THESIS

LABELING OF NONSTRUCTURAL CARBOHYDRATES IN WINTER WHEAT STEMS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY MARY ELLEN HOGAN ENTITLED LABELING OF NONSTRUCTURAL CARBOHYDRATES IN WINTER WHEAT STEMS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

LABELING OF NONSTRUCTURAL CARBOHYDRATES IN WINTER WHEAT STEMS

Fructans are polymers of fructose, which contain a terminal or subterminal glucose moiety. The biosynthetic pathway for the levan-type of fructan, found in wheat, has not been elucidated. These compounds are a significant component of the nonstructural carbohydrate fraction in winter wheat stems. They accumulate when photosynthetic production exceeds their utilization.

Based on a study of the translocation of ^{14}C-sucrose in winter wheat, Robinson (44) suggested that the fructose moiety of translocated sucrose might be used preferentially for biosynthesis of fructans. The present study tested this hypothesis.

Six winter wheat plants were grown to anthesis, then labeled with ^{14}C-glucosyl, ^{3}H-fructosyl-sucrose for three hours. The plants were then dissected and extracted in boiling 80% ethanol for 48 hours. They were subsequently extracted with water (20^{o}C) for 48 hours. Water extracts were hydrolyzed with 2 M trifluoroacetic acid. The hydrolyzed samples were chromatographed using one-way, descending paper chromatography. Chromatograms were cut into one cm strips and eluted with water (20^{o}C). These eluates were analyzed using liquid scintillation spectroscopy.

The same technique was used to analyze aliquots of both the original ethanol and water extracts directly.

Results indicated that a greater amount of the ^{3}H from the fructose moiety of the translocated sucrose appeared in the water extracts, than in the ethanol extracts. Ratios of ^{3}H:^{14}C in the water extracts were greater than those in the supplied sucrose, indicating that the fructose moiety of translocated sucrose was used preferentially in the biosynthesis of water soluble fructans. This suggests that hexoses derived from translocated sucrose were not as readily interconverted in wheat stems as in other plants. The differing ^{3}H:^{14}C ratios of labeled fructans along the stem may indicate the degree of tissue maturity. Older stem sections contained greater amounts of labeled fructans.

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INTRODUCTION

Fructose polymers in grasses accumulate in the extended stem internodes and reach a maximum rate of synthesis near the time of anthesis. These polymers, called fructans, are synthesized from newly formed assimilates translocated to the stem (43,61). Studies of redistribution of these nonstructural carbohydrates in wheat during grain filling (66) show that, although very little is lost via respiration, the final contribution to ear dry weight is, at best, 10%. Ideally, one could increase yields by increasing this contribution to grain filling. In wheat, however, very little is known of either the biosynthesis of these polymers or the mechanism of remobilization. The present study was to determine the ratio of the hexose moieties from translocated sucrose incorporated into these nonstructural carbohydrates, the fructans.

LITERATURE REVIEW

I. Nonstructural Carbohydrates

The term 'nonstructural carbohydrates' refers collectively to monosaccharides, glucose and fructose, and oligosaccharides. The sugars are largely metabolic intermediates. Fructans, along with starch, are considered to be storage polysaccharides (59).

Nonstructural carbohydrates are an important source of reserve energy in perennial plants. These carbohydrates are stored in vegetative organs (stems, crowns, roots, tubers, corms, stolons, rhizomes, etc.) during times in the life cycle when photosynthesis exceeds utilization. Their remobilization becomes essential for winter survival, early spring growth initiation, and production of new tissues following stress. Much research has been done on reserve carbohydrates in grass species, since a knowledge of regrowth potential after fire, cutting, or grazing is vital to range management (59,67). Also, fructan remobilization in grasses during grain development is a possible source of yield improvement.

II. Fructan Accumulators

Taxonomic separations have been made on the basis of the types of nonstructural carbohydrates stored by different species. De Cugnac (59) first separated temperate grasses from tropical ones when he established that starch is the major storage carbohydrate in tropical grasses, while the temperate grasses accumulate fructans. Smith and Groteleuschen (56) separated the temperate grasses further on the basis of accumulation of short-chain versus long-chain fructans. However, the range of chain lengths in wheat fructans was not reported. Smith (60) and Ojima and Isawa (36) have shown that members of the Aveneae, Festuceae and Hordeae (Triticeae) tribes are all fructan accumulators. Hunter et. al. (28) surveyed the water soluble carbohydrates of ten species of tropical pasture grasses and legumes and found that none contained appreciable amounts of fructans. Archbold (3) reviewed all the data up to 1937 on the occurrence of fructans in grasses. He listed many fructan accumulating species, including the commercially important crops of rye, oats, and wheat.Some bacteria and fungal spores are also capable of producing fructans, if they have an adequate sucrose supply (26,65).

III. The Chemistry of Fructans

Fructans are straight chain polymers of fructose that contain a terminal or subterminal glucose residue (1,60).

They exist in two forms, known as the inulins and the levans. Inulins are present primarily in species of the Asteraceae and Campanulaceae families, while the levans occur in temperate grasses (1,3,27,60,67). The difference between the two forms of fructans is in the type of glycosidic linkage that connects the hexose units of the polymer. Inulins contain β-(2-1)-linked D-fructofuranose units and levans contain β-(2-6)-linked D-fructofuranose units (3,51,62,64).

Fructose polymers are unbranched molecules with a relatively low degree of polymerization. Although fructans appear in a homologous series in plant tissues, the range of chain lengths seems to be somewhat species-dependent. The longest chain fructans in grasses are generally found in stem bases and can vary from a degree of polymerization of 26, reported for bromegrass (Bromus inermis), to 260, in timothy (Phleum pratense) (7).

A terminology has developed to describe different types of fructans (53). Inulins are called 1-kestoses or isokestoses because they contain a terminal glucose moiety and a glycosidic linkage connecting C-1 of the first fructose moiety in the polymer to C-2 of the next one (Figure 1). Some levans are 6-kestoses or simply kestoses if the glucose moiety is terminal and glycosidic linkages between the fructose moieties are β-(2-6). Other levans are called neokestoses, if the glucose moiety is subterminal and the glycosidic linkages are β-(6-2), as shown in Figure 1. As the polymer is synthesized the glucose moiety remains near the non-reducing end of the molecule.

INULIN:

C-1 ⟷ C-2

1-kestose = isokestose = glucose-fructose-fructose

glucose-fructose-fructose = 1^{F}-fructosyl sucrose = a trisaccharide

LEVAN:

C-6 ⟷ C-2

A. 6-kestose = kestose = glucose-fructose-fructose

C-6 ⟷ C-2

B. neokestose = fructose-glucose-fructose

fructose-glucose-fructose = 6^{G}-fructosyl sucrose

Figure 1. Fructan nomenclature.

IV. Fructan Enzymology

Biosynthesis of fructans has been studied most extensively in the inulin-containing species *Helianthus tuberosus*. In 1968, Edelman and Jefford (20) presented a biosynthetic scheme in *H. tuberosus* involving two enzymes. The mechanism of action of these enzymes was worked out during $^{14}CO_2$ tracer studies (16). Sucrose-sucrose 1-fructosyltransferase (SST), a β-fructofuranosylase, formed the trisaccharide 1^F-fructosyl sucrose (1-kestose = isokestose, see Figure 1) by transferring the fructose moiety from one sucrose molecule to another via a dismutation reaction. This reaction is irreversible and has a high specificity for sucrose. However, the enzyme is unable to catalyze polymerization beyond the trisaccharide level because this and larger fructan polymers are inactive as acceptors for further transfer of fructose moieties from sucrose. Therefore, Edelman and Jefford proposed the involvement of the second enzyme: β-(2-1) fructan; β-(2-1) fructan 1-fructosyltransferase (FFT) (19). This enzyme transfers a terminal β-(2-1)-linked fructofuranosyl residue to the same position in another growing fructan chain. To date no work has been published indicating the existence of SST or FFT in monocots.

Since the work of Edelman and Jefford in the 1960's, similar enzymes have been isolated from other inulin-containing species, for example, *Asparagus officinalis* (53,54), *Taraxacum officinalis* (48), *Chicorium intybus* (9,10,11,55), and

Agave vera cruz (50,51). Their biosynthetic scheme seems to be corroborated, but so far there is no comparable evidence for the biosynthesis of the grass levans from trisaccharide kestoses.

For a time in the 1960's, Edelman and Jefford's model was challenged by several workers who reported the isolation of the nucleotide sugar UDP-fructose in Dahlia rosea and H. tuberosus (1,60). After further investigation, however, there was no direct evidence to support fructan biosynthesis from UDP-fructose (1,42). Therefore, the biosynthesis of fructans would seem to represent a unique mode of polysaccharide formation in higher plants (9). However, another group of plant oligosaccharides, the sugars of the raffinose family, are also formed via the transfer of a hexose moiety to a growing chain from a non-phosphorylated donor, galactinol (33).

Enzymology studies have also been done to elucidate degradative enzymes of fructans in dicots. Bacon and Edelman (5) first discovered the hydrolytic β-D-fructofuranosidases. These enzymes catalyze degradation of the fructan polymers in H. tuberosus, as well as other species containing β-(2-1)-linked fructosyl residues (47). They exert metabolic control over the concentrations of sucrose and higher fructose polymers in tissues at different times during the growing season. However, these enzymes were inactive in the degradation of bacterial levan, which is a β-(2-6)-linked polymer (18,29,42).

Edelman and Jefford (20) described two of these hydrolytic enzymes from H. tuberosus. They referred to them as β-(2-1)-fructan 1-fructanohydrolases. These enzymes break only the β-(2-1) linkage between a terminal fructosyl group and its adjacent residue. This reaction is faster if the degree of polymerization is lower. It is irreversible, and the final products are a mixture of sucrose and fructose. Sucrose acts as an inhibitor.

V. Fructan Solubility, Distribution, and Fluctuation

All levans are readily extracted in cold or hot water (60). However, fructans (including both inulins and levans) are also differentially soluble in ethanol (59,60). Short-chain fructans can be extracted with fairly high ethanol concentrations (70-95%) while longer chain fructans can be extracted with successively lower concentrations of ethanol. Fructans occur in a homologous series in plant tissues, with the degree of polymerization and the nature of other non-structural carbohydrate components differing among species. These facts must all be considered when designing an extraction procedure to analyze for the total non-structural carbohydrates in a plant.

Among the temperate grass species, the stem usually contains the highest concentration of fructans. These are concentrated in the lower regions of the stems, such as stem bases, stolons, corms and rhizomes (60,67). Concentrations

in leaf sheaths are usually higher than in leaf blades, although neither sheaths nor blades contain large amounts compared to stems.

Studies show both diurnal and seasonal fluctuations in fructan levels in different plant parts. Diurnal fluctuations follow the pattern of a bell-shaped curve; increasing steadily during the morning hours to a high point in mid-afternoon, followed by a decrease into the evening hours (14,60). Seasonal fluctuations seem to be influenced by the ratio of stems to leaves, which is in turn a characteristic of seasonal fluctuation. As a plant grows, the proportion of plant which is stem increases. This results from the photosynthate that is translocated to and accumulated in the stems. Fructan concentrations are related to the balance between photosynthesis, and respiration and growth. They accumulate when photosythetic production exceeds their utilization. Fructan levels may drop, however, if an unusual event (such as defoliation by herbivores or fire) creates a low carbohydrate supply. In this case, the photosynthate that would concurrently be produced would not be sufficient for regrowth and the stored reserves would be remobilized (60,67).

Seasonal fluctuation of fructans is also related to winter dormancy and spring re-growth in perennial plants. In H. tuberosus there is a gradual decrease in degree of polymerization of fructans during winter. In the spring, most fructans are hydrolyzed during tuber sprouting,

providing substrate for early growth (18,20,29). Similar trends occur in Taraxacum officinale (49). Rutherford and Flood (47) showed that activities of enzymes responsible for fructan hydrolysis correlate well with seasonal fluctuations in degree of polymerization. Pollock and Jones (40) and many others showed a comparable pattern of fluctuation for temperate grasses (3,6,21,32,34,40). The major period of fructan accumulation was autumn and again at the time of anthesis, with hydrolysis occurring between January and April. Smith (57) found that long-chain fructans prevail during times of storage, while shorter chains become abundant during periods of rapid growth. Therefore, fructans are stored energy reserves.

Fructan concentrations are also affected by environmental conditions. The most widely studied environmental parameter is temperature. Workers analyzing various inulin-containing species observed hydrolysis of fructans during storage at low temperatures (12,37,46). However, Eagles (17) and Pollock (38,41) found that leaf sheath fructans accumulated at low temperatures in Dactylis glomerata. Smith (58) also observed a greater fructan accumulation at low temperatures in Phleum pratense L., a temperate forage grass. Edelman and Jefford (20) noted that many plants which contain fructans are normally exposed to a cold or dry period during their life cycle. They suggested that a possible stress resistance mechanism is operating. Fructan synthesis and hydrolysis can cause a fairly rapid change

in the size and number of sugar molecules in a cell, thus inducing changes in osmotic potential which afford protection from injury caused by freezing or desiccation (20).

VI. Fructans in Wheat

Archbold (3) cited five studies which showed the existence of fructans in the leaves, stems, and grain of wheat. Other reviewers have mentioned this, but report no work on fructan biosynthesis in the wheat plant.

