage [22,32]. The Peak II glucosamine labeled lipid soluble in chloroform-methanol (2:1) was susceptible to hydrolysis by mild acid but not to mild base treatment. This observation, along with the chromatographic behavior of this lipid on DEAE cellulose (acetate), are similar to properties of the glucosamine lipid assembled *in vitro*, when particulate preparations are incubated with UDP-[^{14}C] GlcNAc [6,16,17,32]. The GlcNAc lipids synthesized *in vitro* have been characterized as GlcNAc polyisoprenol lipids and chitibiosyl polyisoprenoid derivatives [6,16,17,32]. However, the mobility of the Peak II glucosamine labeled lipid formed *in vivo* (R_f .06) is markedly different from the mobility of chitibiose lipid formed *in vitro* [6,16,17,32].

No comparative information is available on the R_f of the GlcNAc lipid formed *in vitro* in solvent system A, so the possibility that the glucosamine lipid formed *in vivo* is this same compound cannot be ruled out.

The radioactivity associated with [^{14}C] mannose labeled Peak II lipids soluble in chloroform-methanol-water (1:1:0.3) migrated with R_f's of .52 and .06 during thin layer chromatography in solvent system A (Table III). These same two lipid components are present in the crude extract (Table II) and can thus be attributed to the presence of these acidic lipids. The observation that the Peak II mannose lipid migrates as two components in thin layer chromatography is consistent with the separation of the Peak II lipids into two acidic components on DEAE cellulose (acetate) by elution with different concentrations of ammonium formate (Figure 2-B). Although the chromatographic behavior of each of these acidic components (Peak IIa and Peak IIb, Figure 2-B) was not determined separately, it was observed that the component with an R_f of .06 was more extensively labeled than the component with an R_f of .52. This implies that the component with an R_f of .06 corresponded to the most extensively labeled Peak II component, Peak IIb (Figure 2-B). The chromatographic behavior of this acidic lipid (.06) is consistent with that observed by Beevers [6] and Hsu [19] for the oligosaccharide lipid formed *in vitro* when particulate preparations were

incubated with [^{14}C] GDP-mannose. The polyisoprenoid nature of the oligosaccharide lipid formed *in vivo* is demonstrated by the alkaline stability and mild acid lability of this fraction [18].

The acidic Peak II [^{14}C] glucosamine labeled lipid soluble in chloroform-methanol-water (1:1:0.3) migrated with an R_f of .06 during thin layer chromatography in solvent system A (Table III). This component is also present in the crude mixture of lipids and therefore this component with an R_f of .06 (Table II) can be designated as an acidic lipid. The chromatographic properties of this Peak II lipid is similar to that reported by Beevers [6] for the oligosaccharide lipid formed *in vitro* from [^{14}C] UDP-GlcNAc. The similarity of the chromatographic properties of the [^{14}C] glucosamine and [^{14}C] mannose labeled oligosaccharide lipids formed *in vitro* (Figure 2 and Table III) is consistent with the concept [22,32] that glucosamine and mannose are incorporated into the same oligosaccharide lipid, which functions as an intermediate in glycoprotein synthesis. The polyisoprenoid nature of the *in vivo* formed [^{14}C] glucosamine oligosaccharide lipids appears to be supported by their resistance to mild base treatment and hydrolysis by mild acid. It was observed, however, that control incubations of acidic Peak II lipids soluble in chloroform-methanol-water (1:1:0.3), heated at 100°C without acid, also liberated considerable radioactivity into the aqueous phase. It therefore appears that these lipids are at least partially heat labile.

Subcellular location of lipids labeled by [^{14}C] mannose and [^{14}C] glucosamine. Membrane fractions, isolated from pea cotyledons which had been injected with [^{14}C] mannose, contained labeled lipid components soluble in chloroform-methanol (2:1). The greatest incorporation of radioactivity occurred into lipids of the mitochondrial fraction, while the rough endoplasmic reticulum (R.E.R.) and a golgi apparatus enriched fraction incorporated radioactivity into lipids to a lesser extent (Table IV). The nature of the [^{14}C] mannose labeled lipids soluble in chloroform-methanol (2:1) was investigated by thin layer chromatography in solvent system A (Table V). Two radioactive components were resolved in this system with R_f's of .44 and .84. These values are consistent with the mobility of [^{14}C] mannose labeled chloroform-methanol (2:1) soluble lipids from extracts of whole cotyledons (Table II and Table III). The compound tentatively identified as a neutral lipid (R_f .44), which was not retained by DEAE cellulose (Peak I, Figure 1-B), was present in greater quantities in both the R.E.R. and golgi apparatus membranes (Table V) than the acidic mannosyl lipid (R_f .84) (Peak II, Figure 1-B). The mitochondrial fraction contained acidic and neutral lipids in similar proportions (Table V). Very little radioactivity from [^{14}C] mannose becomes associated with lipids soluble in chloroform-methanol-water (1:1:0.3) extracted from the subcellular membrane fractions (Table IV). This observation

is consistent with the incorporation of [^{14}C] mannose into lipids isolated from whole cotyledons (Table I).

When [^{14}C] glucosamine was injected into pea cotyledons, radioactivity became associated with lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) extracted from the mitochondria, R.E.R., and a golgi membrane enriched fraction (Table IV). The most extensive incorporation of radioactive was into lipids soluble in chloroform-methanol-water (1:1:0.3). This observation is also consistent with the incorporation of [^{14}C] glucosamine into lipids isolated from whole cotyledons (Table I). Incorporation of radioactivity into the chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) soluble lipids from [^{14}C] glucosamine was greatest into the R.E.R. fraction (Table IV). This finding agrees with the observations of Nagahashi [24,25], who reported that the R.E.R. was the principal site of enzymes responsible for the glycosylation of lipid intermediates from [^{14}C] UDP-GlcNAc in vitro. A large amount of radioactivity was associated with mitochondrial lipids soluble in chloroform-methanol-water (1:1:0.3) and a small amount of radioactivity from [^{14}C] glucosamine was detected in chloroform-methanol-water (1:1:0.3) soluble lipids extracted from the golgi apparatus enriched fraction (Table IV).

The nature of the [^{14}C] glucosamine labeled lipids soluble in chloroform-methanol-water (1:1:0.3), extracted

from the membrane fractions, was determined by thin layer chromatography in solvent system A (Table V).

Both types of chloroform-methanol-water (1:1:0.3) soluble lipids (R_f's .06 and .29) found in the total cotyledonary extract (Table II and Table III) were found associated with golgi membranes (Table V). Slightly more radioactivity is associated with the lipid tentatively identified as the ionic-nonacidic lipid (Peak I, Figure 2-A) with an R_f of .29, than with the acidic lipid (Peak II, Figure 2-A) with an R_f of .06. In contrast, the radioactivity associated with the R.E.R. lipids, soluble in chloroform-methanol-water (1:1:0.3), was found to migrate as a single component with an R_f of .06 (Table V). The lipid of R_f .06 corresponds to the acidic oligosaccharide lipid found in the crude lipid fraction from the total cotyledonary extract (Table II and Table III). This observation is consistent with the site of assembly of the lipid oligosaccharide being the R.E.R. [24,25]. The radioactivity in the chloroform-methanol-water (1:1:0.3) soluble lipids labeled with [^{14}C] glucosamine, which were extracted from mitochondrial membranes, migrated with an R_f of .29 in solvent system A (Table V). This component has been tentatively identified as an ionic-nonacidic lipid in the total cotyledonary extract (Table III and Table IV).

Characterization of the lipid free residue labeled from [^{14}C] glucosamine and [^{14}C] mannose. When the lipid free residues labeled *in vivo* from [^{14}C] glucosamine and [^{14}C] mannose were incubated with protease, 89% and 87% of the radioactivity, respectively, was solubilized. This indicates that the majority of radioactivity from both precursors is associated with proteins in the lipid free residue. These results are in contrast to the *in vitro* studies with [^{14}C] GDP-mannose, in which it is observed that radioactivity associated with the lipid free residue is almost totally resistant to proteolytic digestion [6]. However, the lipid free residue labeled *in vitro* from [^{14}C] UDP-GlcNAc is solubilized by protease digestion [6].

The nature of the radioactivity associated with the proteins in the lipid free residue, labeled *in vivo* from [^{14}C] glucosamine and [^{14}C] mannose, was investigated by subjecting the lipid free residue to total acid hydrolysis. Paper chromatography of the neutralized hydrolysate products of [^{14}C] glucosamine labeled lipid free residue indicated that essentially all of the radioactivity was associated with material which co-migrated with standard glucosamine (Figure 3). In contrast, the radioactivity present in the acid hydrolysate of lipid free residue labeled with [^{14}C] mannose was separated into 3 principal components. Two peaks of radioactivity co-migrated with glucose and mannose, while the third peak had a mobility slightly less than that

of glucosamine (Figure 4). This redistribution of radioactivity from [^{14}C] mannose into other glycosyl components, associated with glycoproteins in the lipid free residue, is strikingly similar to a report by Speake [28], who reported that radioactivity in a lipid free residue labeled by [^{14}C] mannose was redistributed in a way that 40% was associated with mannose, 30% with glucose, and 30% into an unknown compound of slow mobility.

Further work was conducted to determine the nature of the glycopeptide linkage in glycoproteins in the lipid free residue labeled with [^{14}C] glucosamine. The glycopeptides were extensively digested with protease and the products of digestion fractionated by gel filtration on Sephadex G-15. The results (data not shown) show that all of the radioactivity was associated with carbohydrate containing material and no radioactivity was associated with the α-amino nitrogen fractions. Thus, there appears to be no interconversion of [^{14}C] glucosamine into amino acids associated with the peptide portion of the [^{14}C] glucosamine labeled glycoproteins in the lipid free residue.

Dilute alkali treatment (.1 N NaOH) of the [^{14}C] glucosamine labeled glycopeptides, produced by two proteolytic digestions of the lipid free residue, did not alter the mobility of the radioactive glycopeptides on paper chromatograms (data not shown). In contrast, strong alkalai (1.0 N NaOH) treatment of the [^{14}C] glucosamine labeled

glycopeptides markedly increased the mobility of the glycopeptides on paper chromatograms. These observations tend to preclude a glycopeptide linkage to peptidyl serine or threonine, but are consistent with a linkage from N-acetyl glucosamine to asparagine [8]. This supposition was confirmed by the results of a mild acid hydrolysis treatment of glycopeptides isolated from proteolytic digests of [^{14}C] glucosamine labeled lipid free residue. Paper chromatography of the neutralized mild acid hydrolysate of the [^{14}C] glycopeptides indicated that the majority of radioactivity co-migrated with the authentic GlcNAc-Asparagine linkage compound (GlcNAc-Asn) in solvent system E (Figure 5). Similar results were obtained in solvent system F. This actual isolation of this glycopeptide linkage compound, coupled with the B-elimination data (alkaline hydrolysis), provides direct proof that [^{14}C] glucosamine, injected into pea cotyledons, is converted into N-acetyl glucosamine, which is a structural component of the glycopeptide bond of endogenous glycoproteins in the pea cotyledons.

The nature of glycoproteins, isolated from the lipid free residue, labeled <u>in vivo</u> from [^{14}C] glucosamine and [^{14}C] mannose was investigated by SDS polyacrylamide gel electrophoresis. The majority of glycoproteins labeled with [^{14}C] mannose have molecular weights of approximately 57,000, while glycoproteins in the molecular weight range of 15-20,000 are labeled to a much smaller extent (data

not shown). A very similar distribution of glycoproteins is observed when [^{14}C] glucosamine is used as the substrate. The vast majority of proteins labeled with [^{14}C] glucosamine have molecular weights of approximately 57,000 while the proteins in the 15-20,000 molecular weight range are labeled only to a very small extent. The observation that [^{14}C] glucosamine and [^{14}C] mannose are incorporated into proteins of the same molecular weight is consistent with the concept that both of these sugars are components of the core oligosaccharide lipid that transfers the completed oligosaccharide to endogenous proteins.

CONCLUSIONS

The developing pea cotyledon has the capacity to incorporate radioactivity from exogenously applied [^{14}C] glucosamine and [^{14}C] mannose into lipid components soluble in chloroform-methanol (2:1), chloroform-methanol-water (1:1:0.3), and into a lipid free residue.

The majority of radioactivity from [^{14}C] glucosamine and [^{14}C] mannose is incorporated into lipids soluble in chloroform-methanol (2:1) and these lipids have been characterized as neutral lipids on the basis of their behavior of DEAE cellulose (acetate) and their sensitivity to hydrolysis by mild alkalai. The formation of [^{14}C] mannose and glucosamine labeled neutral lipids was not reported in *in vitro* particulate preparations of pea cotyledons incubated with [^{14}C] UDP-GlcNAc and [^{14}C] GDP-mannose [6]. Speake [28], however, has reported the formation of neutral lipids (triglycerides) in *in vivo* preparations of rabbit mammary explants from exogenously applied [^{14}C] mannose and [^{14}C] N-acetyl glucosamine. Apparently these sugars were converted into glycolytic intermediates and finally into the glycerol moiety of the neutral triglycerides [28]. Although we have not demonstrated the operation of this pathway for incorporation of labeled sugars into neutral lipids of pea cotyledons, the extensive *in vivo* interconversion

of [^{14}C] mannose into other glycosyl components present in the carbohydrates of pea cotyledons is consistent with the possibility that this type of pathway is operating in the pea cotyledon as well.

Developing pea cotyledons also incorporate radioactivity *in vivo* from [^{14}C] glucosamine and mannose into acidic lipids (characterized by their retention on DEAE cellulose acetate) which have characteristics consistent with them being polyisoprenol derivatives. Polyisoprenol lipids were also reported to be formed *in vitro* in developing pea cotyledons and have been implicated as glycosyl carrier lipid intermediates, which function in the synthesis of glycoproteins [6].

The [^{14}C] mannose *in vivo* labeled polyisoprenoid lipid soluble in chloroform-methanol (2:1) appears to be identical to the mannosyl lipid produced *in vitro* in developing pea cotyledons incubated in [^{14}C] GDP-mannose. The production of polyisoprenol lipid intermediate sugar derivatives from nucleotide sugar derivate precursors is well documented in the lipid intermediate scheme of glycoprotein biosynthesis [22,32]. If the same mannosyl lipid intermediates are being produced *in vivo* and *in vitro*, this implies that pea cotyledons possess the necessary enzymes to convert mannose into GDP-mannose. The cell fractionation data indicate that the mannosyl lipid is associated *in vivo* with membranes of the mitochondria, R.E.R., and golgi apparatus.

Developing pea cotyledons also incorporate radioactivity *in vivo* from [^{14}C] glucosamine into acidic polyisoprenol lipid derivatives. This observation implies that pea cotyledons also possess the ability to convert glucosamine into UDP-GlcNAc. Roberts reports that glucosamine can be converted into amino sugar derivatives in bean hypocotyls [26] and Mense and Beevers have demonstrated the existence of four of the enzymes necessary for the conversion of glucosamine into UDP-GlcNAc in *in vitro* preparations of developing pea cotyledons [23].