Judel and Mengel (30) analyzed the content of nonstructural carbohydrates in leaves and culms of spring wheat during grain filling. They observed that the fructan concentration increased in leaves and culms until two to three weeks after anthesis. McCraig and Clarke (35) studied nonstructural carbohydrate levels in the vegetative tissues of wheat and oats. The highest concentration of fructans was observed in the stems, and they suggested that these compounds were synthesized between the times of maximum photosynthesis and maximum remobilization during grain filling. However, other workers have regarded the reserve nonstructural carbohydrates in wheat stems to be an unused yield potential under optimal growing conditions. Only under stress conditions would the contribution of fructans to grain filling become significant (31).

At present, our understanding of the biosynthesis and remobilization of the fructans in wheat is incomplete. Further elucidation of these pathways is necessary before nonstructural carbohydrate partitioning can be altered to improve grain yields.

MATERIALS AND METHODS

I. Plant Material, Labeling Experiments and Harvesting

Foundation grain of Triticum aestivum L. var. "Duke" was obtained from the Colorado State Seed Laboratory at Colorado State University, Fort Collins, Colorado 80523. Seeds were germinated in 9 cm petri dishes lined with two layers of Whatman No.1 filter paper. Dishes containing the grain were kept in the dark and watered with a solution of Terra-Coat, a commercial fungicide. When the radicles were 0.5 to 1.0 cm long the seedlings were subjected to an eight week cold treatment ($4^{o}C$) to induce vernalization. Vernalized seedlings were planted in 8 inch pots in a soil mixture containing 33% top soil, 33% peat moss, 12% perlite, 12% vermiculite and 10% sand. The plants were thinned to two or three per pot at tillering.

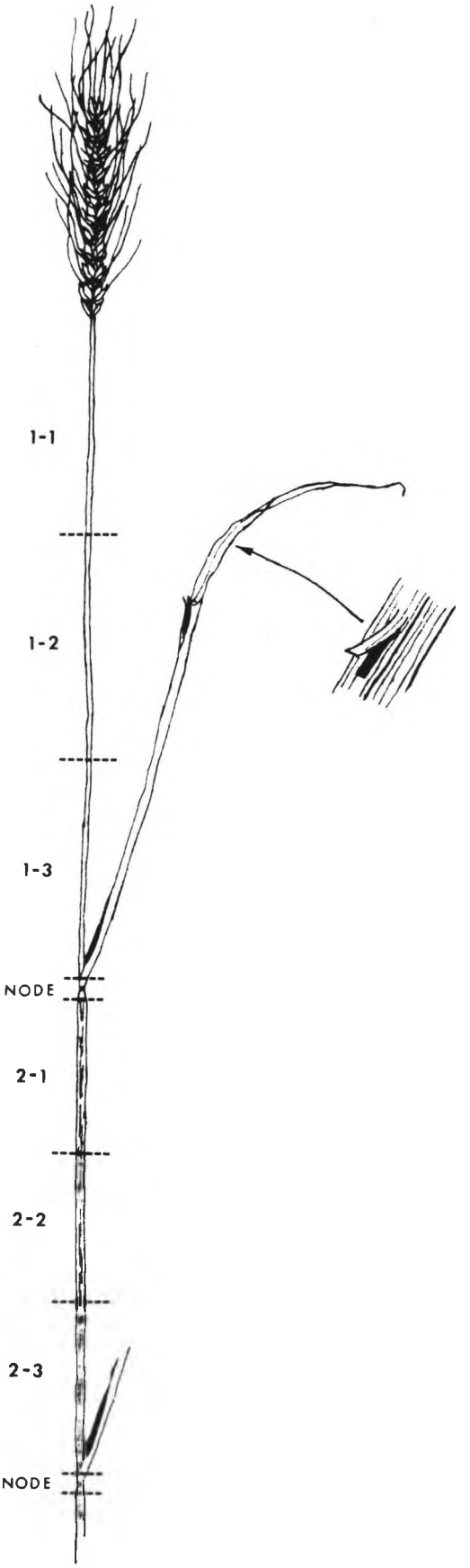
Plants were grown in an Environator Growth Chamber with a 16 hour daylength, a night temperature of $16^{o}C$ and a day-time temperature of $24^{o}C$. The light source was a bank of 12 Westinghouse fluorescent lamps (48T12/CW/HO) plus eight GE 100 watt incandescent bulbs. Irradiance levels were measured with a Li-Cor LI-170 Quantum/Radiometer/Photometer. They ranged from 170 to 200 $\mu E\ m^{-2}\ s^{-1}$ (PAR), at the level of the flag leaves. Plants were watered as needed with a mineral nutrient solution (45).

Six labeling experiments were conducted at anthesis (i.e. anthers were visible above the glumes). The duration of each experiment was three hours, using (glucose-^{14}C (U))-sucrose and (fructose-1-^{3}H(N))-sucrose in combined aliquots, (i.e. asymmetrically-labeled sucrose). The (glucose-^{14}C(U))-sucrose stock solution contained 201.18 μCi/μmol and the (fructose-1-^{3}H(N))-sucrose stock solution contained 11.4 X 10^{3} μCi/μmol. The two were mixed for each experiment in the ratios indicated in Table 1.

All radio-labeled sugar aliquots were dissolved in an equivalent amount of 50 mM N-morpholino ethane sulfonic acid (MES) buffer at a pH of 5.2, to facilitate phloem loading (22). A small aliquot of this supplied radio-label was analyzed to determine the ratio of ^{3}H:^{14}C (see Table 1). Solutions were supplied to the flag leaf of each plant via the reverse flap method of Biddulph (8). The solution was taken up by the plant in less than three hours; thereafter the microcapillary tube was refilled with buffer for the remainder of the three hours.

After each experiment, the plant was harvested by removing it from the pot at ground level. The flag leaf (above the flap) and the spike were discarded and the rest of the plant was dissected as indicated in Figure 2. The leaf sheath of the flag leaf was kept for analyses, but all other leaf tissues were discarded. The length of each internode was measured and cut into two, three, or four separate sections. After excising, each section was placed in boiling 95% ethanol for a few minutes and then stored at -20^{o}C.

Figure 2. Harvesting scheme for dissection of wheat plants. Insert is a detail of the reverse flap method. The same numbering system was followed for internodes 3 and 4 (not shown).


1-1
1-2
1-3
NODE
2-1
2-2
2-3
NODE

II. Extraction, Chromatography and ^{3}H:^{14}C Determinations

Significant ethanol-soluble sugars were extracted initially when the stem sections were placed in boiling 95% ethanol. The excised sections from each plant were further extracted for 48 hours in 80% ethanol using a micro-soxhlet apparatus. Aliquots of the extracts were analyzed. If the amount of radioactivity did not differ significantly from background no plant parts more distant from the supply point were extracted. Extraction beyond 48 hours yielded no additional radioactivity above background. Sufficient extraction of the ethanol-soluble sugars was assumed to have occurred.

A 0.5 ml aliquot of each ethanol extract was removed and placed in 15 ml of dioxane cocktail (4 g PPO, 0.05 g POPOP, and 100 g naphthalene per liter of dioxane). These aliquots were then analyzed on a dual channel Beckman LS 7500 microprocessor controlled liquid scintillation counter.

One ml aliquots of some of the ethanol extracts were batch-extracted with a slurry containing anion and cation exchange resins (Fischer Resyn brand). The aliquots were stirred in this slurry for five minutes on ice, then the beads were filtered off and rinsed three times with 50% ethanol. The resin-cleaned ethanol extracts were evaporated to dryness. The residue was then washed with 0.2 ml of 80% ethanol, and the wash was spotted on Whatman No.1 chromatography paper (18" long). Sucrose, glucose and fructose standards were also spotted on separate chromatograms.

Chromatography was performed using two-dimensional descending chromatography. The first solvent was butanol:acetic acid:water (2:1:1) for 12 hours and the second solvent was t-butanol:methyl ethyl ketone:water:ammonia (4:3:2:1) for 12 hours. Two of these chromatograms were prepared for each extract. One was sprayed with the sugar spray described below. A second was placed on blue brand x-ray film. Some of the chromatograms placed on film were first dipped in a solution of toluene containing 20% (w/v) PPO to enhance the exposure of film in the area of any radioactive spots. After the spots were determined on the chromatograms with sugar spray, comparable, unsprayed chromatograms were matched to them. Sugar spots were cut from the unsprayed chromatograms. Each sample was eluted for 24 hours with 2 ml of distilled water on a shaker bath. A 0.5 ml aliquot of this eluate was placed in dioxane cocktail and analyzed to determine the ratio of ^{3}H:^{14}C in each of the spots.

Each excised plant section that had been previously extracted with ethanol was then extracted for two, 24 hour periods in one ml of distilled water at room temperature. Each small beaker containing the tissue and water was shaken continuously during this extraction. The two extracts were combined and a 0.5 ml aliquot was analyzed in dioxane cocktail. This extract contained water-soluble fructans.

Another 0.5 ml aliquot of each water extract was evaporated to dryness in a hood under a steady air flow. Each sample was then hydrolyzed with one ml of trifluoroacetic acid (TFA) (2) and again evaporated to dryness.

The resulting residue was rinsed three times with 80% ethanol (0.9 ml total), and each wash was streaked onto the origin of Whatman No. 1 chromatography paper (18" X 3.5"). Sidestrip origins on these chromatograms were spotted with standards of glucose, fructose and sucrose.

Descending chromatography was performed in butanol: ethanol:water (9:1:10, v/v/v, organic phase) for five days. After the chromatograms were dry, the sidestrips were cut off and the chromatogram itself was cut into one cm numbered pieces. Each piece was extracted in 2 ml of distilled water for 24 hours on a shaker bath. A 0.5 ml aliquot of each water extract was analyzed in dioxane cocktail, to determine the ratio of ^{3}H:^{14}C in each of the hexose moieties. The sidestrips were sprayed with a reagent to determine the location of the sugar standards. This reagent was sensitive to glucose, fructose, and sucrose and produced yellowish-brown spots. The reagent consisted of 80 ml of 95% ethanol, 10 ml of 40% trichloroacetic acid (w/v), and 10 ml of glacial acetic acid. It was saturated with benzidine hydrochloride.

RESULTS AND DISCUSSION

I. Analysis of Ethanol Extracts

One half ml aliquots were taken from each ethanol extract and analyzed directly. (The H-number method of quench correction was used. H-numbers are a relative measure of the efficiency of detection of radioactivity. H-number values and quench curves for both isotopes are located in Appendix I.) These data provide a profile of the disintegrations per minute (dpm) in each of the two isotopes (^{3}H and ^{14}C) for every section of the plant sampled. The ratio of activity in ^{3}H:^{14}C for every section is listed in Table 1, and the raw data from which these ratios were calculated is listed in Appendix II. It is seen from Table 1 that the ratios of ^{3}H:^{14}C were similar for each stem section within one plant and these ratios match closely the ratio of ^{3}H:^{14}C in the supplied sucrose (Table 1). The standard deviation among these ratios did not exceed 0.32 for any experiment. Therefore, the total ethanol-soluble sugars in this extract were labeled in a pattern that matched closely the pattern of label in the exogenously supplied sucrose. Further analysis by chromatography was necessary to determine the labeling pattern in the individual sugars in this extract.

Table 1. Ratios of $^{3}H:^{14}C$ from the ethanol extracts. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	PLANT					
SAMPLE	1	2	3	4	5	6
Blade	*	1.92	6.67	4.50	4.25	4.01
Leaf Sheath	4.12	2.07	6.71	3.03	4.06	4.04
Stem Sections						
1-1	4.47	1.74	6.94	3.89	4.19	4.25
1-2	4.70	2.46	7.03	4.00	4.31	4.56
1-3	4.49	2.21	7.02	3.85	3.97	4.22
1-4	*	*	*	*	*	4.24
2-1	4.51	2.18	7.00	4.02	3.81	4.34
2-2	4.50	1.79	7.29	4.09	3.74	4.34
2-3	4.48	1.86	7.33	4.02	3.78	4.29
3-1	4.77	**	6.91	3.83	3.75	4.10
3-2	4.69	**	6.72	3.74	3.78	4.19
3-3	*	**	6.65	*	3.79	*
4-1	4.49	**	6.77	3.75	**	4.51
4-2	4.70	**	6.64	3.80	**	**
4-3	**	**	6.63	**	**	**
5-1	**	**	6.33	**	**	**
Supplied Sucrose	5.04	2.67	6.00	3.85	3.62	4.05
Standard Deviation (tissue)	0.21	0.30	0.32	0.32	0.24	0.18

* = No Sample; ** = No Activity

After removal of ions using ion exchange resins, the ethanol extracts were chromatographed, using two-way descending chromatography. Comparable chromatograms were prepared with standards of glucose, fructose, and sucrose. There was not sufficient ^{14}C in the sugars for detection by autoradiography, therefore compounds were located on the chromatograms by means of the sugar spray. Three or four separate spots appeared on each chromatogram. When compared with the standards, the spots corresponded to glucose, fructose, sucrose, and in some cases a fourth, unknown sugar. This fourth sugar was not identified but its location on the chromatogram suggests that it represents a trisaccharide. No short-chain fructans larger than this possible trisaccharide were detected on any chromatogram. This result agrees with the observation that only short-chain fructans are soluble in high concentrations of ethanol (59,60). Upon elution of these compounds, it was found that the radioactivity was not high enough above background to determine accurately the $^{3}H:^{14}C$ ratios in the sugars. This low level radioactivity might be explained by the labeling protocol. There could have been a high turnover rate in these ethanol soluble compounds between the end of the labeling experiment and the time of harvest. Significant label may already have gone into the synthesis of longer chain fructans.

II. Analysis of Water Extracts

A. Unhydrolyzed Samples

As mentioned previously (pg 8) fructans are the only carbohydrates in significant quantity which are extractable with water following an ethanol extraction. One half ml aliquots were taken from the water extracts of the plant sections and analyzed directly for labeled fructans. Table 2 lists the ratio of $^{3}H:^{14}C$ for every section of the plants sampled. (Raw data are listed in Appendix II). Again, the ratios were similar for each stem section within one plant, although they did not match the $^{3}H:^{14}C$ ratio in the supplied sucrose as closely as the unhydrolyzed ethanol samples. The standard deviation between these ratios ranged from 0.18 to 0.94 in these experiments.

In all the experiments (except #5) the greatest amount of label was found in the internode sections directly above and below the node of insertion of the flag leaf (Appendix II). This observation concurs with the work of Robinson (44) for the ethanol fractions, and in the present experiments it was true for the water fractions as well. In Plant 5, however, the greatest amount of label was found in the internode sections surrounding the node of insertion of the second leaf (one node below the flag leaf).

From the work of Robinson (44) one would predict that this water extract would have a greater ratio of $^{3}H:^{14}C$ when compared with the ratios of the supplied sucrose and that of the ethanol extract. Since these data (Table 2)

Table 2. Ratios of $^{3}H:^{14}C$ from the unhydrolyzed water extracts. In stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	PLANT					
SAMPLE	1	2	3	4	5	6
Blade	*	1.03	5.67	4.02	4.17	3.95
Leaf Sheath	2.65	2.00	6.52	3.06	3.07	4.10
Stem Sections						
1-1	3.11	3.61	8.21	3.17	3.87	4.31
1-2	3.84	2.50	6.28	3.19	3.65	4.19
1-3	3.61	1.98	6.14	3.49	3.17	4.25
1-4	*	*	*	*	*	4.24
2-1	4.22	2.03	5.84	4.21	3.11	4.17
2-2	4.20	2.50	6.13	3.96	3.53	4.34
2-3	4.31	2.87	5.41	4.09	3.62	4.28
3-1	4.39	**	5.60	3.66	3.72	4.24
3-2	4.51	**	5.47	4.04	3.67	4.46
3-3	*	**	6.52	**	3.63	**
4-1	3.75	**	6.41	**	**	4.58
4-2	4.04	**	6.52	**	**	**
4-3	**	**	5.46	**	**	**
5-1	**	**	6.48	**	**	**
Supplied Sucrose	5.04	2.67	6.00	3.85	3.62	4.05
Standard Deviation (tissue)	0.94	0.72	0.68	0.60	0.35	0.18

* = No Sample; ** = No Activity

did not conform to this expectation, the water-soluble fructans were hydrolyzed to determine if there was any segregation of radioactivity within the moieties.

B. Hydrolyzed Samples

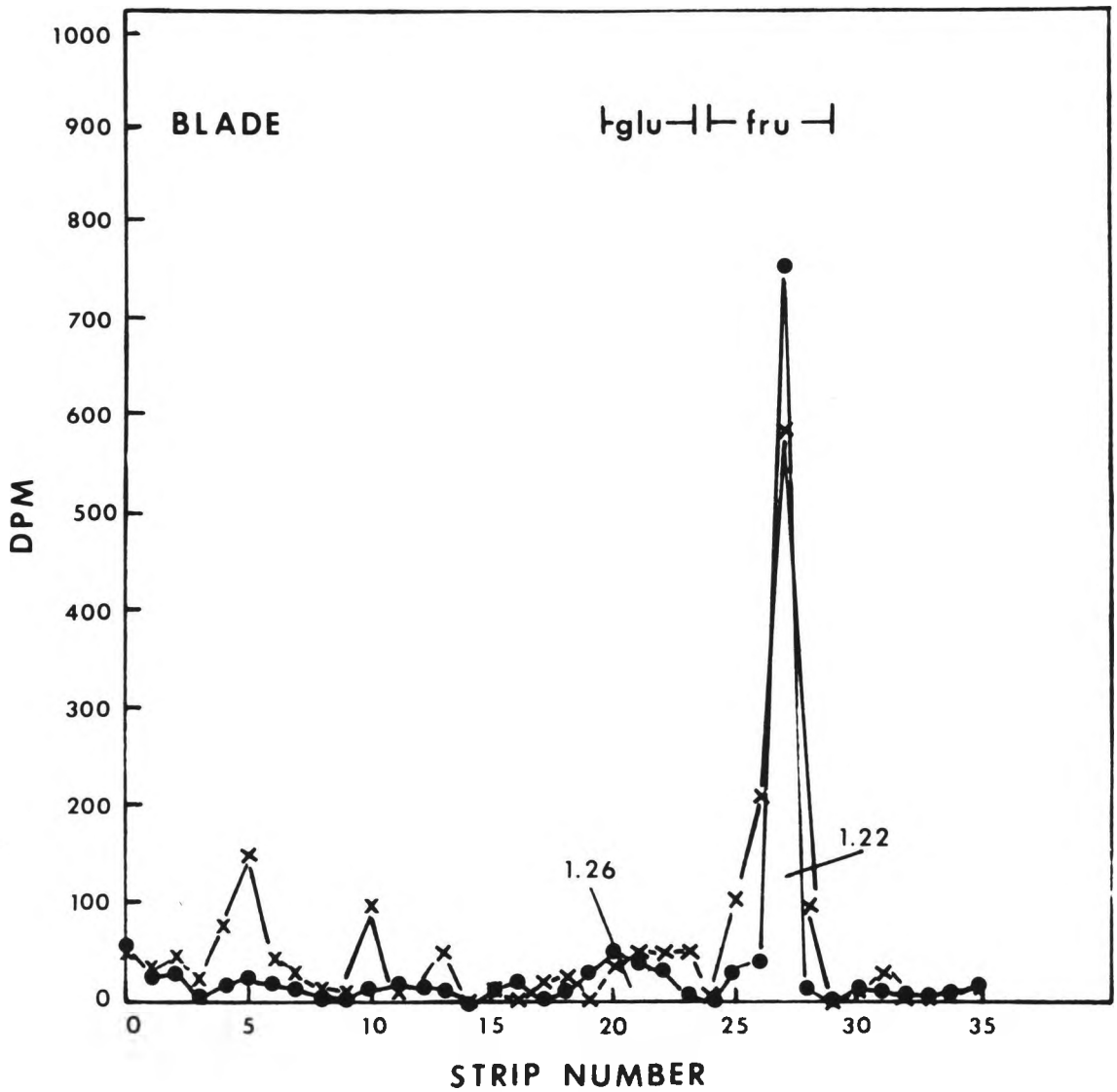
To obtain the labeling pattern in the fructans, aliquots of the water extracts were hydrolyzed, chromatographed, eluted and then analyzed. Figures 3 through 16 represent profiles of the dpm in each one cm chromatogram strip. Data from all of the sections from Plant 3 are shown. Table 3 lists the same type of data for the sections above and below insertion of the flag leaf for the other five experiments. Positions for the two radioactive peaks representing glucose and fructose were determined from the positions of sugar standards on the sidestrips. The ^{3}H:^{14}C ratio in each peak was calculated and is shown on the figures.

It should be noted that in some samples, a significant amount of label remained at the origin on the chromatogram, or in the sections nearer the origin than the glucose peak. A second hydrolysis with 2 M trifluoroacetic acid (TFA) yielded radioactivity only in glucose and fructose, indicating that these compounds are partial hydrolysis products from the first TFA treatment. When this procedure was followed, the ratios in the glucose and fructose peaks were not significantly altered, showing that the compounds at the origin were most likely partially hydrolyzed fructan polymers. When the total dpm for each one cm chromatogram

Table 3. Ratios of $^{3}H:^{14}C$ from the hydrolyzed water extracts. Stem sections 1-3 and 2-1 (above and below the node of insertion of the flag leaf, respectively) are listed for each experiment.

Experiment/ Sample	$^{3}H:^{14}C$ Supplied	$^{3}H:^{14}C$-glucose	$^{3}H:^{14}C$-fructose
Plant 1			
1-3	5.04	0.38	5.02
2-1		0.56	20.63
Plant 2			
1-3	2.67	0.72	2.60
2-1		0.21	44.50
Plant 4			
1-3	3.85	0.49	3.80
2-1		0.87	18.92
Plant 5			
1-3	3.62	0.59	3.63
2-1		0.19	36.55
Plant 6			
1-3	4.05	0.42	4.04
2-1		0.37	27.31

Figures 3-16. DPM in ^{3}H and ^{14}C in the one cm strips of chromatograms of the hydrolyzed water extracts. All the sections of Plant 3 are shown Positions of the sugar standards and ratios of ^{3}H:^{14}C in each peak are indicated. (●——● = ^{14}C, x—— x = ^{3}H) Numbers of sections correspond to the scheme in Figure 2.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glu
fru
1.26
1.22
DPM
STRIP NUMBER
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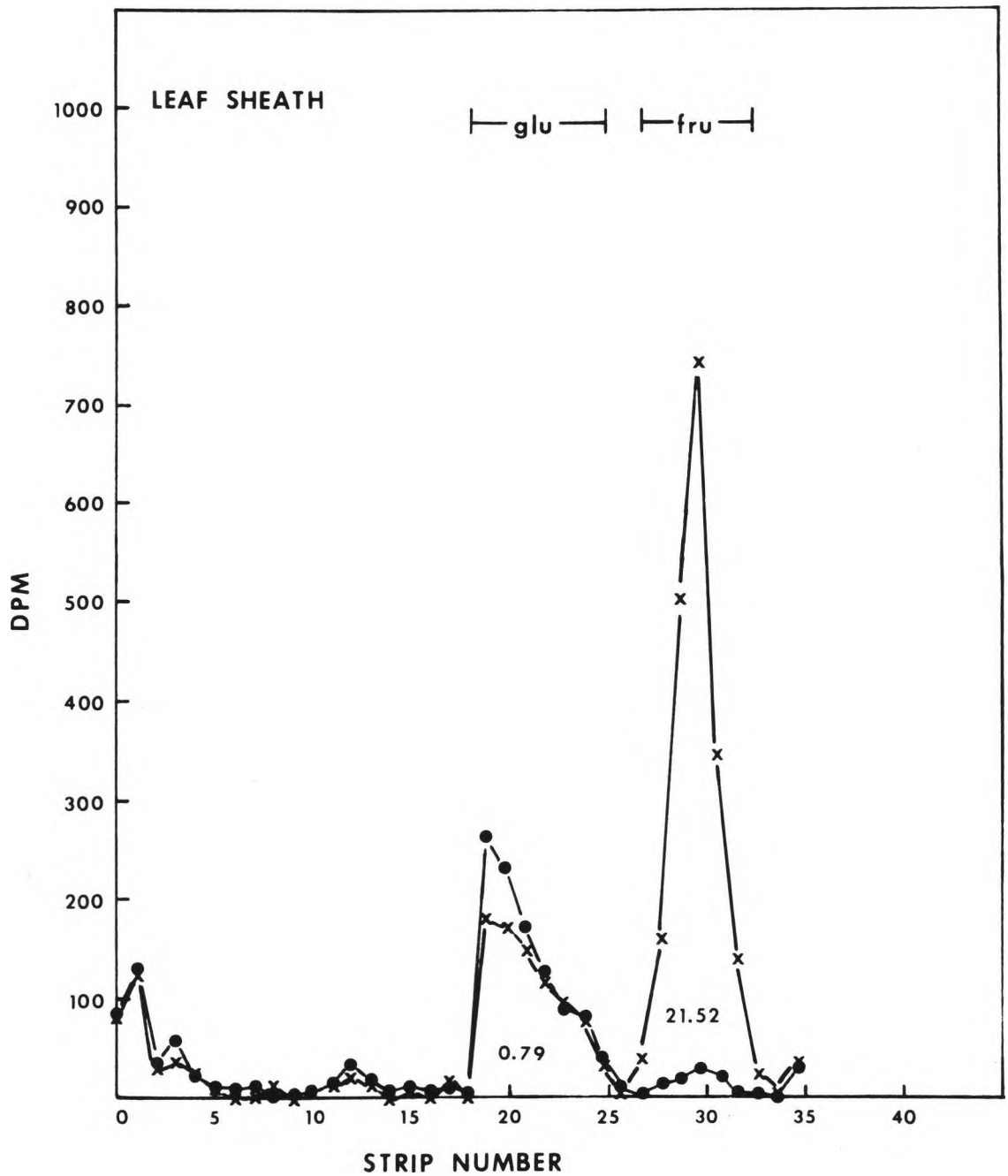
100
200
300
400
500
600
700
800
900
1000
0
5
10
15
20
25
30
35

Figure 3.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glu
fru
1000
900
800
700
600
500
400
300
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100
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5
10
15
20
25
30
35
40
STRIP NUMBER

Figure 4.