The R_f of the [^{14}C] glucosamine labeled polyisoprenoid lipid soluble in chloroform-methanol (2:1) *in vivo* does not agree with this lipid being the chitibiosyl-lipid intermediate that was formed *in vitro* in developing pea cotyledons [6]. Several possibilities arise as to why this discrepancy might exist. Work *in vitro* in several animal systems has indicated that a GlcNAc containing monosaccharide and also a trisaccharide lipid soluble in chloroform-methanol (2:1) may be formed *in vitro* as well as a chitibiosyl lipid [32]. However, the R_f of the *in vivo* labeled compound (.06) is not consistent with the R_f reported for the trisaccharide lipid [10]. No comparative chromatographic data are available to determine whether the *in vivo* labeled glucosamine lipid might be the GlcNAc-lipid, so this possibility cannot be evaluated. In animal systems the mannosyl-lipids and the GlcNAc containing lipids soluble

in chloroform methanol (2:1) are the same lipid component, dolichol [32]. A recent report by Ericscon has indicated that, although the mannosyl lipid in plants may be of the dolichol type, the GlcNAc containing lipids soluble in chloroform methanol (2:1) appears to involve a lipid other than dolichol [13]. Since the chitibiosyl lipid produced *in vitro* in pea cotyledons has the characteristics of a chitibiosyl dolichol lipid [6], the difference in R_f's of the *in vitro* and *in vivo* lipids may be explained by a different lipid acceptor operating *in vivo* for UDP-GlcNAc residues. Since none of these proposed explanations have been adequately tested, the nature of the glycosyl unit(s) associated with the acidic lipid soluble in chloroform methanol (2:1) labeled *in vivo* by [^{14}C] glucosamine remains obscure. The low labeling of this fraction has thus far precluded analysis of the carbohydrate moiety released from this lipid by mild acid hydrolysis.

Radioactivity from [^{14}C] glucosamine and [^{14}C] mannose is incorporated into two different types of lipids *in vivo*, soluble in chloroform-methanol-water (1:1:0.3). Approximately 7% of the radioactivity from [^{14}C] glucosamine and 44% of the radioactivity from [^{14}C] mannose is incorporated into a class of ionic-nonacidic lipids which were not reported formed *in vitro* in pea cotyledons [6]. The majority of the radioactivity from both precursors appears to be incorporated into the same oligosaccharide lipids that were formed *in*

vitro [6]. The structure of these oligosaccharide lipids has not been resolved but the chromatographic behavior of the [^{14}C] glucosamine and [^{14}C] mannose labeled oligosaccharide lipids is consistent with the belief that both of these precursors are incorporated into the same lipid intermediate [32].

In the current investigation it was consistently observed that the lipids soluble in chloroform-methanol-water (1:1:0.3) labeled by [^{14}C] glucosamine were more extensively labeled than those lipids labeled by [^{14}C] mannose. In view of the large amount of neutral lipid labeled by [^{14}C] mannose *in vivo*, the low labeling of the lipid oligosaccharide fraction by [^{14}C] mannose would tend to support the contention that these neutral lipids are not serving as precursors to the oligosaccharide lipid. Significant amounts of the mannose labeled lipid are still synthesized, however, that might be expected to serve as precursors to the mannose labeled lipid oligosaccharide. The low labeling of the mannose labeled lipid oligosaccharide compared to the glucosamine labeled lipid oligosaccharide could be a result of a large endogenous pool of mannosyl precursor for the formation of mannosyl lipid oligosaccharide. Recently, Chambers [10] has suggested that GDP mannose as well as mannosyl lipid may function as a precursor in lengthening the trisaccharide lipid into the oligosaccharide lipid. Our *in vivo* data are consistent with this concept also

and suggests that *in vivo* the mannosyl lipid may not serve as the general precursor for oligosaccharide assembly, as was indicated from *in vitro* studies with pea cotyledons [6]. This contention is also supported by the observation that [^{14}C] mannose labeled lipids associated with defined subcellular membrane fractions also incorporate moderate amounts of radioactivity into lipids soluble in chloroform methanol (2:1) but little into lipids soluble in chloroform-methanol-water (1:1:0.3). These same subcellular membrane fractions incorporate [^{14}C] glucosamine to a small degree into lipids soluble in chloroform-methanol (2:1) and to a much larger degree into lipids soluble in chloroform-methanol-water (1:1:0.3). This is consistent with the pattern of incorporation seen from [^{14}C] glucosamine into lipids of the whole cotyledon. The observation that radioactivity associated with acidic lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) is present in the highest amount in the R.E.R. membranes would appear to be consistent with the localization of glycosyl transferases involved in the synthesis of lipid intermediates in the R.E.R. [24,25]. However, at this time it is uncertain whether the acidic lipids associated with membrane fractions represent lipid intermediates in glycoprotein synthesis or structural lipids, which are integral components of the membranes from which they have been isolated. The observation that the mannose labeled acidic lipids soluble

in chloroform-methanol (2:1) are associated with membranes other than the golgi and R.E.R. indicates that these lipids may have functions in addition to being glycosyl carrying lipid intermediates.

Although a precursor product relationship has not been demonstrated, our findings that acidic lipids, with properties similar to lipid intermediates produced in vitro, are labeled in vivo when [^{14}C] glucosamine or [^{14}C] mannose is provided to pea cotyledons is consistent with the proposed sequence of glycoprotein assembly involving lipid intermediates. The demonstration that radioactivity from [^{14}C] glucosamine becomes associated with a compound identified as the GlcNAc-Asn glycopeptide linkage, isolated from [^{14}C] glucosamine labeled glycoproteins in the lipid free residue, provides direct evidence that pea cotyledons can convert exogenously applied glucosamine into N-acetyl glucosamine. The isolation of the GlcNAc-Asn linkage compound is also significant because glycoproteins with this structural feature are considered to be synthesized through the involvement of the lipid linked intermediate pathway [32]. In contrast to the in vitro studies with pea cotyledons, the [^{14}C] glucosamine labeled glycoproteins are much more extensively labeled in vivo. Speake [28] has reported a similar observation in vivo in rabbit mammary explants and suggests that glycoprotein synthesis in vitro is restricted by the availability of peptidyl acceptors for the glycosyl units.

In the intact pea cotyledon a sustained level of protein synthesis would provide the necessary peptide acceptors for glycosylation to occur.

The nature of the endogenous protein acceptors glycosylated *in vivo* utilizing [^{14}C] glucosamine or [^{14}C] mannose as a substrate has been investigated utilizing SDS polyacrylamide gel electrophoresis. The majority of [^{14}C] glucosamine and mannose is incorporated into endogenous proteins with a subunit molecular weight of approximately 57,000. We have recently demonstrated that exogenously applied [^{14}C] glucosamine is incorporated into the GlcNAc-Asn glycopeptide linkage of the reserve storage protein in pea cotyledons, legumin [8]. This finding implies that legumin may be one of the proteins glycosylated *in vivo*, which is isolated from the lipid free residue. The molecular weight of the glycosylated endogenous protein is consistent with this possibility. The incorporation of radioactivity from either [^{14}C] glucosamine or [^{14}C] mannose into polypeptides of the same molecular weight is consistent with the concept that mannose and glucosamine are both components of the lipid oligosaccharide, which transfers the completed oligosaccharide core to the asparagine residue of the glycosylated protein.

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TABLE I. Incorporation of radioactivity from [^{14}C] glucosamine and [^{14}C] mannose into fractions isolated from developing pea cotyledons.

Fraction	Cpm from [^{14}C] Glucosamine[a)]		Cpm from [^{14}C] Mannose[b)]	
Total Cell Homogenate	7,629,610		3,498,683	
250 g Pellet	632,100	(8.3)*	1,100,000	(31.4)*
Chl-MeOH-H_2O (3/48/47)	1,113,840	(14.6)	135,658	(3.9)
99% MeOH	3,784,853	(49.6)	1,119,620	(32.0)
50% MeOH	977,075	(12.8)	297,024	(8.5)
Chl-MeOH (2:1)	70,400	(1.0)	607,475	(17.4)
Chl-MeOH-H_2O (1:1:0.3)	409,590	(5.4)	72,192	(2.1)
Lipid Free Residue	624,096	(8.2)	309,600	(8.8)
	7,611,954		3,641,569	

*The numbers in parentheses indicate the percentage of cpm in each fraction in relationship to the cpm in the total cellular homogenate.

a) Thirty cotyledons, 21 days post anthesis, were each injected with 3 µl of [^{14}C] glucosamine. The cotyledons were extracted into the fractions listed above as described in Materials and Methods. Radioactivity in each fraction was determined.

b) Fractions were prepared as in a) except that each cotyledon was injected with 3 µl of [^{14}C] mannose.

TABLE II. Distribution of radioactivity following thin layer chromatography of [^{14}C] glucosamine and [^{14}C] mannose labeled lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3).

Fraction Chromatographed	R_f Solvent A	R_f Solvent B	R_f Solvent C
(2:1) from [^{14}C] Mannose	.44,.84	.06,.21,.43,.90	.89
(1:1:0.3) from [^{14}C] Mannose	.06,.43,.52,.94	.03,.35,.90	.75
(2:1) from [^{14}C] Glucosamine	.06,.27,.53,.88	.06,.20,.86	.69
(1:1:0.3) from [^{14}C] glucosamine	.06,.29	.06	.74

TABLE III. Distribution of radioactivity following thin layer chromatography of [^{14}C] glucosamine and [^{14}C] mannose labeled lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3) after fractionation of lipids by DEAE cellulose (acetate) column chromatography. Peak I refers to lipids eluted from DEAE with the starting solvent, Peak II refers to lipids eluted from DEAE with a salt gradient. Refer to Table II for composition of lipid fractions prior to DEAE chromatography.

Fraction Chromatographed	R_f Solvent A	
	Peak I	Peak II
(2:1) from [^{14}C] Mannose	.44	.84
(2:1) from [^{14}C] Glucosamine	.27,.53,.88	.06
(1:1:0.3) from [^{14}C] Mannose	.43,.94	.06,.52
(1:1:0.3) from [^{14}C] Glucosamine	.29	.06

TABLE IV. Incorporation of radioactivity from [^{14}C] glucosamine and [^{14}C] mannose into lipids soluble in chloroform-methanol (2:1) and chloroform-methanol-water (1:1:0.3), extracted from subcellular membrane fractions. Labeled subcellular membrane fractions were prepared and extracted with lipid solvents as described in Materials and Methods.

Membrane Fraction	cpm from [^{14}C] mannose	
	(2:1) lipids	(1:1:0.3) lipids
Golgi	1,660	0
R.E.R.	2,516	0
Mitochondria	5,264	200
Membrane Fraction	**cpm from [^{14}C] glucosamine**	
	(2:1) lipids	(1:1:0.3) lipids
Golgi	1,092	3,112
R.E.R.	6,784	13,564
Mitochondria	636	9,820

TABLE V. Distribution of radioactivity following thin layer chromatography of lipids soluble in chloroform-methanol (2:1) labeled by [^{14}C] mannose and lipids soluble in chloroform-methanol-water (1:1:0.3) labeled by [^{14}C] glucosamine. Lipids were extracted from subcellular membrane fractions as described in Materials and Methods.

Membrane Fraction Chromatographed	R_f's of (2:1) Soluble Lipids From [^{14}C] Mannose in Solvent A
Golgi	.44, .84 (2.1:1)[a]
R.E.R.	.44, .84 (2.9:1)[a]
Mitochondria	.44, .84 (1:1)[a]
Membrane Fraction Chromatographed	**R_f's of (1:1:0.3) Soluble Lipids From [^{14}C] Glucosamine in Solvent A**
Golgi	.29, .06 (1.4:1)[b]
R.E.R.	.06
Mitochondria	.29

a) Ratio of cpm in lipid with an R_f of .44 to the cpm in the lipid with an R_f of .84.

b) Ratio of cpm in lipid with an R_f of .29 to the cpm in the lipid with an R_f of .06.

LEGENDS FOR FIGURES

Figure 1. DEAE cellulose (acetate) column chromatography of [^{14}C] glucosamine and [^{14}C] mannose labeled lipids soluble in chloroform-methanol (2:1).

A) 2:1 soluble lipids labeled from [^{14}C] glucosamine

B) 2:1 soluble lipids labeled from [^{14}C] mannose.

2:1 soluble lipid fractions were applied to DEAE cellulose (acetate) columns in the appropriate extracting solvent. Elution of the columns and collection of fractions was performed as described in Materials and Methods. Arrows represent the point at which each eluting solvent was applied to the column.

Figure 2. DEAE cellulose (acetate) column chromatography of [^{14}C] glucosamine and [^{14}C] mannose labeled lipids soluble in chloroform-methanol-water (1:1:0.3).

A) 1:1:0.3 soluble lipids labeled from [^{14}C] glucosamine.

B) 1:1:0.3 soluble lipids labeled from [^{14}C] mannose.

Chromatography was performed as in Figure 1.

Figure 3. Paper chromatography of the total acid hydrolysate of the [^{14}C] glucosamine labeled lipid free residue.

[^{14}C] Glucosamine labeled lipid free residue was acid hydrolyzed and then spotted on Whatman 3 MM paper and developed in solvent system D for 24 hr. The chromatograms

were cut into 1 cm strips and counted for radioactivity. Various standard marker compounds were chromatographed and detected as described in Materials and Methods. GlcN = glucosamine, Glu = glucose, Mann = mannose.

Figure 4. Paper chromatography of the total acid hydrolysate of the [^{14}C] mannose labeled lipid free residue.

Hydrolysis and chromatography were performed as described in Figure 3.

Figure 5. Paper chromatography of a partial acid hydrolysate of [^{14}C] glucosamine labeled glycopeptides.

[^{14}C] Glucosamine labeled glycopeptides, isolated from the [^{14}C] glucosamine labeled lipid free residue as described in Materials and Methods, were hydrolyzed in 2 N HCl for 20 min at 100°C. Following neutralization, the hydrolysate was spotted on Whatman 3 Mm paper and developed in solvent system F for 24 hr. The chromatogram was cut into 1 cm sections and counted for radioactivity. Standard marker compounds were chromatographed and detected as described in Materials and Methods. GlcN = glucosamine, GlcNAc = N-acetyl glucosamine, GlcNAc-Asn = 1-N-B-L-aspartyl-2-deoxy-B-D-glycopyranosylamine.