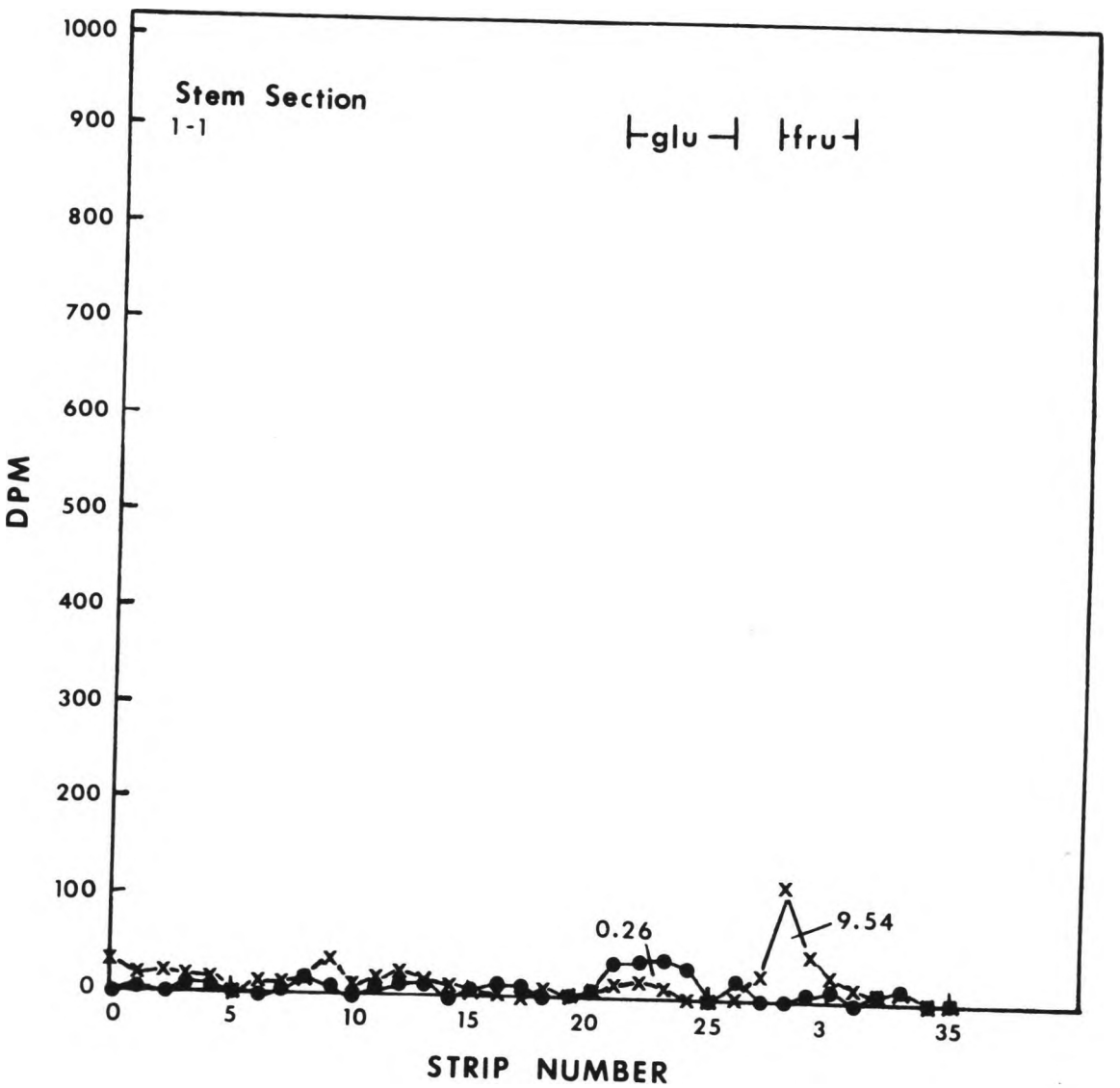

Stem Section
1-1
glu
fru
DPM
1000
900
800
700
600
500
400
300
200
100
0
0.26
9.54
0
5
10
15
20
25
3
35
STRIP NUMBER

Figure 5.

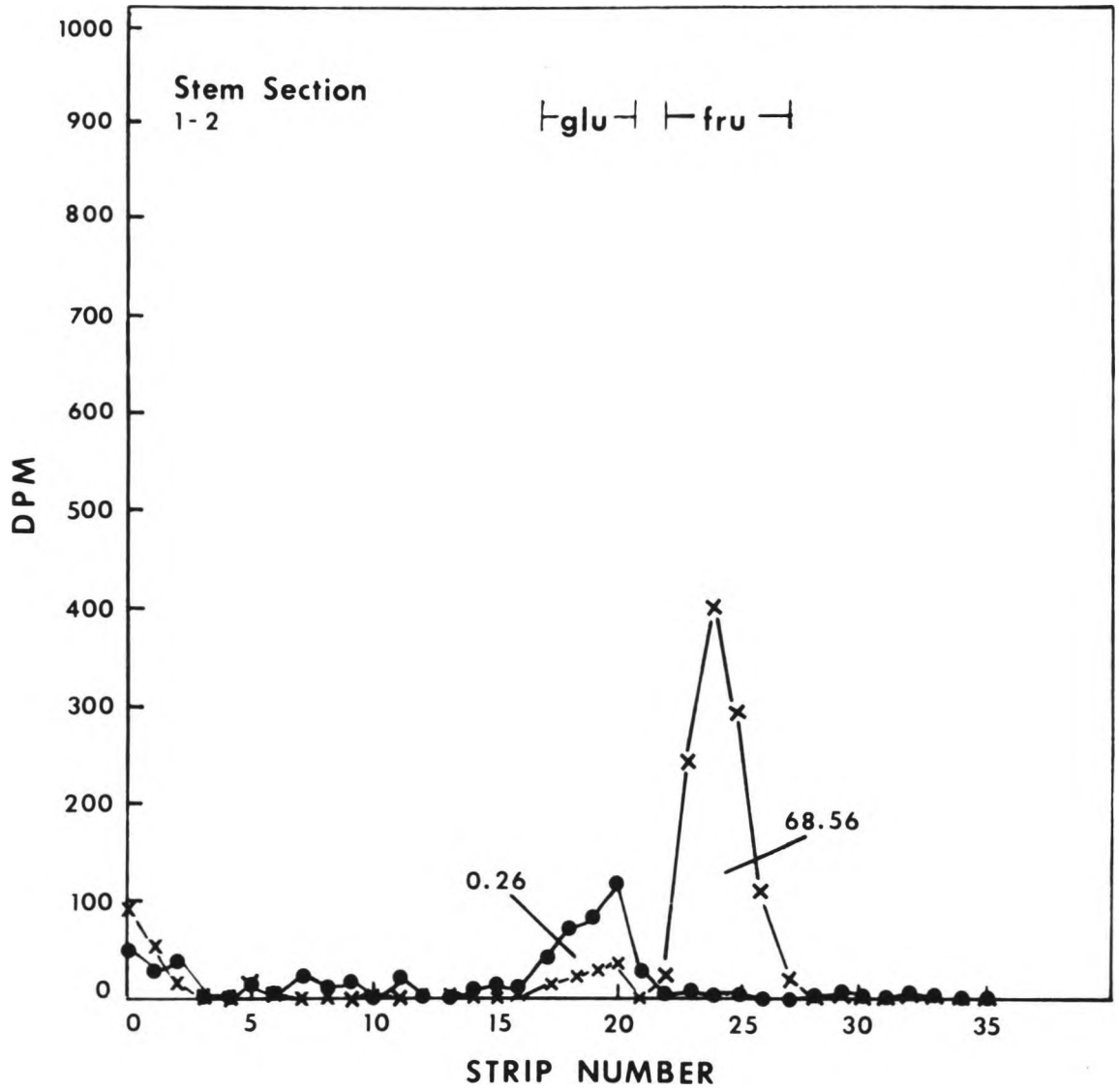

Stem Section
1-2
glu
fru
1000
900
800
700
600
500
400
300
200
100
0
DPM
0.26
68.56
0
5
10
15
20
25
30
35
STRIP NUMBER

Figure 6.

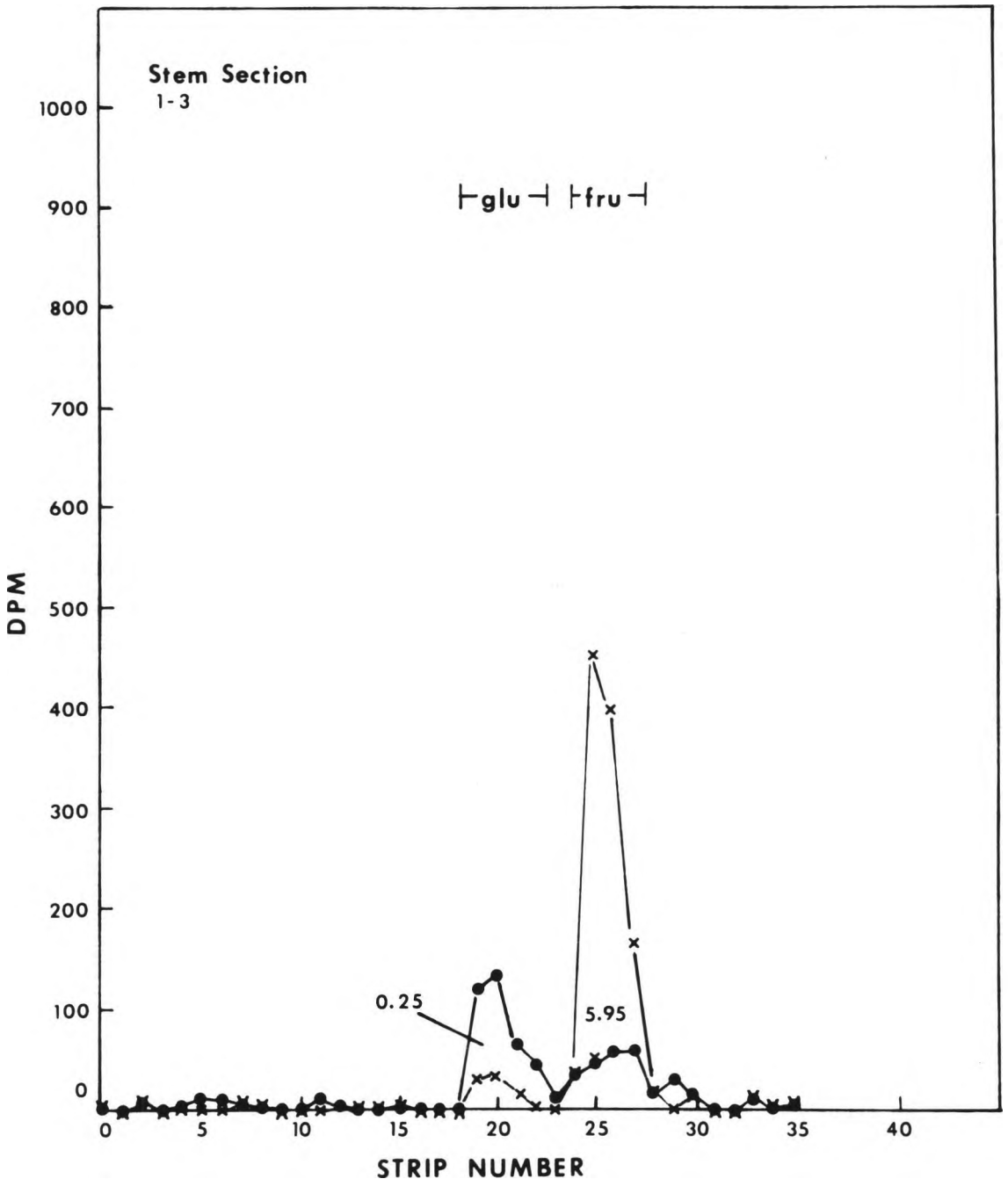

Stem Section
1-3
glu
fru
1000
900
800
700
600
500
400
300
200
100
0
DPM
0.25
5.95
0
5
10
15
20
25
30
35
40
STRIP NUMBER

Figure 7.

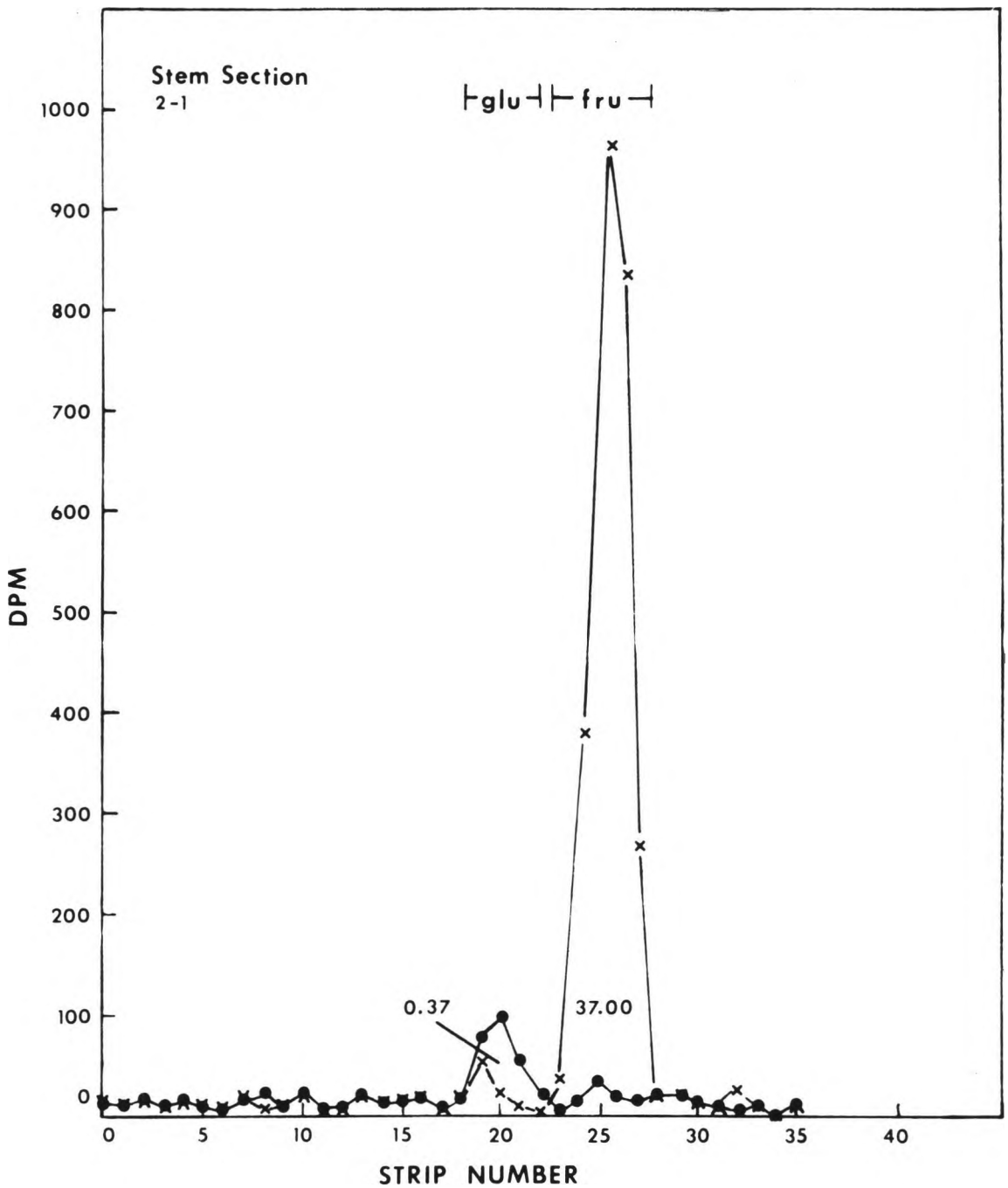

Stem Section
2-1
glu
fru
1000
900
800
700
600
500
400
300
200
100
0
DPM
0.37
37.00
0
5
10
15
20
25
30
35
40
STRIP NUMBER

Figure 8.