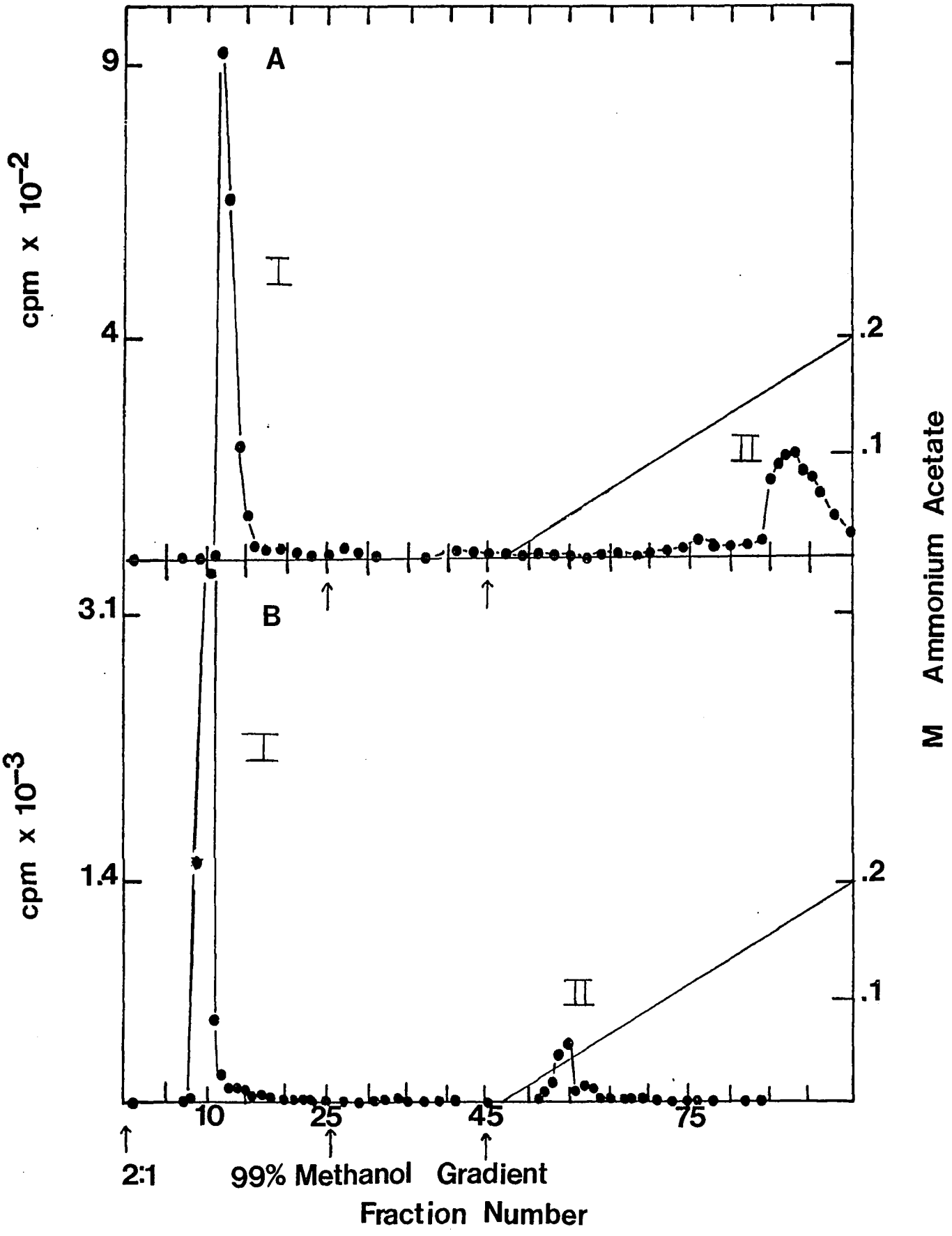
A
B
I
II
I
II
9
4
3.1
1.4
cpm x 10^{-2}
cpm x 10^{-3}
.2
.1
.2
.1
M Ammonium Acetate
10
25
45
75
2:1
99% Methanol Gradient
Fraction Number

FIGURE 1

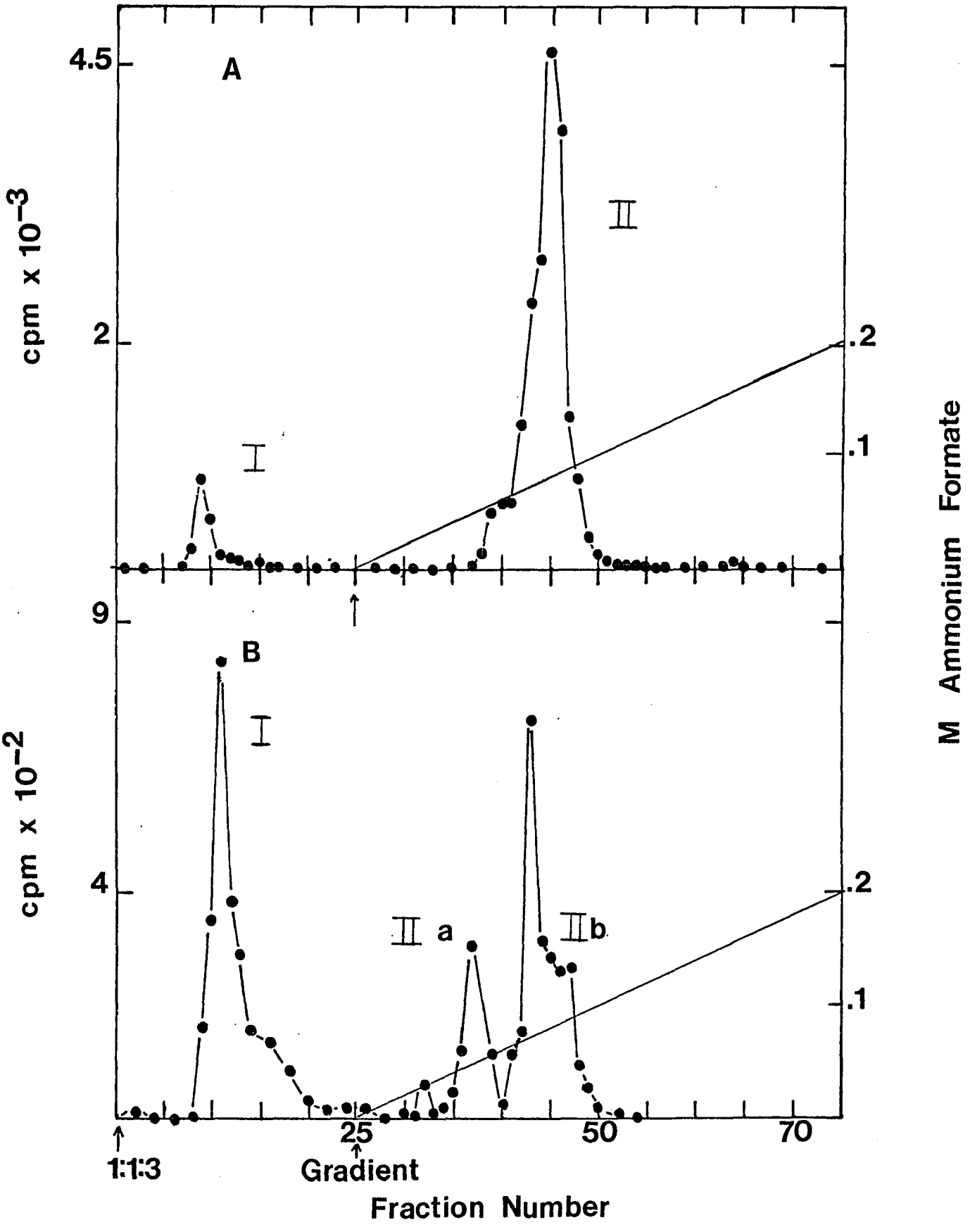
A
B
I
II
II a
II b
4.5
2
9
4
cpm x 10⁻³
cpm x 10⁻²
.2
.1
.2
.1
M Ammonium Formate
25
50
70
1:1:3
Gradient
Fraction Number

FIGURE 2

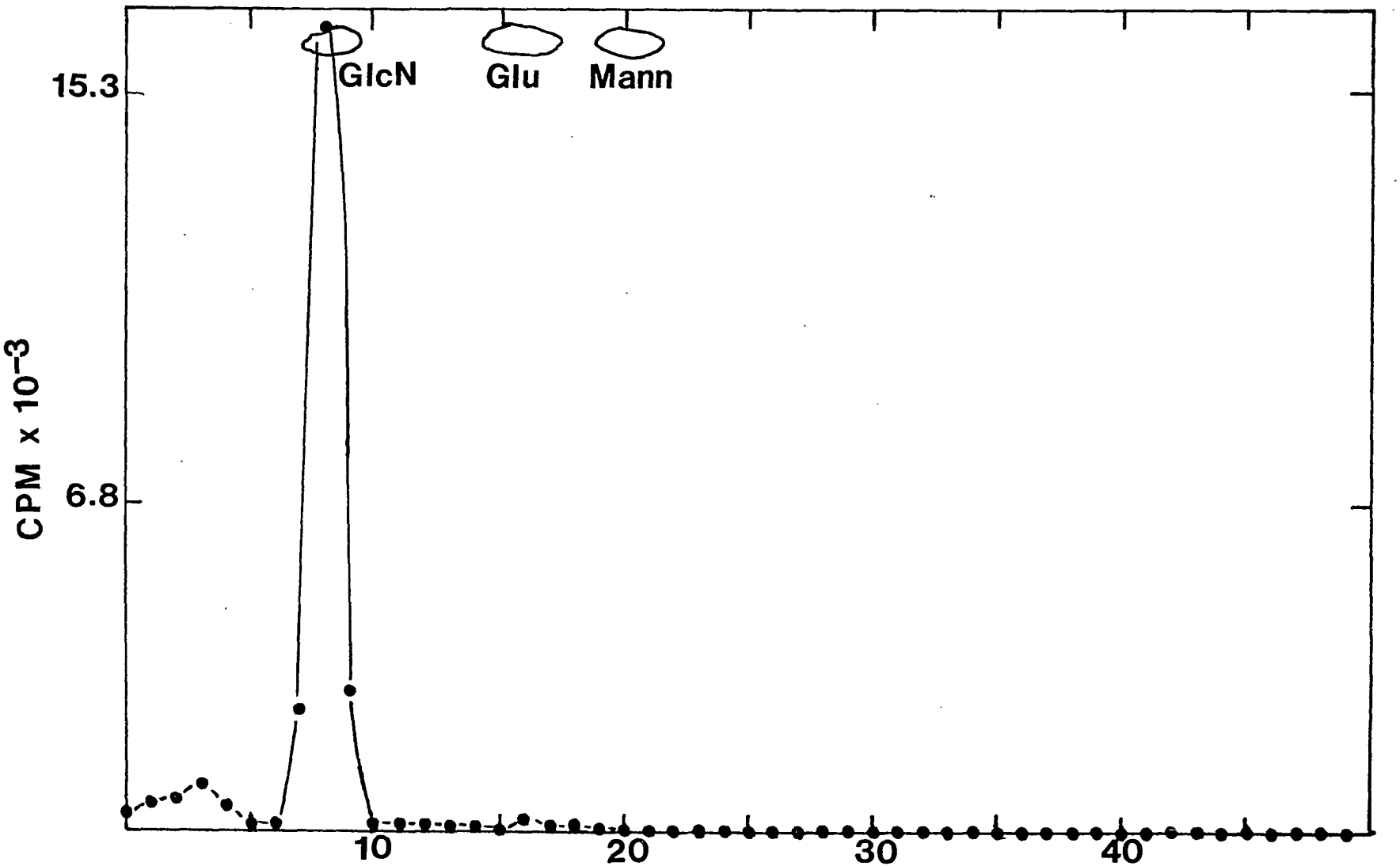

FIGURE 3

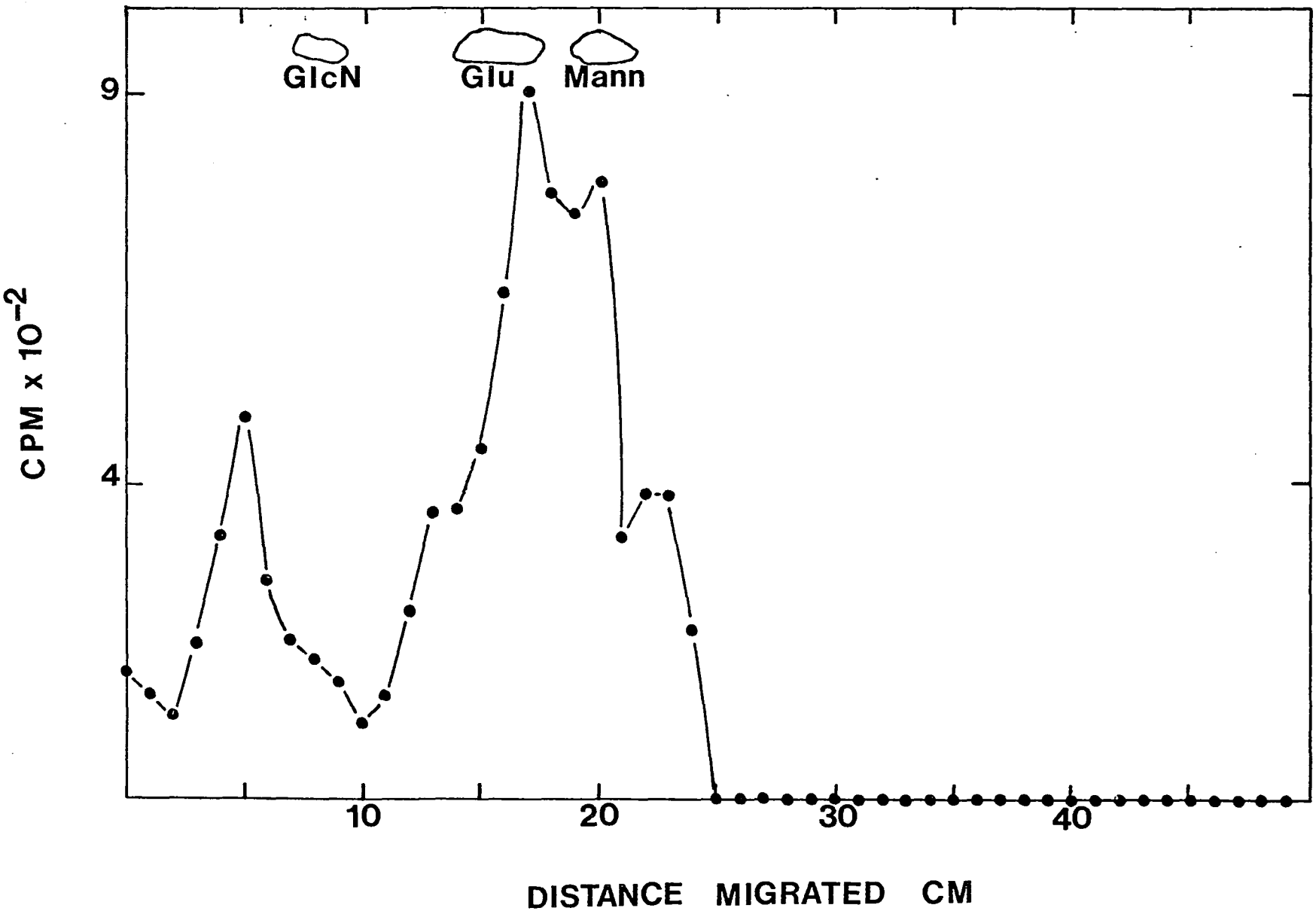

FIGURE 4

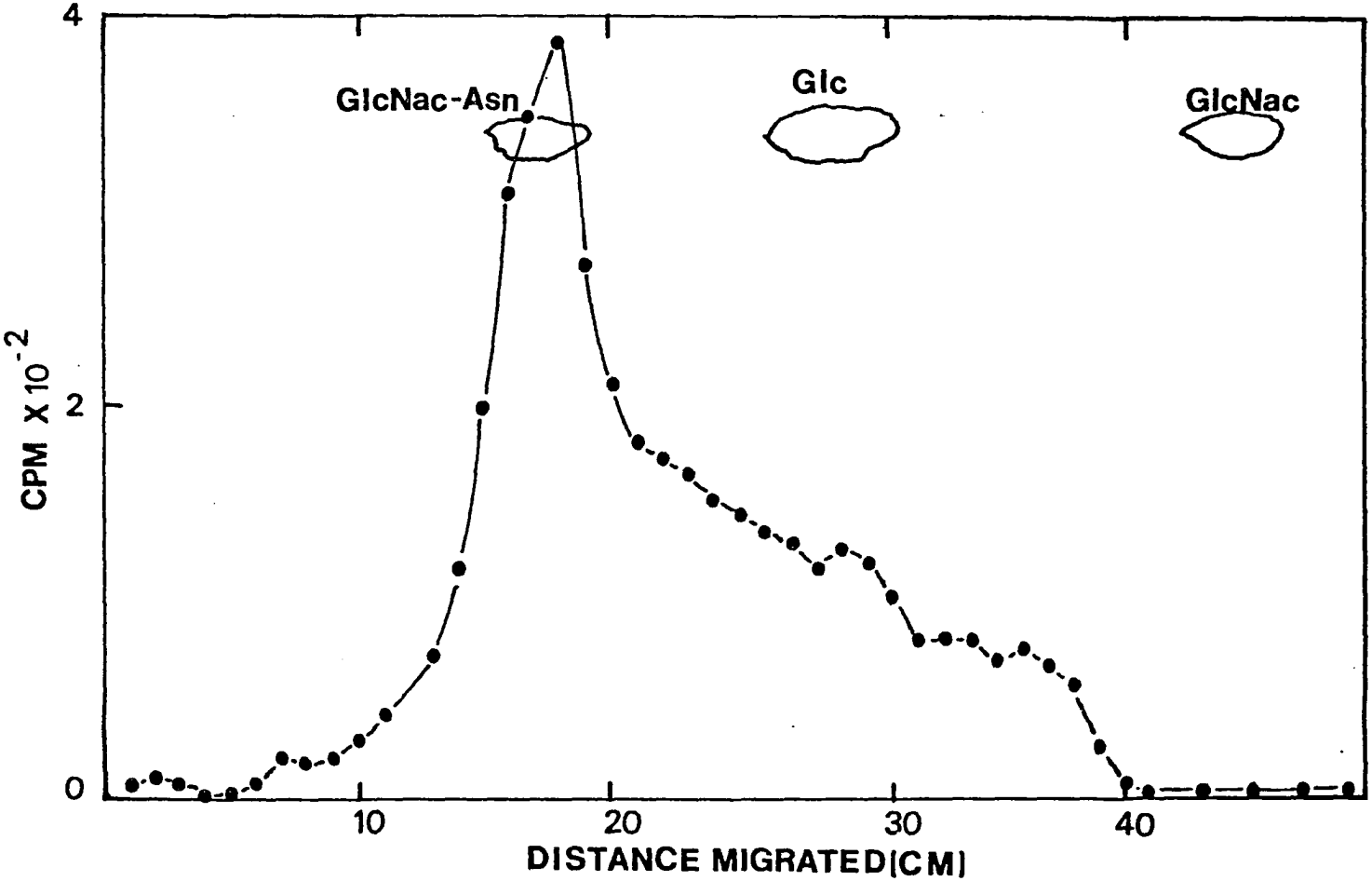
4
2
0
CPM X 10^-2
GlcNac-Asn
Glc
GlcNac
10
20
30
40
DISTANCE MIGRATED(CM)

FIGURE 5

CHAPTER IV

PAPER 3

A DEVELOPMENTAL STUDY OF MEMBRANE BOUND N-ACETYLGLUCOSAMINYL AND MANNOSYL TRANSFERASES IN COTYLEDONS FROM *PISUM SATIVUM* L.

ABSTRACT

The distribution of UDP-N-acetylglucosamine N-acetylglucosaminyl transferase and GDP-mannose mannosyl transferase activity, assayed in the presence of Mg^{2+} or Mn^{2+}, has been determined in subcellular membrane fractions of developing pea cotyledons 15 days and 29 days post anthesis. The distribution of transferases at these two extremes of development has been compared to the distribution of these same glycosyl transferases previously reported in pea cotyledons 21 days post anthesis (Nagahashi, J., R. C. Mense, L. Beevers, Plant Physiol. 62: in press).

With the following exceptions, the distribution of glycosyl transferases in cotyledons 15 and 29 days post anthesis was very similar to that reported for day 21 cotyledons. GDP-mannosyl transferase activity was not detected in the presence of Mg^{2+} at 1.20 g/cc in day 15 cotyledons and UDP-GlcNAc transferase activity was not detected in the presence of Mn^{2+} at 1.12 g/cc in day 29 cotyledons. In addition to these differences, protein bodies (1.25 g/cc) are not present in day 15 cotyledons and the chloroplasts and mitochondria sediment at a greater density from day 29 cotyledons than from either day 15 or day 21 cotyledons.

The observation that no glycosyl transferase activity was associated with plasma membranes, mitochondria, or protein bodies during either day 15 or day 29 is consistent with the data reported for day 21 cotyledons.

INTRODUCTION

Lipid linked intermediates have been implicated as glycosyl carriers in the synthesis of glycoproteins in animals [18], plants [2,5-7,11] and fungi [12]. Earlier work in our laboratory established the role of lipid intermediates in glycoprotein synthesis from particulate fractions of developing pea cotyledons of Pisum sativum L. [2]. Subsequent studies by Nagahashi and Beevers [14] represented the first conclusive report of the identity of the intracellular location of UDP-GlcNAc and GDP-Man transferases involved in the glycosylation of lipid intermediates and glycoproteins. The association of GDP-Man transferases with the rough endoplasmic reticulum (R.E.R.) has recently been confirmed in hen oviduct [4] and yeast cells [10]. Recently, the differential influence of the divalent cations Mg^{2+} and Mn^{2+} on the membrane associated UDP-GlcNAc and GDP-Man transferase activities in pea cotyledons has been reported by Nagahashi and Beevers [15].

In microsomal fractions from developing pea cotyledons 21 days post anthesis, the R.E.R. appears to be the principal site of the UDP-GlcNAc and GDP-Man transferases involved in the synthesis of lipid intermediates and glycoproteins. Both Mg^{2+} and Mn^{2+} stimulated UDP-GlcNAc and GDP-Man transferases are associated with E.R. membranes, although

the Mg^{2+} and Mn^{2+} dependent GDP-Man transferases appear to be located on different sites of the R.E.R. [15]. In these studies, the R.E.R. was characterized by its density (1.165 g/cc), its association with the marker enzyme antimycin A insensitive NADH-Cyt c reductase, and by morphological examination of these membranes by electron microscopy. The association of glycosyl transferases with R.E.R. membranes was confirmed by "shifting" experiments in which both glycosyl transferases and the R.E.R. marker enzyme were converted to a lower density (from 1.165 to 1.10 g/cc) by EDTA treatment. Apparently, EDTA removes ribosomes from the R.E.R. by chelating the Mg^{2+} ions, which are necessary in maintaining the association of ribosomes to E.R. membranes [14], thus producing a less dense denuded or smooth form of E.R. Since EDTA treatment does not alter the density of plasma membranes, shifting experiments also provide a mechanism for the differentiation of transferases associated with the R.E.R. (1.165 g/cc) and activities that may have been associated with plasma membranes (1.16-1.18 g/cc) [14].

UDP-GlcNAc and GDP-Man transferases are also associated with membranes equilibrating at a density of 1.12 g/cc, which have associated pH 7.5 IDPase activity. IDPase has been used as a marker enzyme for the Golgi apparatus [16]. However, since morphological examination of the pea cotyledon membranes sedimenting at 1.12 g/cc revealed both Golgi apparatus membranes and plastid envelopes, the glycosyl

transferase activities at 1.12 g/cc could not be unequivocally associated with Golgi membranes [14]. The Mg^{2+} dependent GDP-Man transferase at 1.12 g/cc appears to be involved in the formation of polymannans [14]. A Mn^{2+} stimulated UDP-GlcNAc transferase activity is also found at 1.12 g/cc and may be involved in attaching glycosyl residues to the core oligosaccharide of glycoproteins, which were initially synthesized in the R.E.R. [15].

A Mg^{2+} stimulated GDP-Man transferase activity was also observed in microsomal fractions at a density of 1.20 g/cc. The unidentified membranes at this density were speculated to be derived from the nuclear envelopes of developing pea cotyledons and the associated transferase appeared to be involved in the formation of polymannans [14].

Low levels of both UDP-GlcNAc and GDP-Mann transferase activities were also reported in sucrose density gradients of 250-13,000 g pellets from developing pea cotyledons 21 days post anthesis [14,15]. With the exception of low levels of GDP-Man transferase activities associated with chloroplast thylakoid membranes at 1.15 g/cc, all of the transferase activities detected in these pellets were similar in distribution, although much lower in magnitude, to those activities found in gradients of microsomal pellets [14,15]. This observation was consistent with results which indicated that the 250-13,000 g pellets were contaminated by R.E.R. unknown, and pH 7.5 IDPase associated membrane fractions [14].

Contrary to prior reports from animal systems [3], glycosyl transferase activities in day 21 pea cotyledons were not associated with mitochondrial membranes (which equilibrate at 1.185 g/cc with associated antimycin A sensitive NADH-Cyt c reductases activity and Cyt-c oxidase activity) [14,15]. There also was no transferase activity observed in membranes of protein bodies at a density of 1.25 g/cc, which were identified by morphological examination of these membranes by electron microscopy [14].

The possibility that the association of glycosyl transferases with specific intracellular membranes might change during development of the pea cotyledon was investigated in the present study. Developing pea cotyledons at early, 15 days post anthesis, and late stages of development, 29 days post anthesis, were assayed to determine the distribution of Mg^{2+} and Mn^{2+} stimulated UDP-GlcNAc and GDP-Man transferase activity. Particular attention was focused on the possibility that membrane associated transferases not reported in day 21 cotyledons (e.g., mitochondrial, plasma membrane and protein body glycosyl transferases) might be present at either extreme of development. The distribution of glycosyl transferases reported in day 21 cotyledons was also compared to that of day 15 and day 29 cotyledons to determine if there were any changes in these enzymes during development. Treatments with EDTA, shifting experiments, were employed to confirm the association of transferases with the R.E.R.

and to investigate the possible association of transferases with plasma membranes.

MATERIALS AND METHODS

Preparation of material for sucrose density gradient centrifugation. Pea plants (Pisum sativum L. cv Burpeeana) were grown as previously described [1]. Cotyledons 15 or 29 days post anthesis were harvested, the testae and embryonic axis removed, and the cotyledons were weighed (10-20 grams fresh weight were routinely used for each experiment). The cotyledons were homogenized in grinding mix (0.5 M sucrose, 5 mM 2-mercaptoethanol, and 30 mM Tris-MES (pH 7.5)) as described previously [14]. The homogenate was filtered through cheesecloth and centrifuged at 250 g for 5 min to remove cell wall fragments, starch, and intact chloroplasts and nuclei. The 250 g pellet was discarded and the supernatant was centrifuged at 13,000 g for 15 min to obtain the crude mitochondrial pellet. Centrifugations up to 13,000 g were performed in an SS-34 rotor in a Sorvall RC-2B centrifuge. The supernatant from the 13,000 g spin was centrifuged at 40,000 g for 35 min in a Beckman 42.1 rotor to obtain the crude microsomal pellet.

Samples were prepared for linear sucrose gradient centrifugation in the following manner. The 250-13,000 g (crude mitochondrial) pellet and the 13,000-40,000 g (crude microsomal) pellet were each split into equal halves. One half, the control treated pellet, was washed in grinding mix and the other half was washed in grinding mix plus 5 mM

EDTA to produce the EDTA treated pellet. Each pellet was then repelleted at its initial force and suspended in 2 ml of buffered sucrose (0.25 M sucrose, 5 mM 2-mercaptoethanol, and 30 mM Tris-MES (pH 7.5)).

Sucrose density gradient centrifugation. Mitochondrial or microsomal "control" or "EDTA treated" pellets were overlaid on 15-60% buffered sucrose gradients, prepared as described previously [14], and centrifuged to equilibrium at 82,500 g for 15 hr in a Beckman SW 27 rotor. The gradients were fractionated into 30 fractions (1.2 ml/fraction), the fractions monitored for absorbance at 280 Nm, and the percent sucrose in each fraction determined as described previously [14]. The fractions were then assayed for various enzyme activities.

Enzyme assays. NADH-Cyt c reductase and Cyt-c oxidase activities were assayed spectrophotometrically by determining the reduction or oxidation of Cyt-c at 550 Nm [8]. Inosine diphosphotase (IDPase) activity at pH 7.5 was determined as described by Leonard and Van Der Woude [13]. Chlorophyll was extracted into 80% acetone and the absorbance at 652 Nm converted to µg of chlorophyll [9]. The activity of glycosyl transferases was determined by measuring the incorporation by membrane fractions of radioactivity from [^{14}C] nucleoside diphosphate sugars into trichloroacetic acid percipitable material. UDP-[U^{14}C]-GlcNAc (300 mCi/mmol, 24 µCi/ml) and

GDP-[$U^{14}C$]- mannose (179 mCi/mmol, 25 μCi/ml), purchased from Amersham Searle, were the nucleoside diphosphate substrates used to measure the activities of UDP-GlcNAc and GDP-Mann transferases respectively. Membrane fractions (0.3 ml) were incubated with .1 ml of substrate mixture (final concentrations of 50 mM Tris, 10 mM KCl, 10 mM $MgCl_2$ or $MnCl_2$, and 2.5 mM 2-mercaptoethanol brought to pH 7 with HCl) plus 1 μl of the appropriate radioactive substrate in a shaking water bath at 37°C for 1 hr as described previously [14]. The reaction was stopped by the addition of trichloroacetic acid (5% w/v, final concentration) and the percipitated material filtered through glass fiber filters (Whatman GF/A). The radioactive percipitate retained by the filter was washed with cold 5% trichloroacetic acid to remove unincorporated counts, the filters were removed and placed into scintillation vials, and the vials dried at 100°C for 1 hr. The vials were then filled with scintillation fluid (8.0 gms PPO, 2 liters toluene, 1 liter Triton-X-100) and radioactivity determined in a Beckman LS-100 scintillation counter.

RESULTS AND DISCUSSION

Linear sucrose density gradient analyses of the 13,000-40,000 g pellet from cotyledons 15 days post anthesis. When the distribution of UDP-GlcNAc transferase activity in the presence of Mg^{2+} or Mn^{2+} was determined in sucrose gradients, a broad peak of activity was detected in the center of the gradient (Figure 1-A). A portion of this broad peak was centered around a density of 1.165 g/cc and was associated with a peak of NADH-Cyt-c reductase activity and also a peak of pH 7.5 IDPase activity. Another area of UDP-GlcNAc transferase activity was located around the area of a density of 1.175 g/cc and was coincident with the major peak of 280 Nm absorbance. This area of activity was quite spread out and followed the distribution of the 280 Nm absorbance peak. In previous studies [14,15] the peak of NADH-Cyt-c reductase activity and the peak of UDP-GlcNAc transfer activity was coincident with the highest peak of absorbance at 280 Nm. The disperse distribution of UDP GlcNAc transferase activity in the presence of Mg^{2+} and Mn^{2+} in this study and the observation that the peak of NADH-Cyt-c reductase does not correspond to the major peak of absorbance at 280 Nm suggest that different forms of R.E.R., possibly differing in the number of ribosomes attached to the membranes, are present in this gradient.

If this supposition were correct, it would be consistent with the previous report that UDP-GlcNAc transfer activity in the presence of Mg^{2+} and Mn^{2+} is associated with the R.E.R. [15]. The activity of the UDP-GlcNAc transferase associated with the R.E.R. is slightly higher in the presence of Mg^{2+} than in the presence of Mn^{2+}. In the presence of Mn^{2+}, another peak of UDP-GlcNAc transferase activity was detected at a density of 1.12 g/cc, coincident with the major peak of pH 7.5 IDPase activity (Figure 1-A).

The R.E.R. was converted to a less dense or smooth form of E.R. when the microsomal pellet was treated with 5 mM EDTA, prior to application to the sucrose density gradient (Figure 1-B). The R.E.R. marker enzyme was also shifted to an area of lighter density with one peak of activity equilibrating at around 1.10 g/cc and another peak of activity around 1.12 g/cc. The major peak of absorbance at 280 Nm was also shifted to a lighter density of 1.12 g/cc. The observation that the NADH-Cyt-c reductase activity and the peak of absorbance at 280 Nm shifts to an area of lower density following EDTA treatment confirms that this enzyme is associated with the R.E.R. The observation that 2 peaks of NADH-Cyt-c reductase activity are detected following EDTA treatment is consistent with our previous observation that different forms of R.E.R. were initially present in the gradient. The peak of pH 7.5 IDPase activity detected at 1.165 g/cc in the control gradient was relocated to a

density of 1.12 g/cc in the EDTA treated pellet, confirming the association of this enzyme with the R.E.R. The UDP-GlcNAc transferase activity detected in the presence of Mg^{2+} and Mn^{2+} was shifted from a density of 1.165 (Figure 1-A) to a density of 1.10-1.12 in the EDTA treated pellet (Figure 1-B), which was consistent with the behavior of the R.E.R. marker enzyme and the major peak of absorbance at 280 Nm.

The peak of UDP-GlcNAc transferase activity detected at 1.12 g/cc in the presence of Mn^{2+} remained at a density of 1.12 g/cc following EDTA treatment (Figure 1-B). The pH 7.5 IDPase activity associated with this transferase activity also did not change density following EDTA treatment.