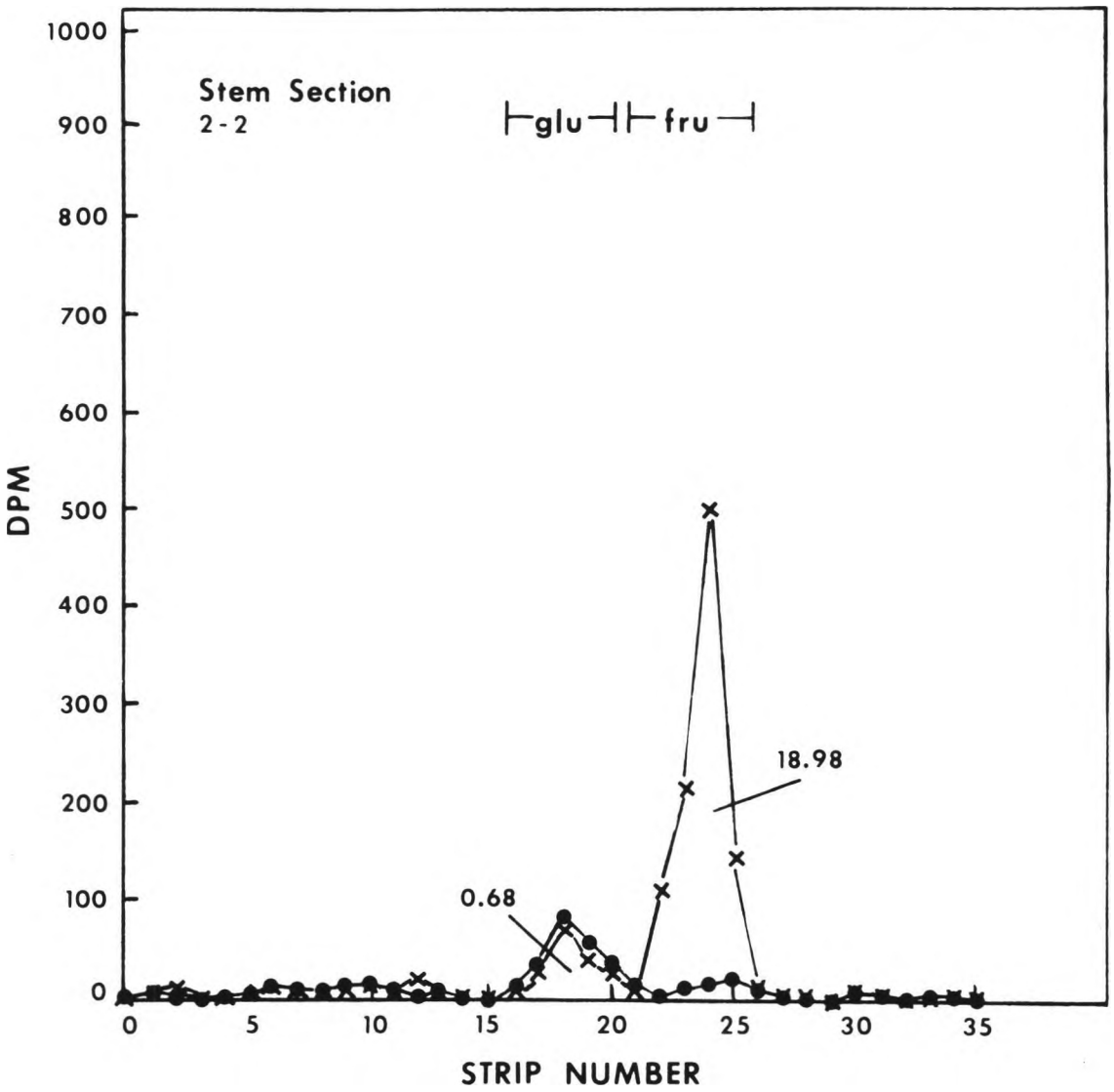
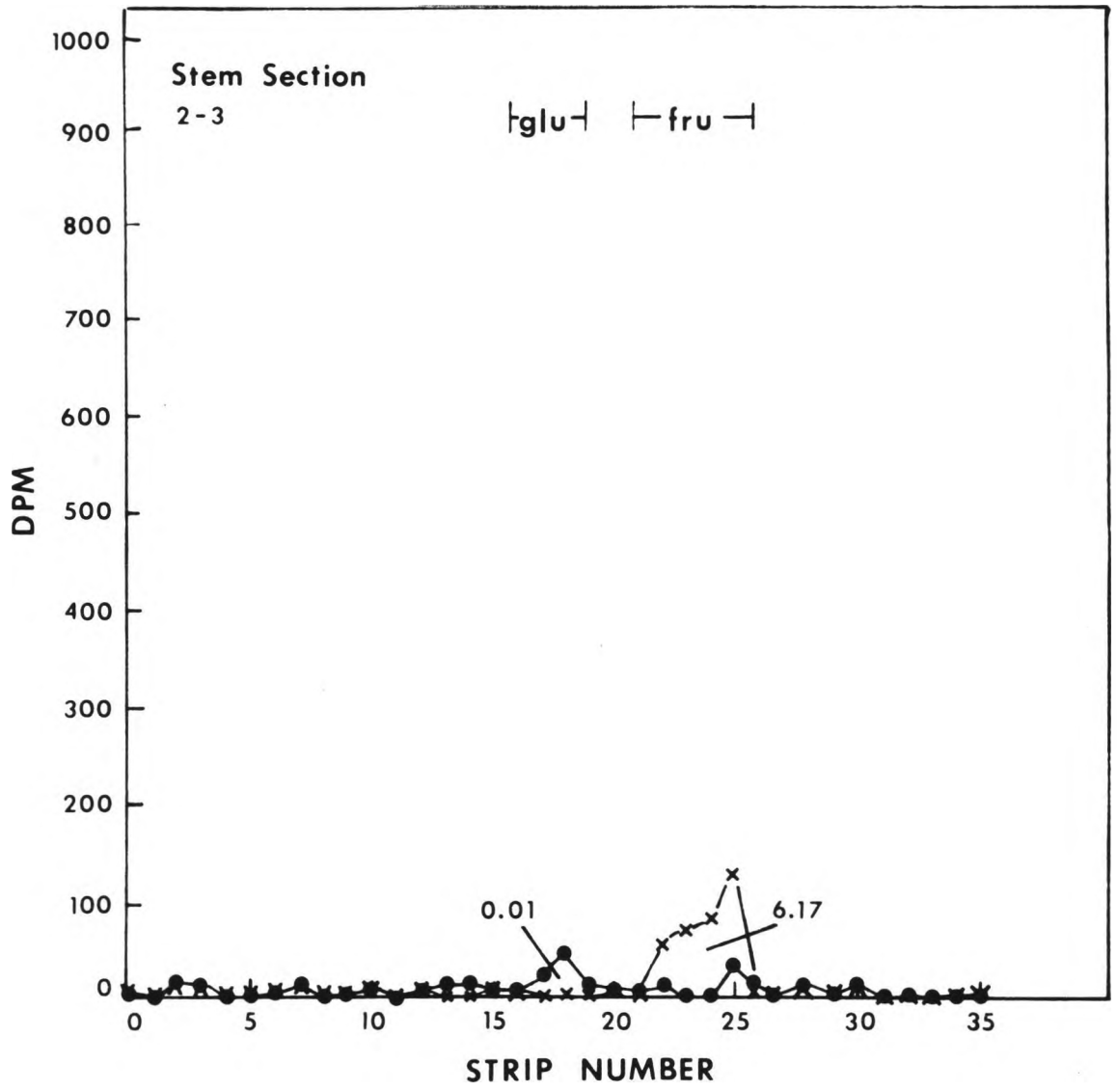

Stem Section
2-2
glu
fru
DPM
1000
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
STRIP NUMBER
18.98
0.68

Figure 9.


Stem Section
2-3
glu
fru
1000
900
800
700
600
500
400
300
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DPM
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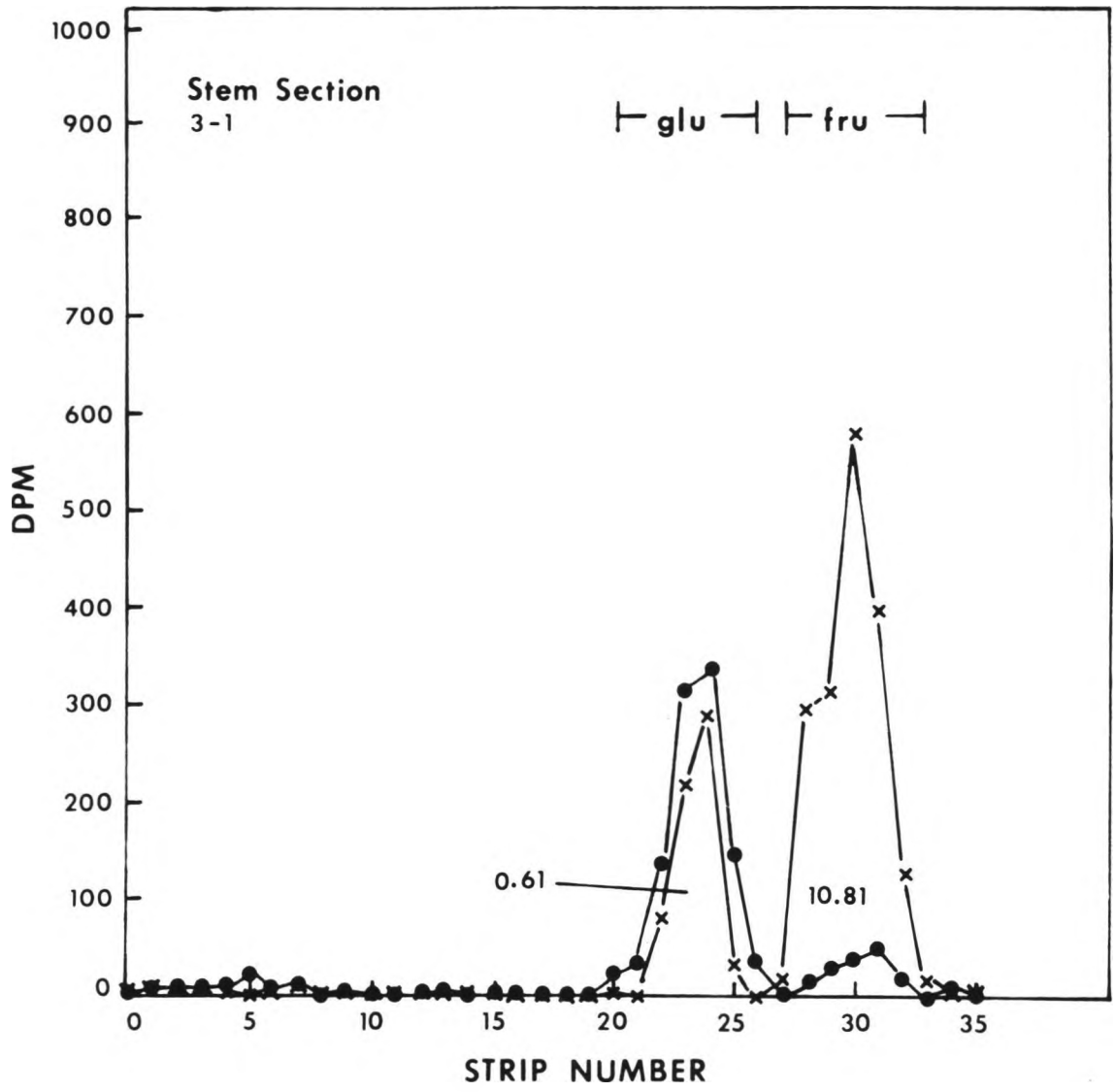
6.17
0
5
10
15
20
25
30
35
STRIP NUMBER

Figure 10.