When the distribution of GDP-Mann transferase was determined in the presence of either Mg^{2+} or Mn^{2+}, two areas of activity were observed (Figure 2-A). One peak, at a density of 1.12 g/cc, coincided with a peak of pH 7.5 IDPase activity. The activity of the GDP-Mann transferase was slightly higher in this peak in the presence of Mn^{2+} than in the presence of Mg^{2+}. A second area of activity, present as a shoulder of the peak of transferase activity at 1.12 g/cc, was detected in the presence of either Mg^{2+} or Mn^{2+} in the region of the R.E.R. marker enzyme. The density at which the GDP-Mann transferase activity and the associated R.E.R. marker equilibrates (1.145 g/cc) was less dense than that reported for the R.E.R. and its associated UDP-GlcNAc

transferase activity in Figure 1 (1.165 g/cc). Nagahashi has also noted that R.E.R. equilibrates at different densities on different dates experiments were run, and attributed these differences to changes in endogenous levels of Mg^{2+} in the pea cotyledons [15].

Following treatment of the microsomal pellet with EDTA (Figure 2-B), the GDP-Mann transferase in the presence of Mg^{2+} or Mn^{2+}, which was previously located at a density of around 1.145 g/cc (Figure 2-A), was relocated to a lighter density of around 1.10-1.12 g/cc. The R.E.R. marker enzyme and major peak of absorbance at 280 Nm were also shifted to a density of 1.10-1.12 g/cc following EDTA treatment, confirming the association of GDP-Mann transferase activity in the presence of Mg^{2+} or Mn^{2+} with the R.E.R. membranes (Figure 2-B). The GDP-Mann transferase activity in the presence of Mg^{2+} or Mn^{2+}, as well as the associated pH 7.5 IDPase activity, equilibrating at a density of 1.12 g/cc (Figure 2-A), did not appear to change location after EDTA treatment (Figure 2-B). It was difficult to determine, however, which transferase activity was associated with smooth E.R. (1.10-1.12 g/cc) and which was associated with the pH 7.5 IDPase activity at 1.12 g/cc, since these two enzyme activities overlapped considerably following EDTA treatment.

Following treatment of the microsomal pellet with EDTA (Figures 1-B and 2-B), no UDP-GlcNAc or GDP-Mann

transferase activity remained in the center of the gradient (1.16-1.18 g/cc). These results indicate that under the assay conditions employed, neither transferase activity was associated with the plasma membrane, which equilibrates at a density of 1.16-1.18 g/cc [14].

Linear sucrose gradient analyses of the 250-13,000 g pellet from cotyledons 15 days post anthesis. When UDP-GlcNAc transferase activity was assayed in the presence of Mg^{2+}, a broad peak of activity was located around a density of 1.18 g/cc (Figure 3-A). This peak was also coincident with peaks of pH 7.5 IDPase and NADH-Cyt-c reductase activities. When the UDP-GlcNAc transferase activity was assayed in the presence of Mn^{2+} (Figure 3-A), three areas of activity could be detected. A broad shallow area of activity was centered around a density of 1.18 g/cc, but was of a much lower magnitude than the UDP-GlcNAc transferase activity in the presence of Mg^{2+}. Another peak of UDP-GlcNAc activity in the presence of Mn^{2+} was detected at a density of 1.12 g/cc in the same region as a very small peak of pH 7.5 IDPase activity (Figure 3-A). The third peak of UDP-GlcNAc transferase activity in the presence of Mn^{2+} was found at a density of 1.10 g/cc, which is the same density at which denuded E.R. has been reported to equilibrate [14,15].

UDP-GlcNAc transferase activity detected in the presence of Mg^{2+} or Mn^{2+}, previously located around a density of 1.18 g/cc (Figure 3-A), was shifted to an area of lighter

density (1.12 g/cc) following EDTA treatment of the mitochondrial pellet (Figure 3-B). This shift of UDP-GlcNAc transferase activity indicates that the transferase was not associated with mitochondrial membranes, since the peak of absorbance at 280 Nm and the peak of NADH-Cyt-c reductase activity associated with the mitochondria did not change density following EDTA treatment (Figure 3-B). The shift of UDP-GlcNAc transferase activity from 1.18 g/cc to 1.12 g/cc is consistent with previous reports that mitochondrial pellets are contaminated with R.E.R. membranes [14,15]. The observation that R.E.R. shifted to a density of 1.12 g/cc instead of 1.10 g/cc as was reported previously [14] may be explained by the partial retention of ribosomes to the E.R. membranes following EDTA treatment. It appears that the majority of the pH 7.5 IDPase activity at 1.18 g/cc is associated with R.E.R., rather than mitochondrial membranes, since this enzyme activity shifts to a lighter density following EDTA treatment (Figure 3-B). This observation is consistent with our earlier contention that pH 7.5 IDPase activity was associated with R.E.R. membranes in the microsomal fractions.

It is difficult to assess whether the peak of UDP-GlcNAc transferase activity detected in the presence of Mn^{2+} at 1.12 g/cc (Figure 3-A) changes location following EDTA treatment, since denuded E.R. and its associated glycosyl transferase equilibrates at this density as well

following EDTA treatment (Figure 3-B). No UDP-GlcNAc transferase activity appears to be associated with chloroplast membranes, which equilibrate at 1.15 g/cc and are associated with a large peak of 280 Nm absorbance as well as a peak of pH 7.5 IDPase activity (Figure 3). Following EDTA treatment, no UDP-GlcNAc transferase activity was associated with mitochondrial membranes, which equilibrate at 1.18 g/cc and are associated with a peak of 280 Nm absorbance and a peak of NADH-Cyt-c reductase activity (Figure 3).

When GDP-Mann transferase activity was assayed in the presence of Mg^{2+} and Mn^{2+}, the only large peak of activity was detected in the presence of Mg^{2+} at a density of 1.12 g/cc. This peak was also associated with a small shoulder of pH 7.5 IDPase activity (Figure 4-A). This peak of activity remained at a density of 1.12 g/cc following EDTA treatment of the mitochondrial pellet (Figure 4-B). It also appears that additional GDP-Mann transferase activity, assayed in the presence of Mg^{2+} or Mn^{2+}, was shifted to a density of 1.12 g/cc. This activity was probably associated with a small amount of R.E.R. contaminating the mitochondrial pellet, which was shifted to this density following EDTA treatment. This shift in GDP-Mann transferase activity was accompanied by a slight shift of pH 7.5 IDPase activity from a density of 1.18 g/cc to 1.12 g/cc. Experiments with the EDTA treated pellets indicated that no GDP-Mann

transferase activity was associated with mitochondrial membranes at 1.18 g/cc. No GDP-Mann transferase activity was detected with chloroplast membranes at 1.15 g/cc (Figure 4).

A striking difference is observed in the absorbance at 280 Nm with 21 day old cotyledons [14] and 15 day cotyledons (Figures 3 and 4). A large peak of absorbance at 280 Nm equilibrating at 1.25 g/cc, which has been previously identified as protein bodies [14], is not present in day 15 cotyledons but is present in day 21 cotyledons [14]. This observation is consistent with a report by Basha [2] that the major storage proteins located in the protein bodies of pea cotyledons, legumin and vicilin, are synthesized in small amounts 15 days post anthesis.

Linear sucrose density gradient analyses of the 13,000-40,000 g pellet from cotyledons 29 days post anthesis. When the distribution of UDP-GlcNAc transferase activity in the presence of Mg^{2+} or Mn^{2+} was assayed from sucrose gradients of microsomal pellets, a peak of activity was detected coincident with the R.E.R. marker at a density 1.16 and 1.175 g/cc (Figure 5-A). The transferase activity in the presence of Mg^{2+} was slightly greater than in the presence of Mn^{2+}. In contrast to a previous report [15], no UDP-GlcNAc transferase activity in the presence of Mn^{2+} was detected at a density of 1.12 g/cc, associated with a peak of pH 7.5 IDPase activity (Figure 5-A).

When the microsomal pellet was treated with EDTA (Figure 5-B), the R.E.R. marker and a large peak of absorbance at 280 Nm were shifted to an area of lighter density. It appeared, as was discussed earlier, that different types of R.E.R. were present in the microsomal gradient (at 1.16 and 1.175 g/cc), which were converted to different forms of denuded E.R. (1.11 and 1.13 g/cc) following EDTA treatment. The peak of absorbance at 280 Nm was also shifted to an area of 1.11 to 1.13 g/cc (Figure 5-B). The behavior of the R.E.R. marker, following EDTA treatment, is exactly mirrored by the shift of the UDP-GlcNAc transferase activity detected in the presence of Mg^{2+} or Mn^{2+}. These results confirm the association of this transferase with the R.E.R.

When the distribution of GDP-Mann transferase was determined in the presence of Mg^{2+}, three peaks of activity were detected. Only two of these peaks of activity were detected in the presence of Mn^{2+} (Figure 6-A). The GDP-Mann transferase activity at a density of 1.12 g/cc, which was associated with a peak of pH 7.5 IDPase activity, was much greater in the presence of Mg^{2+} than Mn^{2+}. A peak of GDP-Mann transferase activity was also detected at a density of 1.17 g/cc, coincident with a peak of NADH-Cyt-c reductase activity and the large peak of absorbance at 280 Nm, in the presence of either Mg^{2+} or Mn^{2+}. The activity in the presence of Mg^{2+} is slightly higher at this density than in the presence of Mn^{2+} (Figure 6-A). A third major peak of activity was

detected at a density of 1.20 g/cc in the presence of Mg^{2+} and corresponded to membranes associated with the unknown fraction, which was reported by Nagahashi in day 21 cotyledons [14,15].

Following treatment of the day 29 microsomal pellet with EDTA, the GDP-Mann transferase activity in the presence of Mg^{2+} or Mn^{2+} is shifted to an area of lighter density (1.11 g/cc) (Figure 6-B). The R.E.R. marker as well as the peak of absorbance at 280 Nm were also shifted to this lighter density. The GDP-Mann transferase activity in the presence of Mg^{2+} and Mn^{2+} at 1.12 g/cc, as well as the associated pH 7.5 IDPase activity, does not appear to change density following treatment with EDTA (Figure 6-B). The GDP-Mann transferase activity detected at 1.20 g/cc in the presence of Mg^{2+} (Figure 6-A) seems to disappear following EDTA treatment (Figure 6-B). A similar behavior of this transferase has been reported by Nagahashi in day 21 cotyledons [14] and was thought to be a result of lowering the Mg^{2+} concentration by the chelating agent EDTA below that required for the optimal activity of this Mg^{2+} stimulated enzyme.

Experiments with EDTA treatment of microsomal pellets indicate that no UDP-GlcNAc or GDP-Mann transferase activities are associated with plasma membranes in day 29 cotyledons (Figures 5 and 6).

Linear sucrose gradient analyses of the 250-13,000 g pellet from cotyledons 29 days post anthesis. When the distribution of UDP-GlcNAc transferase activity in the presence of Mg^{2+} or Mn^{2+} was assayed in sucrose gradients of microsomal pellets, low levels of incorporation into membranes at densities of 1.19 and 1.14 g/cc were observed (Figure 7-A). The transferase activity at 1.19 g/cc was associated with a peak of NADH-Cyt-c reductase activity and a peak of absorbance at 280 Nm, while the transferase activity at 1.14 g/cc was associated with a small shoulder of NADH-Cyt-c reductase activity (Figure 7-A).

When the mitochondrial pellet was treated with EDTA (Figure 7-B), the UDP-GlcNAc transferase activity detected in the presence of Mg^{2+} and Mn^{2+} at 1.19 g/cc (Figure 7-A) was relocated to a density of 1.14 g/cc. A portion of the NADH-Cyt-c reductase activity also was converted to a density of 1.14 g/cc by EDTA treatment. These results confirm prior observations (Figures 3 and 4) and previous results [14] that R.E.R. membranes and their associated glycosyl transferases contaminate mitochondrial gradients. The observation that R.E.R. marker enzymes are converted to a density of 1.14 g/cc suggests that the transferase activity and the small shoulder of NADH-Cyt-c reductase activity equilibrating at 1.14 g/cc from the control pellet was due to the presence of small amounts of smooth E.R. present in the control pellet. Analysis of the mitochondrial

gradient following EDTA treatment indicates that no UDP-GlcNAc transferase activity is associated with mitochondrial membranes, which equilibrate at 1.19 g/cc and are associated with NADH-Cyt-c reductase activity and a peak of absorbance at 280 Nm (Figure 7-B). No UDP-GlcNAc transferase activity appears to be associated with chloroplast membranes (1.17-1.18 g/cc) or protein bodies (1.25 g/cc) (Figure 7).

When GDP mannosyl transferase activity was determined in gradients of mitochondrial pellets in the presence of Mg^{2+}, only a very disperse area of activity was detected in the center of the gradient between 1.17-1.19 g/cc (Figure 8-A). A very small peak of activity of 1.12 g/cc, coincident with a small peak of pH 7.5 IDPase activity, was also detected in the presence of Mg^{2+}. Following treatment of the mitochondrial pellet with EDTA, the transferase activity at 1.17-1.19 g/cc was shifted to a density of 1.14 g/cc (Figure 8-B), coincident with the peak of the relocated R.E.R. marker enzyme (Figure 7-B). The peak of glycosyl transferase activity at 1.12 g/cc as well as the associated pH 7.5 IDPase activity, was not shifted following EDTA treatment (Figure 8-B).

When Mn^{2+} was included in the assay mixture rather than Mg^{2+}, two areas of GDP-Mann transferase activity were detected (Figure 9-A). One area of activity was located in the center of the gradient, around a density of 1.19 g/cc, and this area of transferase activity was shifted to an

area of lighter density (1.14 g/cc), along with the R.E.R. marker, following treatment of the mitochondrial gradient with EDTA (Figure 9-B). The peak of GDP-Mann transferase activity at 1.14 g/cc in the control pellet (Figure 9-A) appeared to be due to the presence of smooth E.R. in the mitochondrial control pellet. The contamination of mitochondrial pellets by smooth E.R. has been detected in other mitochondrial pellets as well (Figure 7-A).

EDTA treatment of mitochondrial pellets indicates that no GDP-Mann transferase activity is associated with mitochondrial membranes. No GDP-Mann transferase activities appear to be associated with either chloroplast membranes (1.17 g/cc) or protein bodies (1.25 g/cc) (Figure 7-9).

In pea cotyledons 29 days post anthesis (Figures 7-9), both the chloroplasts (1.17-1.18 g/cc) and mitochondria (1.19 g/cc) equilibrate at heavier densities than the same organells from pea cotyledons either 15 days (Figures 3 and 4) or 21 days [14,15] post anthesis. Possibly, changes in these organells accompany the dehydration of the pea cotyledon, which begins to reach a very pronounced state at 29 days post anthesis [17].

CONCLUSIONS

From the present and previous studies [14,15], a rudimentary picture has developed concerning the distribution of glycosyl transferases at early, mid, and late days of development of pea cotyledons.

During each of the three days of development examined, the principal location of UDP-GlcNAc transferase activity detected in the presence of Mg^{2+} or Mn^{2+} appears to be the R.E.R. membranes (Figures 1-A and 5-A) and [14,15]. The association of this activity with the R.E.R. is confirmed by the observation that the UDP-GlcNAc transferase activity detected in the presence of either Mg^{2+} or Mn^{2+} shifted to a lower density following EDTA treatment (Figures 1-B and 5-B), and [14,15]. It has previously been suggested that in 21 day cotyledons Mn^{2+} stimulates the transfer of glycosyl units to glycoprotein and that Mg^{2+} stimulates the transfer of sugars to lipid intermediates [15]. The observation that UDP-GlcNAc transferase activity in the presence of Mg^{2+} is greater than the activity in the presence of Mn^{2+} in the R.E.R. membranes on day 15 and 29 may reflect differing endogenous concentrations of Mg^{2+} and Mn^{2+} on the days the experiments were performed. GDP-Mann transferase activity in the presence of Mg^{2+} or Mn^{2+} is also associated with the R.E.R. on each day of development (Figures 2-A and 5-A) and

[14,15]. On the basis of studies of day 21 cotyledons, the GDP-Mann activity in the presence of Mg^{2+} stimulates the transfer of sugars to glycoprotein and Mn^{2+} stimulates transfer to lipid intermediates [15].

On day 15 and day 21, UDP-GlcNAc transferase activity in the presence of Mn^{2+} is associated with membranes equilibrating at a density of 1.12 g/cc, associated with pH 7.5 IDPase activity (Figure 1-A) and 15 . The location of this transferase activity and the associated marker enzyme does not shift to a lighter density following EDTA treatment (Figure 1-B) and [15]. This transferase activity has been tentatively associated with Golgi apparatus membranes and appears to function in adding side branches to core oligosaccharides originally synthesized in the R.E.R. [15]. This transferase activity is not present during day 29 in development and suggests a change of enzymology in the Golgi apparatus membranes during the course of development.

GDP-Mann transferase activity in the presence of Mg^{2+} or Mn^{2+} is associated with Golgi apparatus membranes at a density of 1.12 g/cc during each day of development (Figures 2-A and 5-A) and [15]. The location of this transferase and the associated marker enzyme does not shift following EDTA treatment (Figures 2-B and 5-B) and [15]. It has been suggested that in day 21 cotyledons, the Mg^{2+} stimulated activity is responsible for transfer of glycosyl units to polymannans while Mn^{2+} stimulates transfer to

glycolipids (although it has not been established if these glycolipids are lipid intermediates involved in glycoprotein synthesis) [15]. The observation that GDP-Mann Mn^{2+} stimulated activity is greater than Mg^{2+} stimulated activity during day 15 in golgi apparatus membranes (Figure 2-A), while the reverse of this behavior is seen during day 21 [15] and day 29 (Figure 5-A), may either reflect differing endogenous levels of Mg^{2+} and Mn^{2+} on these particular days or may indicate that more polymannans are synthesized during later stages of development of the pea cotyledon.

During 21 days [14,15] and 29 days post anthesis (Figure 5-A), a Mg^{2+} stimulated GDP-Mann transferase is associated with an unknown fraction, which equilibrates at a density of 1.20 g/cc. This activity is not detected during day 15 of development (Figure 2-A). The report in day 21 cotyledons that the Mg^{2+} activity stimulated transfer of glycosyl units to polymanns [15], would be consistent with our speculation that polymannans might be synthesized in greater amounts during later days of development. In agreement with previous reports [14,15] is the observation that no transferase activity appears to be associated with plasma membranes during either day 15 (Figures 1 and 2) or day 29 (Figures 5 and 6).

With a relatively few exceptions, all of the UDP-GlcNAc and GDP-Mann transferase activity sedimenting in the mitochondrial fraction can be accounted for by the contamination

of the mitochondrial pellet by R.E.R. and Golgi apparatus membranes [14] (Figures 3, 4, and 7-9). The contaminating R.E.R. was sometimes shifted, following EDTA treatment, to a heavier density than that reported [14,15] in day 21 cotyledons (Figures 3-B, 4-B, 7-B, and 9-B). This behavior may be explained by the partial retention of ribosomes to the E.R. membranes after EDTA treatment. In day 15 cotyledons a Mn^{2+} stimulated UDP-GlcNAc transferase activity (Figure 3-A) and on day 29 a Mn^{2+} stimulated GDP-Mann transferase activity (Figure 9-A) is detected in control mitochondrial fractions at a density expected for smooth E.R. The activities were not reported in day 21 cotyledons [14,15].

Although low levels of GDP-Mann transferase activity were reported associated with chloroplasts in mitochondrial preparations from day 21 cotyledons [14,15], this activity was not detected in extracts from cotyledons 15 days (Figure 4) or 29 days post anthesis (Figures 8 and 9). In agreement with results from day 21 cotyledons [14,15], no UDP-GlcNAc or GDP-Mann transferase activity could be detected associated with mitochondrial or protein body membranes on either day 15 (Figures 3 and 4) or day 29 (Figures 7-9) of development.

The density at which mitochondria and chloroplasts sediment in gradients of mitochondrial extracts appears to increase during day 29 of development (1.19 and 1.17-1.18 g/cc respectively) as compared to the density during day 15

or day 21 of development (1.18 and 1.15 g/cc respectively). This change in density may be due to changes in these organelle membranes associated with the rapid dehydration of the cotyledons, which begins to become quite pronounced on day 29 of development [17]. Protein bodies do not appear to be present in day 15 cotyledons (Figures 3 and 4) but are present in large amounts on day 21 [14,15] and day 29 (Figures 7-9) of development. This observation is in agreement with a report by Basha [1] that legumin and vicilin, the primary reserve proteins found in protein bodies, are synthesized in low amounts at early days of development of pea cotyledons.

LITERATURE CITED

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LEGENDS FOR FIGURES

Figure 1. Sucrose density gradient analyses of membrane-associated UDP-GlcNAc transferase activities isolated from pea cotyledons 15 days post anthesis and sedimented between 13,000 and 40,000 g.

A. A 2 ml sample of a control washed and suspended pellet was layered over a 36 ml sucrose gradient (15 to 60% sucrose, w/w). Centrifugation was for 15 hr at 82,500 g. Fraction size was 1.2 ml. UDP-GlcNAc transferase activity was assayed in the presence of 10 mM divalent cation.

B. The isolated crude pellet was washed in 5 mM EDTA and pelleted at the initial force. All further manipulations were described in 1-A. Transferase assays were performed in the presence of 10 mM divalent cation.

Figure 2. Sucrose density gradient analyses of membrane-associated GDP-Mann transferase activities isolated from pea cotyledons 15 days post anthesis and sedimented between 13,000 and 40,000 g.

A. Preparation of the gradient was identical to 1-A. GDP-Mann transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 3. Sucrose density gradient analyses of membrane-associated UDP-GlcNAc transferase activities isolated from pea cotyledons 15 days post anthesis and sedimented between 250-13,000 g.

A. Preparation of the gradient was identical to 1-A. UDP-GlcNAc transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 4. Sucrose density gradient analyses of membrane-associated GDP-Mann transferase activities isolated from pea cotyledons 15 days post anthesis and sedimented between 250-13,000 g.

A. Preparation of the gradient was identical to 1-A. GDP-Mann transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 5. Sucrose density gradient analyses of membrane-associated UDP-GlcNAc transferase activities isolated from pea cotyledons 29 days post anthesis and sedimented between 13,000-40,000 g.

A. Preparation of the gradient was identical to 1-A. UDP-GlcNAc transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 6. Sucrose density gradient analyses of membrane associated GDP-Mann transferase activities isolated from pea cotyledons 29 days post anthesis and sedimented between 13,000-40,000 g.

A. Preparation of the gradient was identical to 1-A. GDP-Mann transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 7. Sucrose density gradient analyses of membrane-associated UDP-GlcNAc transferase activities isolated from pea cotyledons 29 days post anthesis and sedimented between 250-13,000 g.

A. Preparation of the gradient was identical to 1-A. UDP-GlcNAc transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 8. Sucrose density gradient analyses of membrane-associated GDP-Mann transferase activities isolated from pea cotyledons 29 days post anthesis and sedimented between 250-13,000 g.

A. Preparation of the gradient was identical to 1-A. GDP-Mann transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

Figure 9. Sucrose density gradient analyses of membrane-associated GDP-Mann transferase activities isolated from pea cotyledons 29 days post anthesis sedimented between 250-13,000 g.

A. Preparation of the gradient was identical to 1-A. GDP-Mann transferase was assayed in the presence of 10 mM divalent cation.

B. Preparation was the same as 1-B. Transferases were assayed in the presence of 10 mM divalent cation.

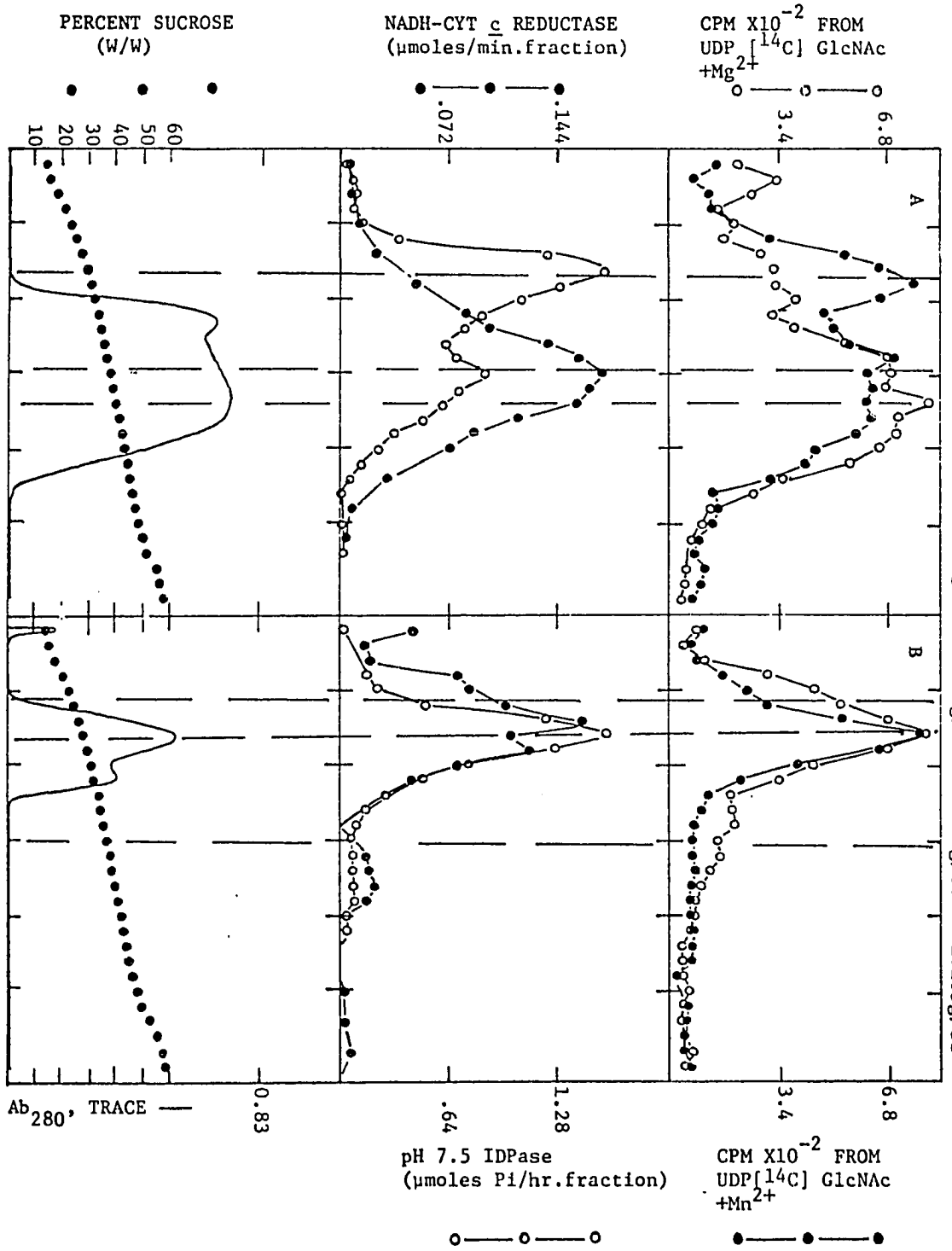
PERCENT SUCROSE
(W/W)
NADH-CYT c REDUCTASE
(μmoles/min.fraction)
CPM X10^{-2} FROM
UDP [^{14}C] GlcNAc
+Mg^{2+}
A
B
1.12g/cc
1.165g/cc
1.175g/cc
1.10g/cc
1.12g/cc
1.165g/cc
FRACTION NUMBER
Ab$_{280}$, TRACE
pH 7.5 IDPase
(μmoles Pi/hr.fraction)
CPM X10^{-2} FROM
UDP[^{14}C] GlcNAc
+Mn^{2+}

FIGURE 1

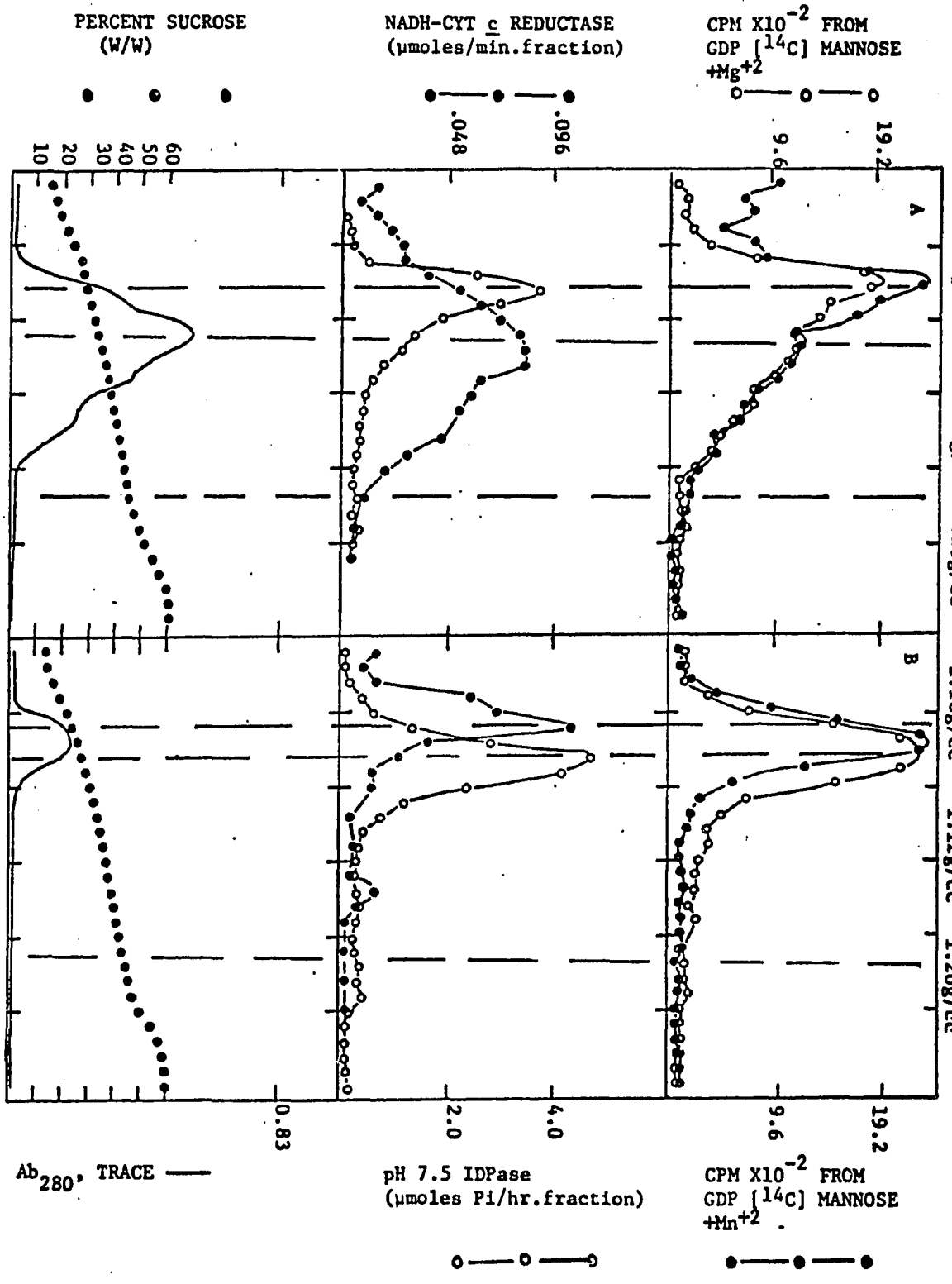
PERCENT SUCROSE
(W/W)
10
20
30
40
50
60
NADH-CYT c REDUCTASE
(μmoles/min.fraction)
.048
.096
CPM X10^{-2} FROM
GDP [^{14}C] MANNOSE
+Mg^{+2}
9.6
19.2
A
1.12g/cc
1.145g/cc
1.20g/cc
B
1.10g/cc
1.12g/cc
1.20g/cc
10
20
FRACTION NUMBER
10
20
0.83
2.0
4.0
9.6
19.2
Ab$_{280}$, TRACE
pH 7.5 IDPase
(μmoles Pi/hr.fraction)
CPM X10^{-2} FROM
GDP [^{14}C] MANNOSE
+Mn^{+2}