Stem Section
3-1
glu
fru
DPM
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900
800
700
600
500
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300
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100
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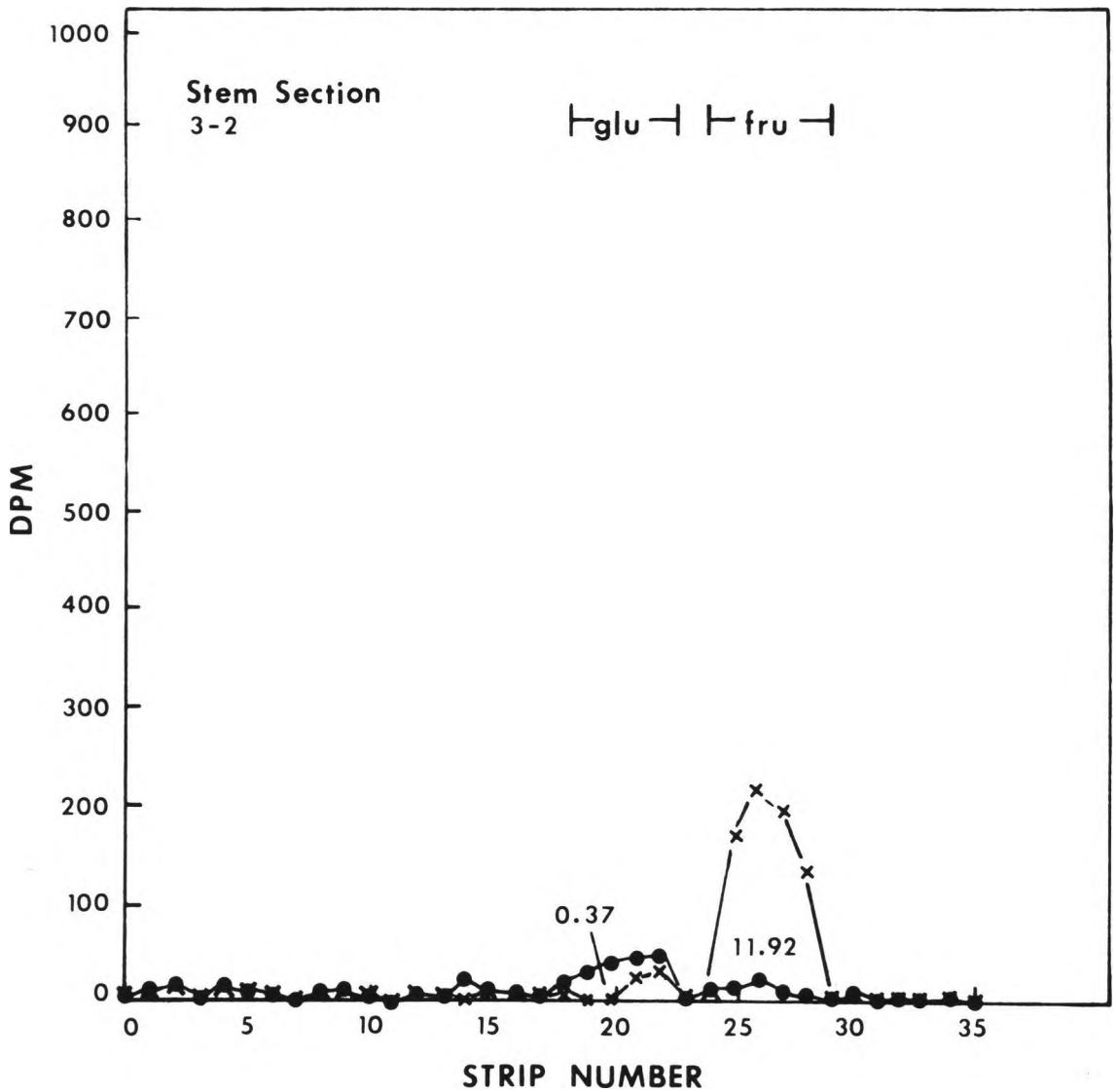
0
5
10
15
20
25
30
35
STRIP NUMBER

Figure 11.


Stem Section
3-2
glu
fru
0.37
11.92
DPM
STRIP NUMBER
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800
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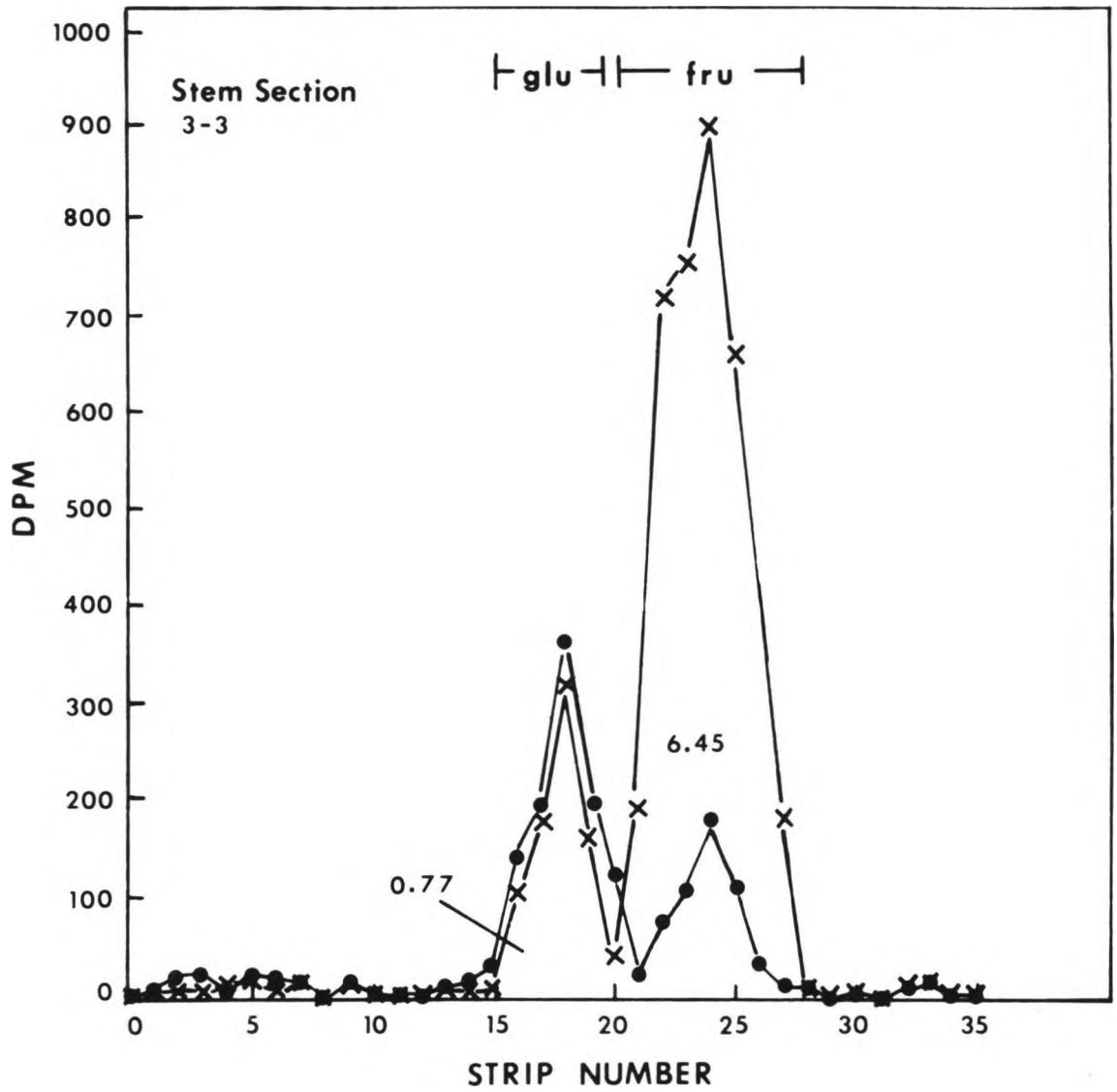
500
400
300
200
100
0
0
5
10
15
20
25
30
35

Figure 12.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glu
fru
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0.77
DPM
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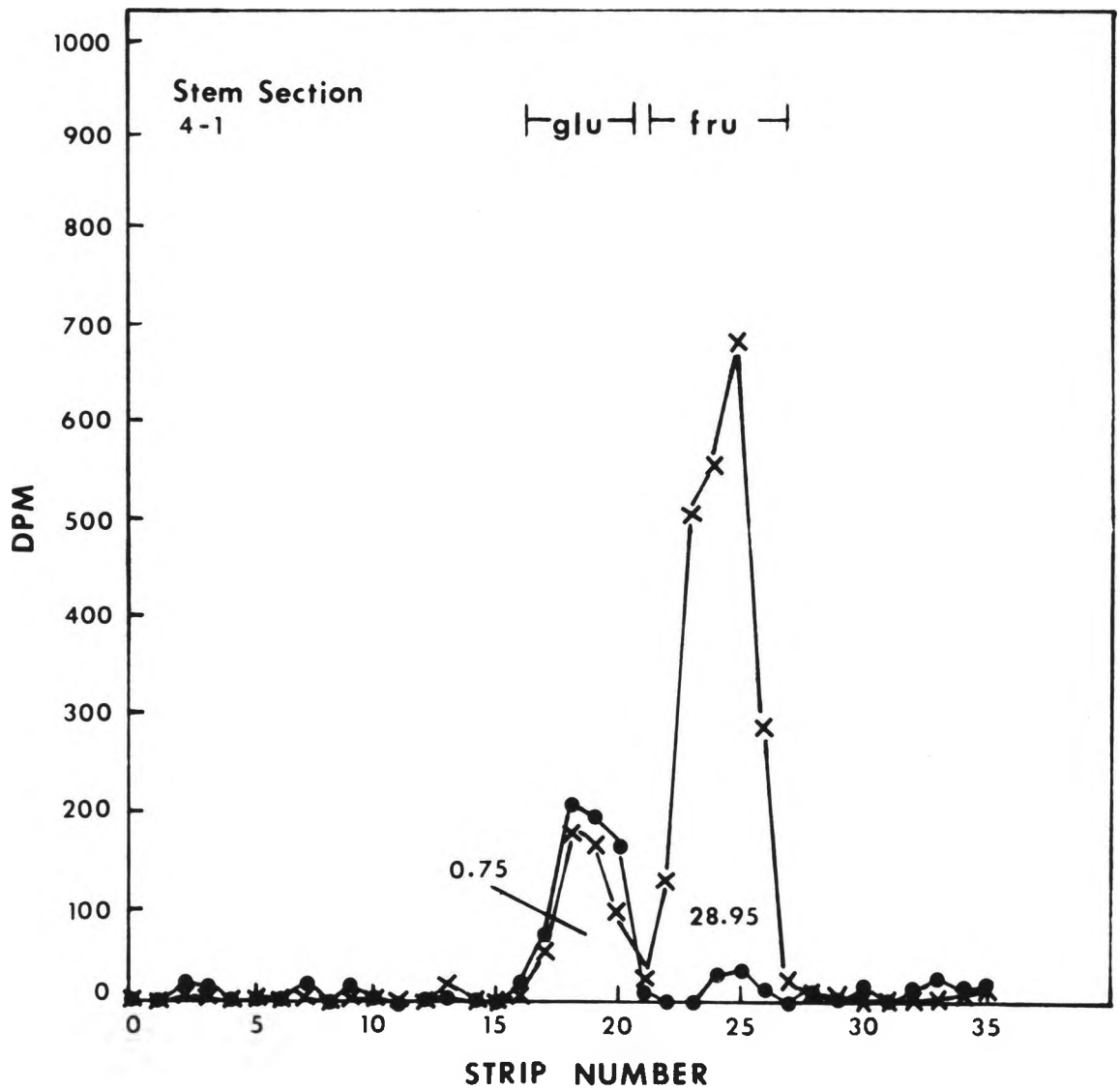
500
600
700
800
900
1000
0
5
10
15
20
25
30
35

Figure 13.


Stem Section
4-1
glu
fru
DPM
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900
800
700
600
500
400
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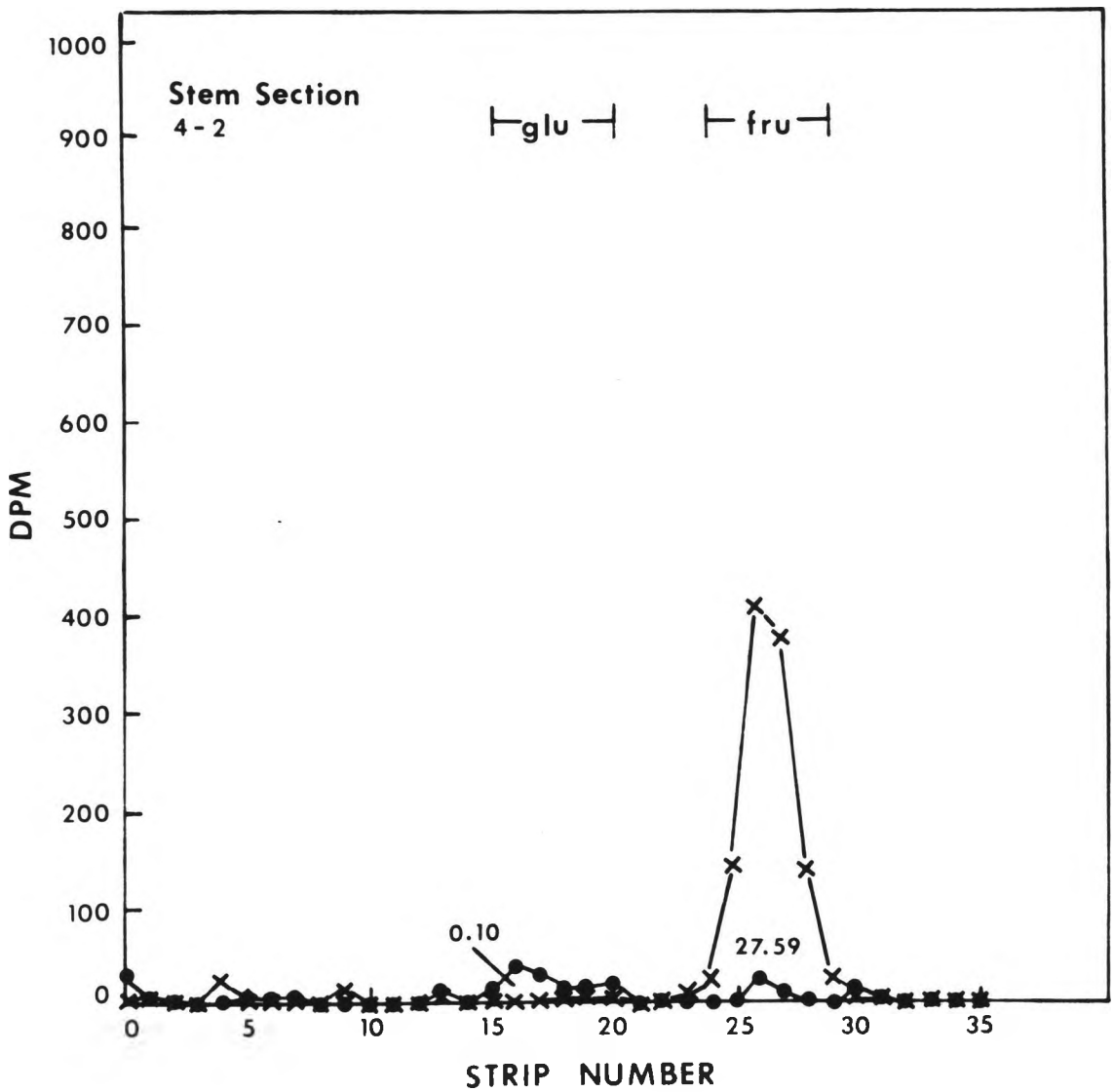
0
0.75
28.95
0
5
10
15
20
25
30
35
STRIP NUMBER

Figure 14.


Stem Section
4-2
glu
fru
1000
900
800
700
600
500
400
300
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DPM
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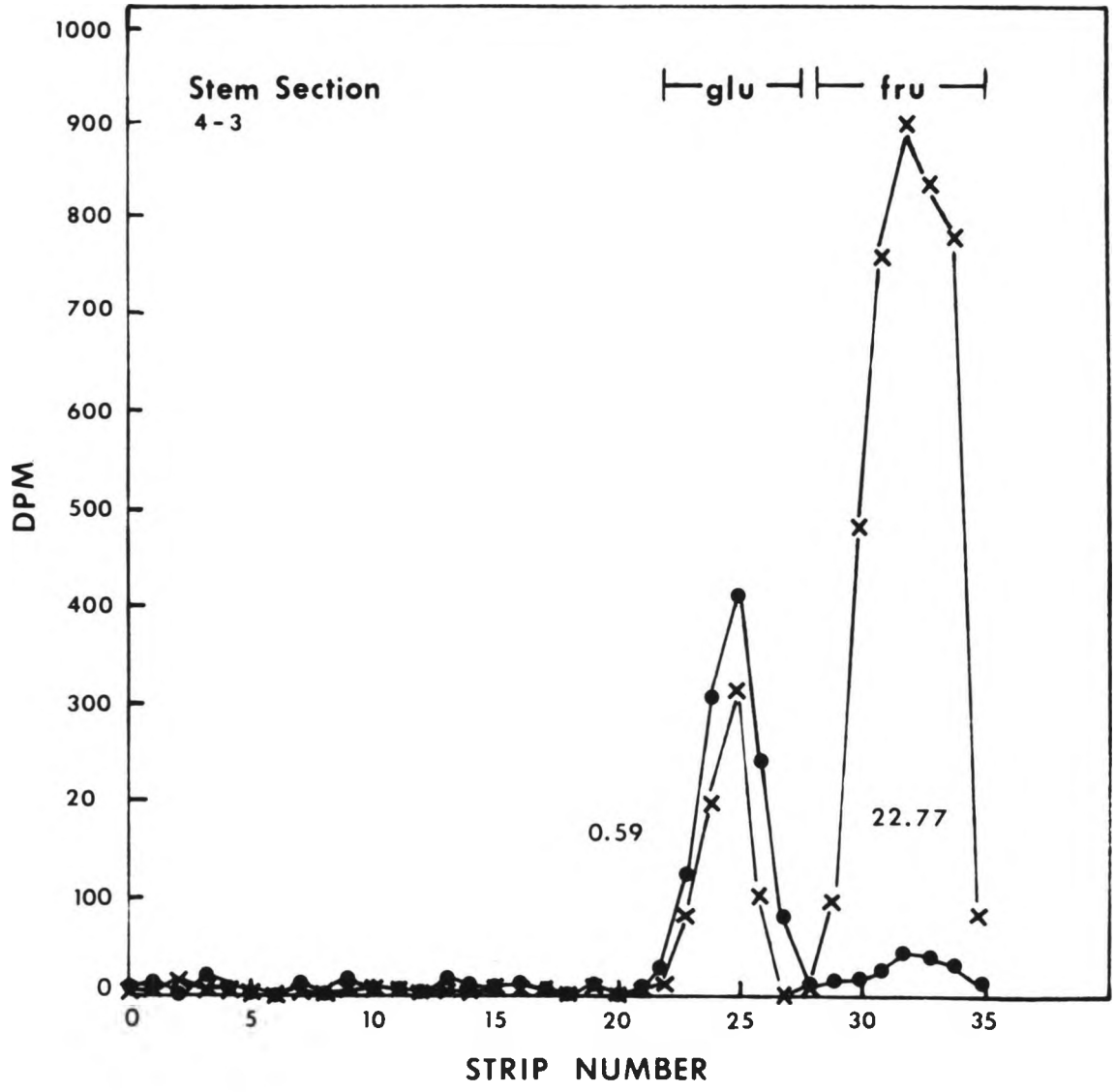
27.59
0
5
10
15
20
25
30
35
STRIP NUMBER

Figure 15.


Stem Section
4-3
glu
fru
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900
800
700
600
500
400
300
20
100
0
DPM
0.59
22.77
0
5
10
15
20
25
30
35
STRIP NUMBER

Figure 16.

strip were added together they yielded the same ratios of $^{3}H:^{14}C$ reported for the unhydrolyzed water extracts (Table 2).

It is unlikely that the amount of label in these peaks represents the amount of fructan biosynthesis in that portion of the stem. Amount of label seems more likely to be related to the proximity of the stem section to the supply point. By separating the two hexose moieties via hydrolysis of these water-soluble fructans, it was possible to observe the labeling pattern in glucose and fructose, i.e. the ratio of $^{3}H:^{14}C$ in each peak. This could not be observed in the unhydrolyzed samples (Table 2). It is easily observed from these data that the water-soluble fractions from these winter wheat stems at anthesis contain more dpm in fructose than in glucose. In each experiment, the ratio of $^{3}H:^{14}C$ in these fructose peaks is much greater than the ratio of $^{3}H:^{14}C$ in the supplied sucrose. This strongly suggests the preferential use of the ^{3}H-labeled fructose moiety from the supplied sucrose in the synthesis of that portion of these water-soluble fructans. Hatch and Glasziou (24) have demonstrated complete randomization of the hexose moieties of parenchyma sucrose in sugarcane leaves. Robinson (44), however, measured the ratios of specific activities of ^{14}C-labeled sugars in wheat stem extracts. She reasoned that the ratio of specific activities of the hexose moieties from the supplied sucrose should be 1:1 if both moieties are utilized in sucrose resynthesis. However, the ratios reported were greater than expected, suggesting a

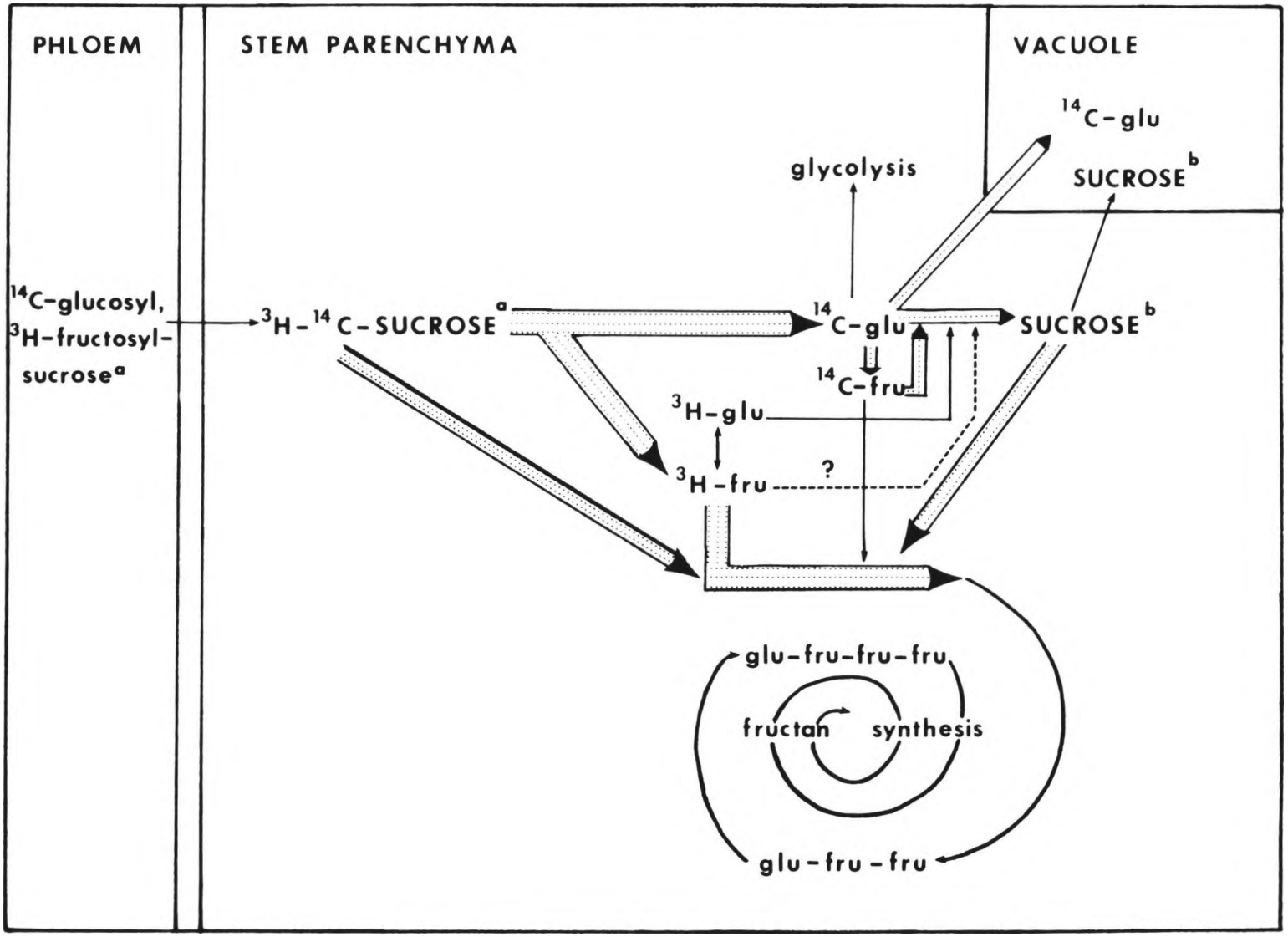
preferential use of the fructose moiety in an alternate pathway or pathways. Suggested alternate pathways for the fructose moiety of the supplied sucrose in her model included preferential use of fructose in either respiration or fructan biosynthesis. The extent to which each cell utilized these alternate pathways would determine the magnitude of the specific activity ratios.

Figures 7, 10, and 13 represent data from the three most immature sections of tissue from each internode. Section 3 (Figure 7) is the only section from this plant with a ratio of $^{3}H:^{14}C$ in the fructose peak that does not exceed 6.0 (the ratio in the supplied sucrose). This is also the least mature section of the youngest internode. Sections 2-3 and 3-3 also had $^{3}H:^{14}C$ ratios in the fructose peak that did not exceed the ratio in the supplied sucrose as greatly as the older tissues. (The ratios for these relatively young sections were 6.17 and 6.45, respectively). The same pattern was observed in the other five plants (Table 3). This trend may indicate that sucrose metabolism is a function of the maturity of the stem tissue. In the younger tissues, metabolism is directed towards reactions contributing to active growth, i.e. greater degradation of translocated sucrose via invertases and pyrophosphorylases. These reactions would lead to a greater amount of randomization, producing $^{3}H:^{14}C$ ratios closer to that in the sucrose supplied. This can be seen by comparing Figures 7 and 8 (sections 1-3 and 2-1 of Plant 3). Section 1-3, the most immature stem section of

the plant, has a ratio of $^{3}H:^{14}C$ in the fructose peak almost identical to that of the supplied sucrose. Section 2-1, an older stem section, had completed its active growth and was diverting a greater proportion of assimilate into fructans. This is reflected in a $^{3}H:^{14}C$ ratio in the fructose peak which is much greater than that in the supplied sucrose.