FIGURE 2

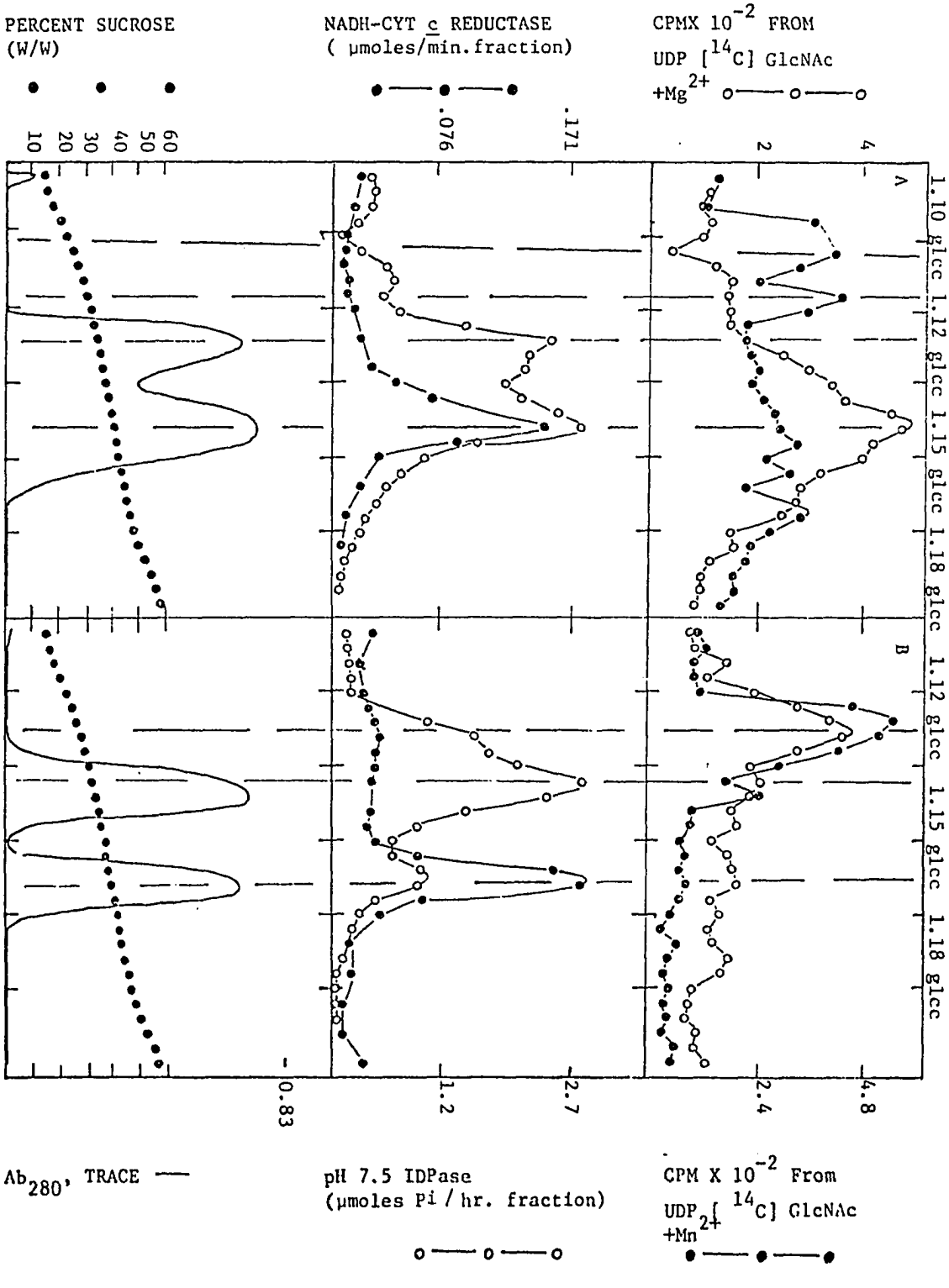
PERCENT SUCROSE
(W/W)
NADH-CYT c REDUCTASE
(μmoles/min. fraction)
CPMX 10^{-2} FROM
UDP [^{14}C] GlcNAc
+Mg^{2+}
1.10 glcc 1.12 glcc 1.15 glcc 1.18 glcc
1.12 glcc 1.15 glcc 1.18 glcc
Fraction Number
Ab_{280}, TRACE
pH 7.5 IDPase
(μmoles Pi / hr. fraction)
CPM X 10^{-2} From
UDP [^{14}C] GlcNAc
+Mn^{2+}

FIGURE 3

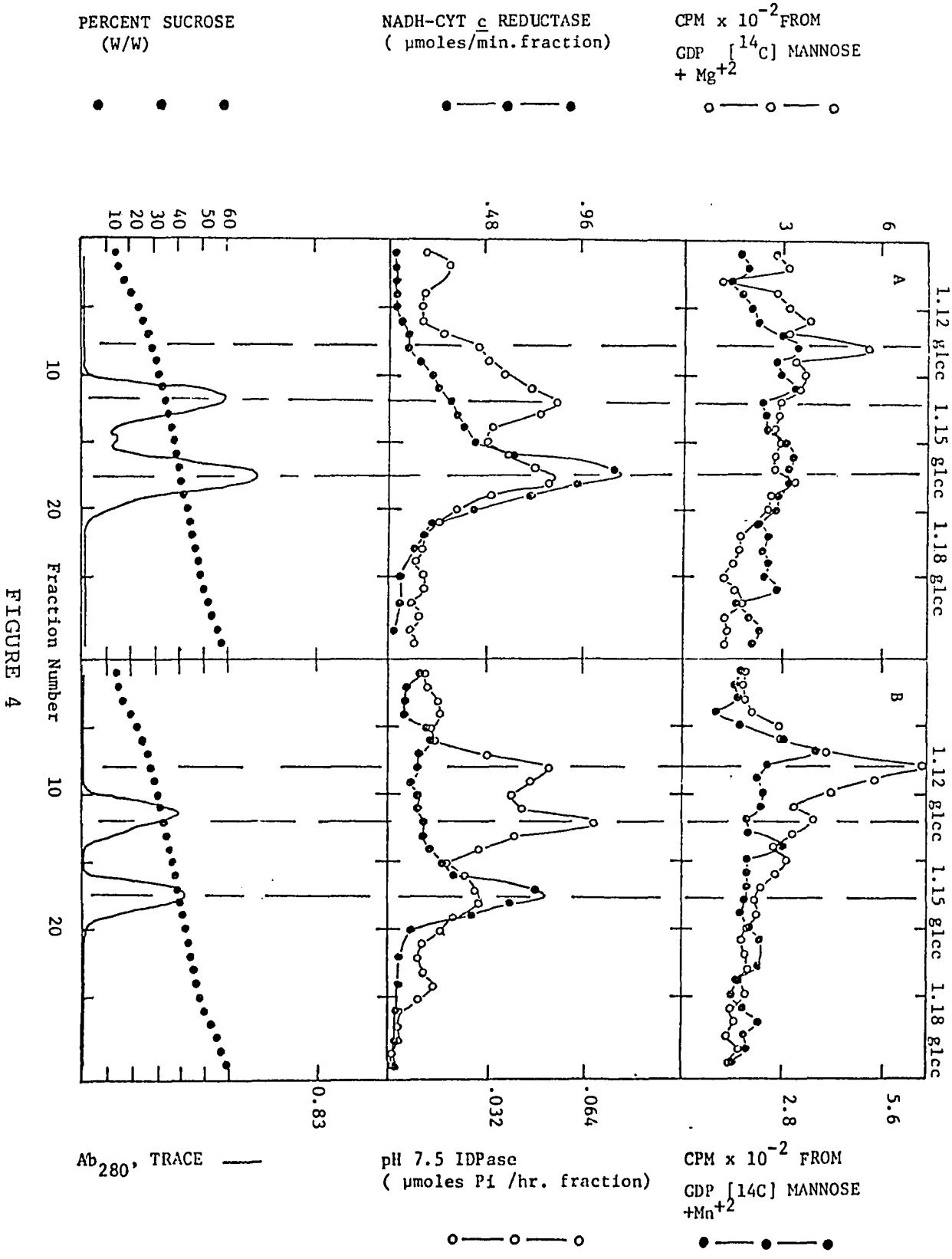
PERCENT SUCROSE
(W/W)
NADH-CYT c REDUCTASE
(µmoles/min.fraction)
CPM x 10^{-2} FROM
GDP [^{14}C] MANNOSE
+ Mg^{+2}
10 20 30 40 50 60
.48 .96
3 6
A
1.12 glcc 1.15 glcc 1.18 glcc
10 20 Fraction Number 10 20
B
1.12 glcc 1.15 glcc 1.18 glcc
0.83
.032 .064
2.8 5.6
Ab_{280}, TRACE
pH 7.5 IDPase
(µmoles Pi /hr. fraction)
CPM x 10^{-2} FROM
GDP [14C] MANNOSE
+Mn^{+2}

FIGURE 4

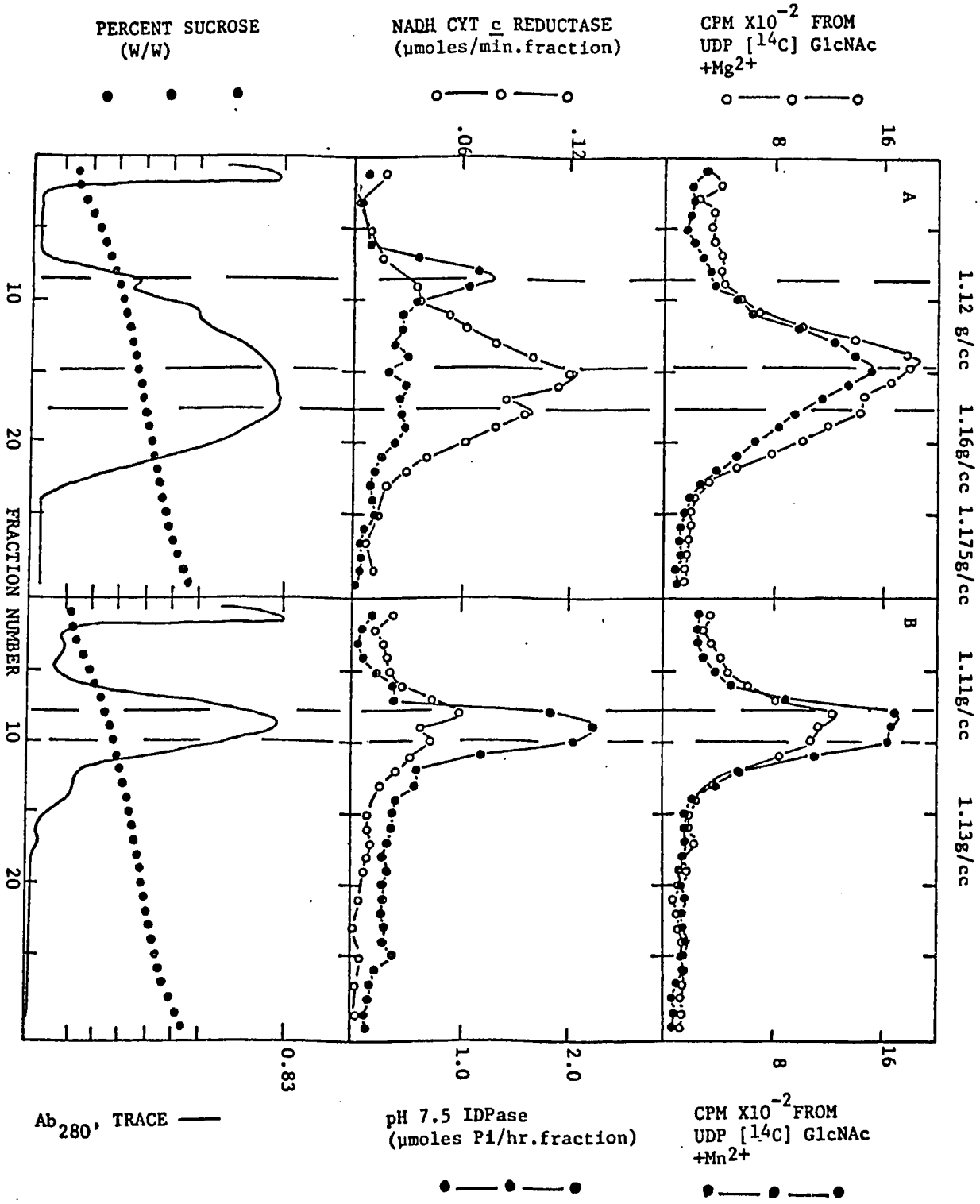
PERCENT SUCROSE
(W/W)
NADH CYT c REDUCTASE
(μmoles/min.fraction)
CPM X10^{-2} FROM
UDP [^{14}C] GlcNAc
+Mg^{2+}
.06
.12
8
16
A
1.12 g/cc
1.16g/cc
1.175g/cc
1.11g/cc
1.13g/cc
10
20
FRACTION NUMBER
10
20
B
0.83
1.0
2.0
8
16
Ab$_{280}$, TRACE
pH 7.5 IDPase
(μmoles Pi/hr.fraction)
CPM X10^{-2}FROM
UDP [^{14}C] GlcNAc
+Mn^{2+}