Figure 17 integrates the data presented into a model to describe the pathway for hexoses from asymmetrically-labeled sucrose partitioning into fructans in winter wheat stems. Once the asymetrically-labeled sucrose was unloaded from the phloem into the stem parenchyma cells, the labeled hexose moieties became involved in various metabolic pathways. It appears that the ^{3}H-fructose from the labeled sucrose was transferred directly to a growing fructan polymer. This direct addition of fructose units to fructans was shown to be catalyzed in certain dicots by the enzyme FFT. A similar mechanism may occur here. This transfer would leave ^{14}C-glucose. This ^{14}C-glucose may in turn randomize to form ^{14}C-fructose, as was seen by the small but distinct amount of ^{14}C found in the fructose peaks (Figures 3 through 16). The ^{14}C-glucose may have also become involved in sucrose resynthesis. Based upon studies of fructan structures, involvement of sucrose (SUCROSE[a] in Figure 17) in fructan biosynthesis would contribute the ^{14}C-labeled glucose unaltered into the terminal or subterminal position of a fructan polymer. Evidence for this contribution is also indicated in Figures 3 through 16, where the ^{14}C-glucose peaks

Figure 17. Proposed pathway for hexoses partitioning into fructans from asymetrically-labeled SUCROSE[a] (as it was supplied to the plant). 'b' indicates resynthesized sucrose, after randomization. The width of the arrows is a relative indication of the degree to which each of these reactions takes place.


PHLOEM
STEM PARENCHYMA
VACUOLE
^{14}C-glu
SUCROSE^{b}
glycolysis
^{14}C-glucosyl,
^{3}H-fructosyl-
sucrose^{a}
^{3}H-^{14}C-SUCROSE^{a}
^{14}C-glu
SUCROSE^{b}
^{14}C-fru
^{3}H-glu
^{3}H-fru
?
glu-fru-fru-fru
fructan synthesis
glu-fru-fru

are present, although small in total activity (dpm). The ^{3}H-glucose component of this peak would be contributed upon randomization of ^{3}H-fructose and subsequent sucrose resynthesis. The resynthesized sucrose could then be a donor in fructan polymer synthesis (Figure 17). The ratios of ^{3}H:^{14}C in these glucose peaks (always less than the supply ratio) support the conclusion that the hexoses randomized, somewhat in all sections, but much more in the more immature tissue.

After randomization of the label, the hexoses can be utilized in several different metabolic pathways in the stem. As was explained previously, evidence indicates that fructan biosynthesis does not involve the use of phosphorylated fructose intermediates or free fructose (1,42). Therefore, if the fructose moiety of translocated sucrose is to be used preferentially in the biosynthesis of the fructans, enzymes such as the FFT discovered in H. tuberosus must be present, and their activities must be greater than the enzymes that catalyze competing reactions with the translocated sucrose.

Once the fructose moiety from translocated sucrose is transferred to a fructan polymer there must be an alternate sink for the remaining glucose. This would insure that the fructan biosynthesis reactions continue. Some free glucose molecules can go directly into glycolysis, others can become sequestered in the vacuole, while others can be converted to sucrose. As a result of these reactions, as well as randomization of hexose sugars from translocated sucrose,

it is possible to explain the labeling pattern of the hexose moieties of the fructans observed in these experiments.

The width of each arrow in Figure 17 shows the relative rates to which each reaction is assumed to take place. A large arrow indicates the preferential use of ^{3}H-fructose in fructan biosynthesis. Labeled sucrose may also be used directly as the base subunit in fructan biosynthesis. It is shown from the data that both hexoses randomize to some degree. The randomized hexoses may then be directly involved in fructan biosynthesis or they may recombine in sucrose synthesis and subsequently partition into the vacuole (44) or into fructans.

CONCLUSION

The fundamental purpose of the plant breeder's research has been to increase the yields of the world's important agronomic crop plants. New varieties are constantly introduced whose productivity has been improved as a result of genetic changes. However, these genetic changes are expressed through morphological and physiological alterations, which in turn may be factors directly influencing yield. For example, Austin, et. al. (4) recently analyzed changes associated with genetic improvements in twelve winter wheat varieties. The new, high yielding varieties had shorter stem lengths, earlier anthesis dates and lower stem weights. These morphological changes correlate with the accompanying physiological changes affecting yield. The present study was devoted to one potential yield-altering metabolic pathway in winter wheat - the biosynthesis of fructans.

The present data support other reports that fructans are the major nonstructural storage carbohydrate in winter wheat stems (3,30,35,43,60,61,67). It was shown that the fructose moiety of translocated sucrose is used preferentially in the synthesis of these nonstructural storage carbohydrates. Therefore, this study is an early step in the elucidation of the pathway of fructan synthesis. Once

this pathway is defined completely research may be done to examine the possibility of altering source-sink relationships so that a greater amount of this carbohydrate may be remobilized. Increased contribution to grain filling could ultimately increase crop yields in winter wheat.

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APPENDIX I

Table I-1. Quench curve data obtained from a sealed, quenched series of ^{14}C and ^{3}H samples.

Sample	cpm	Efficiency (%)
1	155,633	78
2	149,846	75
3	141,562	71
4	134,214	67
5	128,953	64
6	118,801	59
#1-6 = quenched ^{14}C standards (199,300 dpm)		
7	173,534	35
8	159,176	32
9	136,796	28
10	102,936	21
11	55,885	11
12	15,191	3
#7-12 = quenched ^{3}H standards (485,000 dpm)		

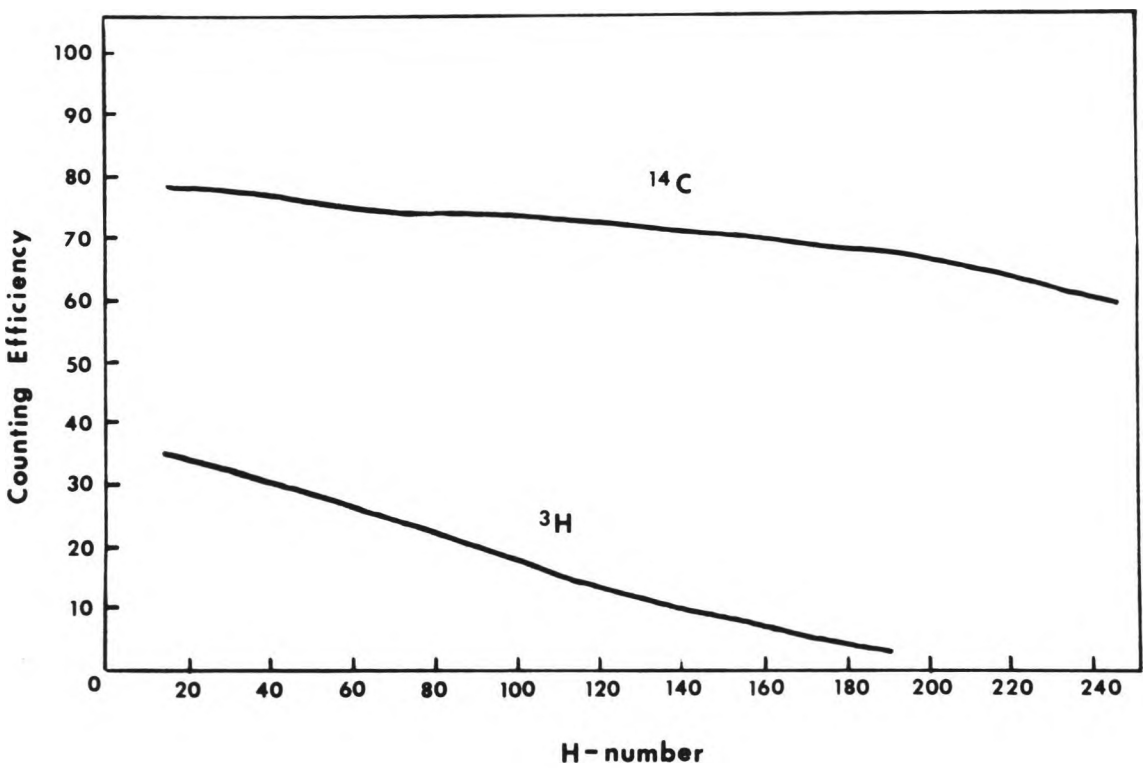

Figure I-1. Quench curves for ^{14}C and ^{3}H.

APPENDIX II

Table II-1. DPM for extracts from Plant 1. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	Ethanol		Water	
Sample	^{3}H	^{14}C	^{3}H	^{14}C
Blade	*	*	*	*
Leaf Sheath	30,539	7 435	1,555	585
Stem Sections				
1-1	4,079	913	153	49
1-2	17,974	3,824	638	166
1-3	61,265	13,639	2,169	601
2-1	26,866	5,958	12,816	3,038
2-2	12,054	2,679	13,766	3,276
2-3	8,694	1,939	13,954	3,235
3-1	7,808	1,636	2,471	564
3-2	4,591	978	963	214
3-3	**	**	**	**
4-1	1,058	235	873	233
4-2	714	152	485	120
4-3	**	**	**	**
5-1	**	**	**	**
Supplied Sucrose	1,776,014	352,241	1,776,014	352,241

* = No Sample; ** = No Activity

Table II-2. DPM for extracts from Plant 2. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	Ethanol		Water	
Sample	^{3}H	^{14}C	^{3}H	^{14}C
Blade	101,687	52,830	9,072	8,850
Leaf Sheath	72,398	35 058	6,262	3,134
Stem Sections				
1-1	23	13	36	10
1-2	214	87	36	14
1-3	1,277	577	183	93
2-1	4,231	1,937	320	157
2-2	29	16	11	4
2-3	14	7	19	7
3-1	**	**	**	**
3-2	**	**	**	**
3-3	**	**	**	**
4-1	**	**	**	**
4-2	**	**	**	**
4-3	**	**	**	**
5-1	**	**	**	**
Supplied Sucrose	3,312,961	1,237,468	3,312,961	1,237,468

** = No Activity

Table II-3. DPM for extracts from Plant 3. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	Ethanol		Water	
Sample	^{3}H	^{14}C	^{3}H	^{14}C
Blade	16,180	2,420	681	120
Leaf Sheath	9,378	1,398	9,637	1,479
Stem Sections				
1-1	4,011	578	254	31
1-2	16,893	2,405	2,004	319
1-3	38,562	5,494	1,855	302
2-1	171,907	24,544	3,355	573
2-2	89,366	12,258	2,051	334
2-3	69,333	9,459	927	171
3-1	15,969	2,309	974	168
3-2	12,329	1,834	148	27
3-3	5,131	772	277	42
4-1	6,963	1,031	394	64
4-2	3,141	473	255	39
4-3	5,302	799	198	36
5-1	2,357	372	101	16
Supplied Sucrose	1,578,337	263,056	1,578,337	263,056

Table II-4. DPM for extracts from Plant 4. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	Ethanol		Water	
Sample	^{3}H	^{14}C	^{3}H	^{14}C
Blade	17,224	3,824	522	130
Leaf Sheath	290,879	96,001	7,651	2,501
Stem Sections				
1-1	1,484	382	516	163
1-2	18,961	4,745	1,260	395
1-3	151,982	39,433	6,371	1,825
2-1	50,808	12,651	54,713	12,985
2-2	41,689	10,002	21,140	5,335
2-3	28,342	7,055	12,034	2,946
3-1	35,047	9,142	3,620	990
3-2	17,302	4,632	1,102	272
3-3	*	*	*	*
4-1	4,231	1,130	**	**
4-2	4,278	1,125	**	**
4-3	**	**	**	**
5-1	**	**	**	**
Supplied Sucrose	1,145,864	297,380	1,145,864	297,380

* = No Sample; ** = No Activity

Table II-5. DPM for extracts from Plant 5. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	Ethanol		Water	
Sample	^{3}H	^{14}C	^{3}H	^{14}C
Blade	14,269	3,357	2,087	501
Leaf Sheath	38,636	9 512	318	104
Stem Sections				
1-1	8,653	2,066	492	127
1-2	8,956	2,081	575	157
1-3	10,173	2,560	881	278
2-1	8,391	2,202	694	223
2-2	1,015	272	1,986	563
2-3	29,661	7,845	17,194	4,751
3-1	4,366	1,164	37,294	10,037
3-2	3,207	849	5,840	1,593
3-3	1,900	501	2,592	714
4-1	**	**	**	**
4-2	**	**	**	**
4-3	**	**	**	**
5-1	**	**	**	**
Supplied Sucrose	102,145	28,216	102,145	28,216

** = No Activity

Table II-6. DPM for extracts from Plant 6. In the stem sections, the first number represents the internode, and the second number represents the section within the internode (see Figure 2).

	Ethanol		Water	
Sample	^{3}H	^{14}C	^{3}H	^{14}C
Blade	8,729	2,172	7,478	1,892
Leaf Sheath	15,499	3,836	33,130	8,086
Stem Sections				
1-1	7,768	1,827	8,810	2,045
1-2	331	73	28,879	6,894
1-3	11,400	2,703	23,433	5,513
1-4	20,100	4,736	18,959	4,476
2-1	20,357	4,693	24,724	5,927
2-2	17,879	4,126	21,602	4,977
2-3	18,302	4,267	14,850	3,468
3-1	1,994	487	1,751	413
3-2	1,362	325	580	130
3-3	*	*	*	*
4-1	1,062	235	241	53
4-2	**	**	**	**
4-3	**	**	**	**
5-1	**	**	**	**
Supplied Sucrose	173,069	42,748	173,069	42,748

* = No Sample; ** = No Activity