FIGURE 5

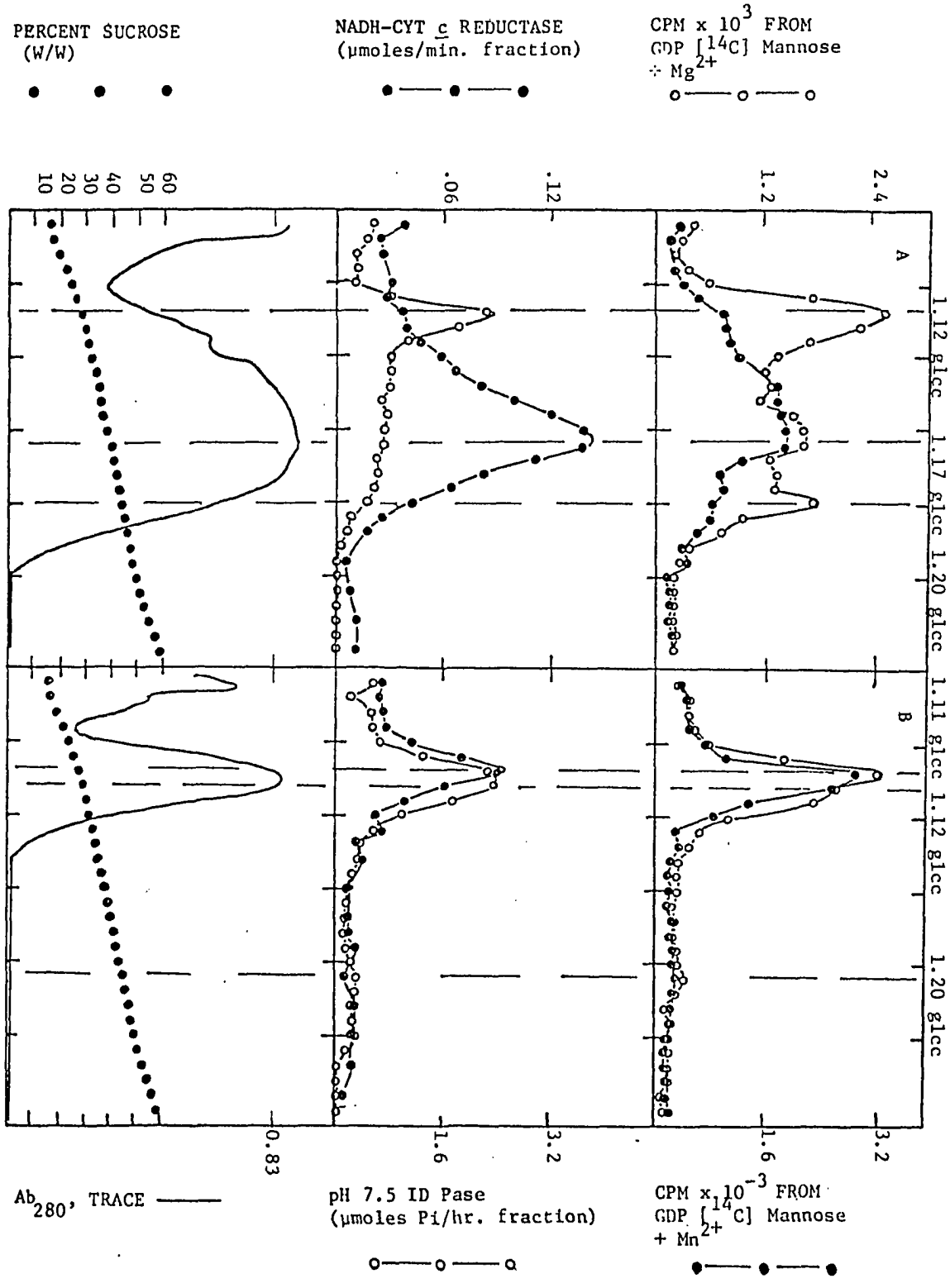
PERCENT SUCROSE
(W/W)
NADH-CYT c REDUCTASE
(μmoles/min. fraction)
CPM x 10^3 FROM
GDP [14C] Mannose
+ Mg2+
A
B
1.12 g/cc
1.17 g/cc
1.20 g/cc
1.11 g/cc
1.12 g/cc
1.20 g/cc
Fraction Number
Ab280, TRACE
pH 7.5 ID Pase
(μmoles Pi/hr. fraction)
CPM x 10^-3 FROM
GDP [14C] Mannose
+ Mn2+

FIGURE 6

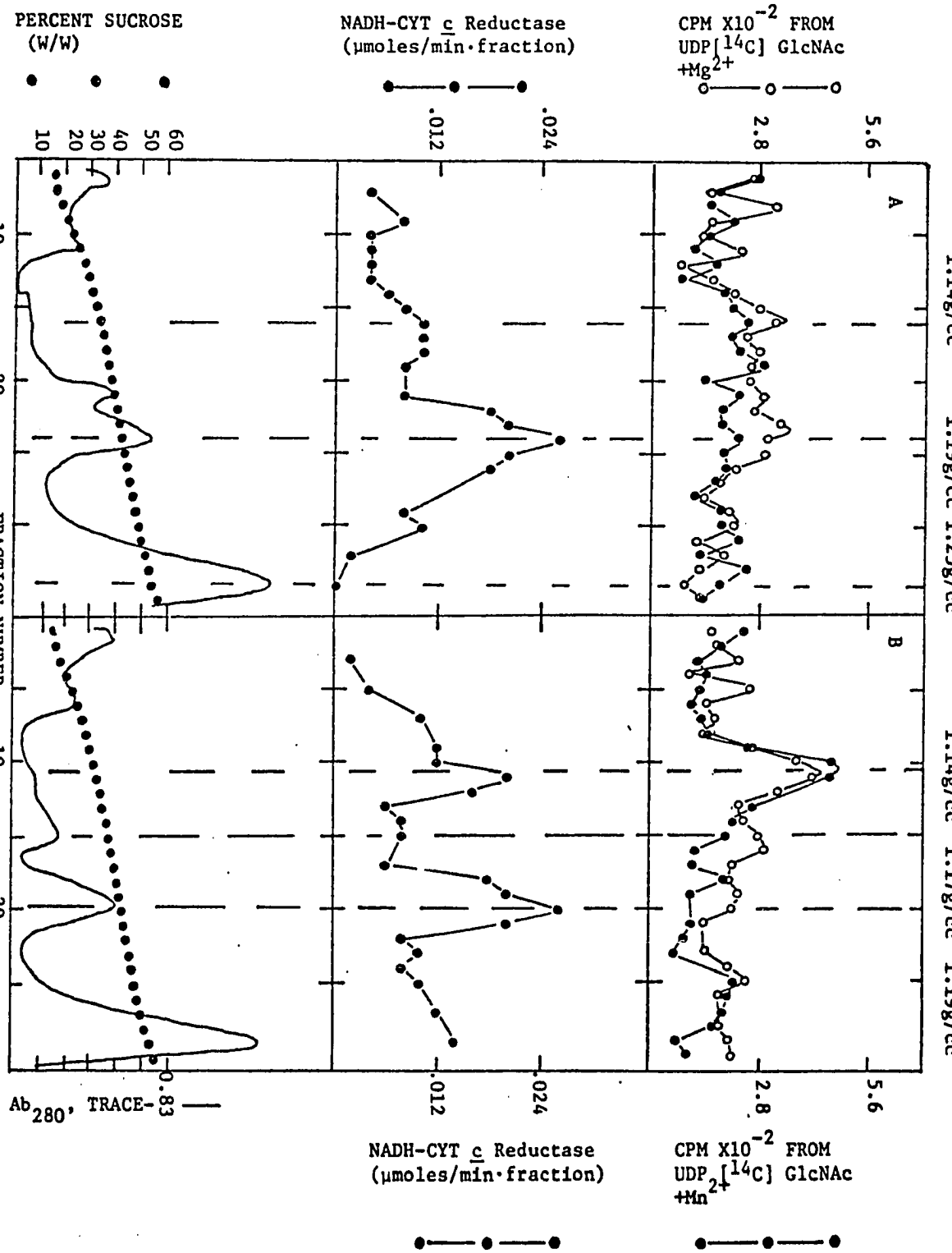
PERCENT SUCROSE
(W/W)
NADH-CYT c Reductase
(μmoles/min·fraction)
CPM X10^{-2} FROM
UDP[^{14}C] GlcNAc
+Mg^{2+}
10
20
30
40
50
60
.012
.024
2.8
5.6
A
B
1.14g/cc
1.19g/cc
1.25g/cc
1.14g/cc
1.17g/cc
1.19g/cc
FRACTION NUMBER
10
20
10
20
Ab$_{280}$, TRACE-
0.83
.012
.024
2.8
5.6
NADH-CYT c Reductase
(μmoles/min·fraction)
CPM X10^{-2} FROM
UDP$_2$[^{14}C] GlcNAc
+Mn^{2+}

FIGURE 7

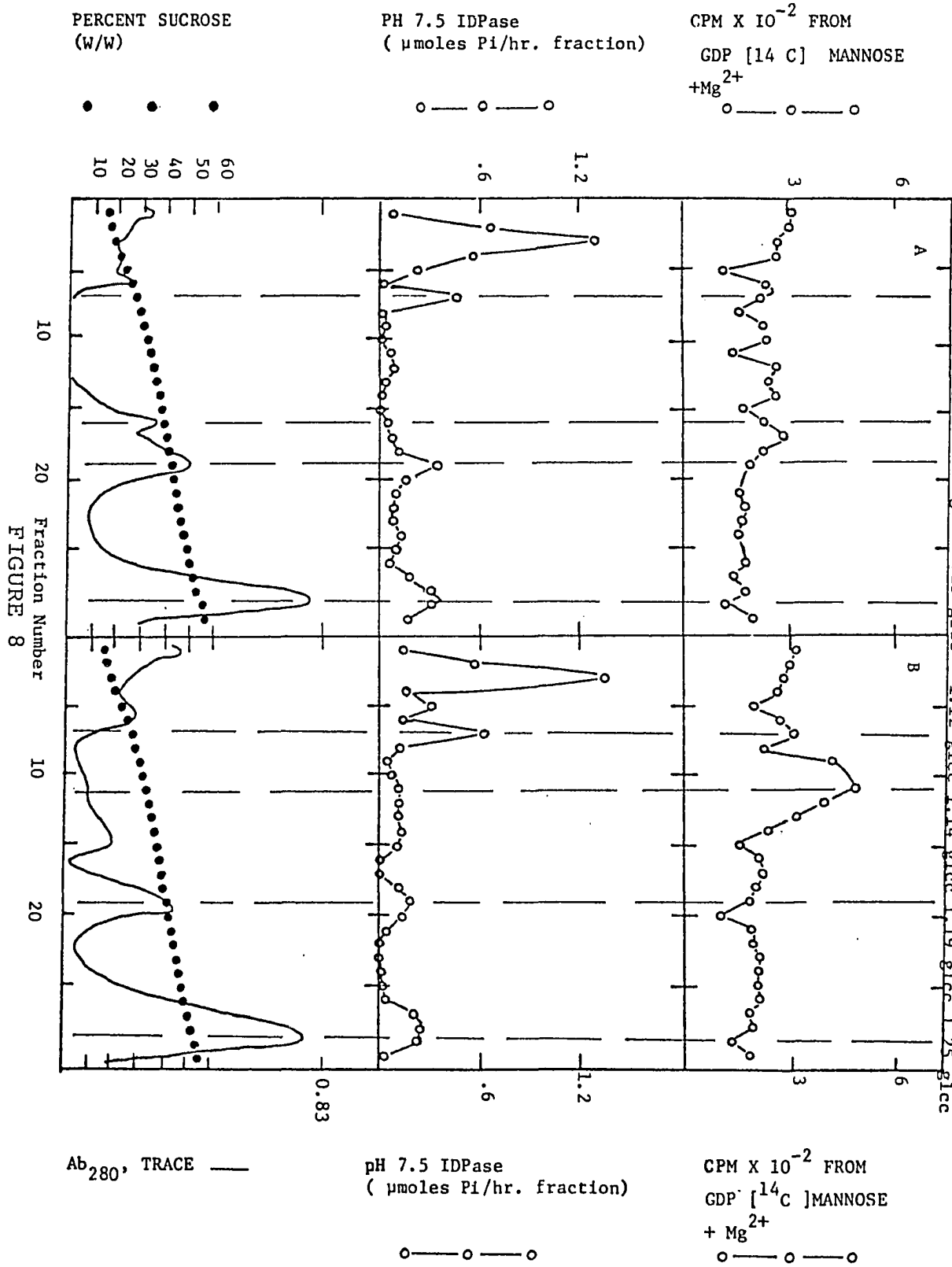
PERCENT SUCROSE (W/W)
PH 7.5 IDPase (µmoles Pi/hr. fraction)
CPM X IO^{-2} FROM GDP [14 C] MANNOSE +Mg^{2+}
10 20 30 40 50 60
.6 1.2
3 6
A
B
1.12 g/cc 1.17 g/cc 1.19 g/cc 1.25 g/cc
1.12 g/cc 1.14 g/cc 1.19 g/cc 1.25 g/cc
10 20 Fraction Number 10 20
0.83
Ab$_{280}$, TRACE
pH 7.5 IDPase (µmoles Pi/hr. fraction)
CPM X 10^{-2} FROM GDP [^{14}C]MANNOSE + Mg^{2+}

FIGURE 8

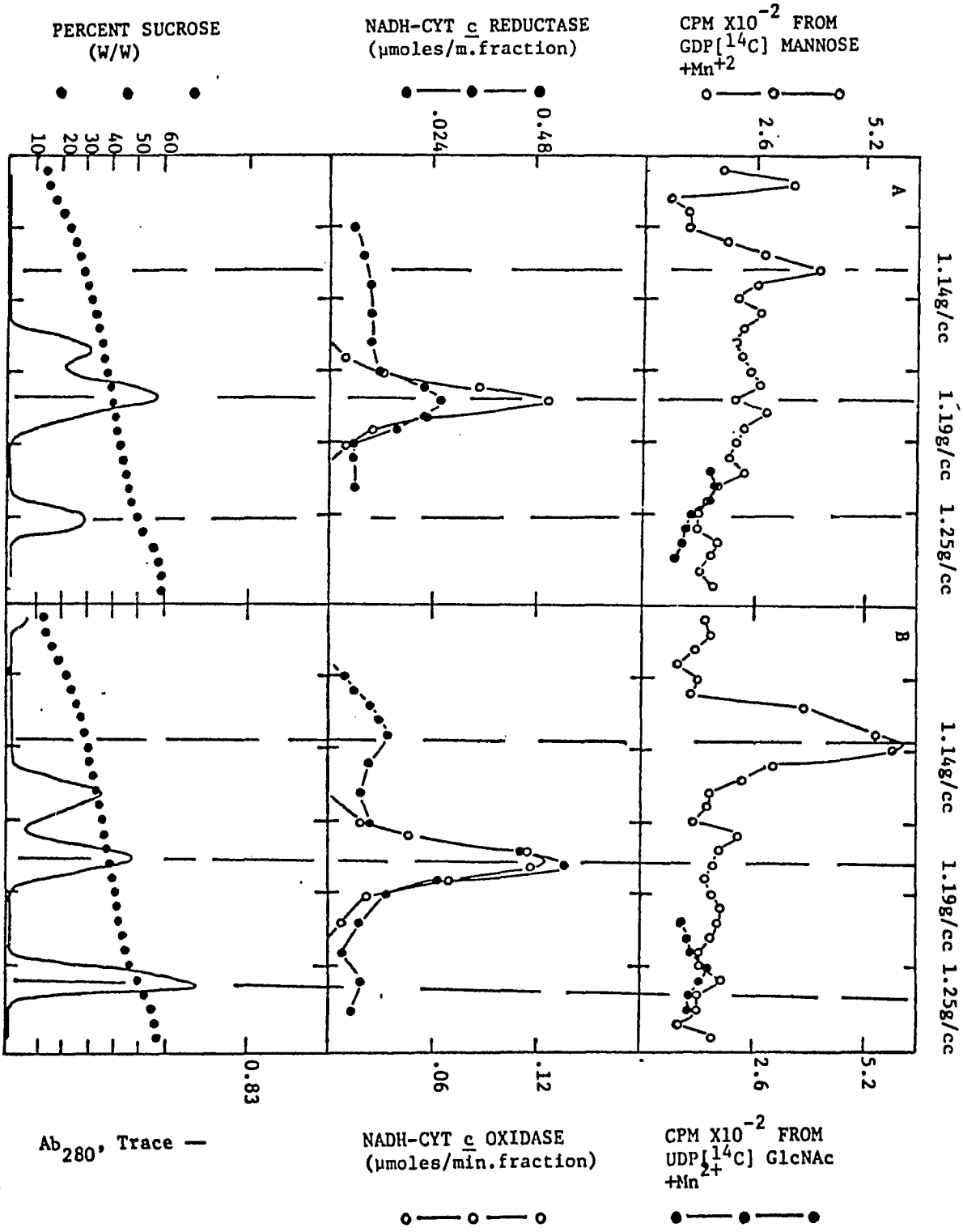
PERCENT SUCROSE
(W/W)
NADH-CYT c REDUCTASE
(μmoles/m.fraction)
CPM X10^{-2} FROM
GDP[^{14}C] MANNOSE
+Mn^{+2}
10
20
30
40
50
60
.024
0.48
2.6
5.2
A
B
1.14g/cc
1.19g/cc
1.25g/cc
FRACTION NUMBER
10
20
0.83
.06
.12
Ab$_{280}$, Trace —
NADH-CYT c OXIDASE
(μmoles/min.fraction)
CPM X10^{-2} FROM
UDP[^{14}C] GlcNAc
+Mn^{+2}

FIGURE 9