THE RELATIONSHIP OF L-GLUTAMINE TO SEEDLING VIGOR IN WHEAT (TRITICUM AESTIVUM L.) AND THE INTERACTION WITH INORGANIC NITROGEN SUPPLY

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ABSTRACT

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By

John Edward Stuurwold

The history of the research on the correlation of seed protein content and seedling vigor in wheat is discussed, with special reference to the work relating glutamine, glutamic acid and proline to increases in seedling dry weight and protein content. The use of glass fistulas for supplying material to a seed and for injecting small amounts of liquid directly into the endosperm is described.

The relationship of seedling vigor of Logan wheat (Triticum aestivum L.) with glutamine content of the endosperm proteins was studied by adding glutamine to wheat seeds. Glutamine added to wheat seeds by means of a glass fistula increased seedling dry weight.

Tracer studies in which L-glutamine-[^{14}C(U)] was injected directly into the seed showed that utilization of endosperm-derived glutamine was related both to endosperm protein content and to the nitrogen supply in the media. Similar studies with L-glutamic-[^{14}C(U)] acid showed that there was little difference in the use of this amino acid by seedlings grown from low or high protein seed either with or without a source of inorganic N.

Nitrate uptake from solution was found to be related primarily to

the nitrate content of the media in the days immediately preceding the test, not to the protein content of the seed.

L-glutamine was metabolically most active in seedlings grown from low protein seeds when inorganic nitrogen was not supplied in the media. In seedlings grown from high protein seeds and when inorganic N was supplied in the media, catabolism of L-glutamine dropped off. There were no differences in the amounts of either L-glutamic acid or its amide incorporated into various plant fractions regardless of endosperm protein content or inorganic N supply.

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Finally, but certainly not least, I would like to thank my wife for putting up with all the inconveniences while I finished this thesis and my parents for the moral support all along the way.

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INTRODUCTION

The research that led to this present study was initiated in the early 1960's. The first of these studies explored the influence of the s-triazine herbicide simazine on the nitrogen nutrition of several plant species both in the laboratory and in field experiments (41,42,43,46,55). Later studies were concerned with the ability of the s-triazines and substituted uracils to increase protein content of both forage and seed and with the relationship of seed protein content to subsequent growth and yield (30,40,44,45,48).

The increased protein content in the seed of wheat and other small grains, whether chemically induced or produced by high N fertilizer rates, increased dry matter accumulation, protein in the forage, and in some cases yields (13,20,28,52). The results were not consistent, however (44).

The yield of grain has been shown to be related to seed size (1,5,21, 22,23), but in some of these and in all subsequent studies, seed size was removed as a variable by using seed within a narrow weight range.

In an attempt to find ways to obtain more consistent results, several studies were undertaken to determine which part of the wheat seed was responsible (2,29,31).

Lowe, et al., (29), working with a Mexican semi-dwarf wheat, Inia 66, found that amino acid composition expressed as micromoles of amino acid per gram of whole grain meal was positively correlated with seedling dry weight for all amino acids. When the amino acid composition was

expressed on a mole percent basis, however, some amino acids were found to be negatively correlated, some not correlated, and a few positively correlated with seedling dry weight. Among those positively correlated were proline and glutamic acid (including the amide). These amino acids comprise a large part of the major storage proteins in wheat and they increase to a greater degree than the other amino acids when N fertilizer applications or subtoxic applications of herbicides are used to obtain higher protein seed. Glutamic acid (and the amide) content on a mole percent basis was also positively correlated with subsequent yield in Michigan (29).

In a second series of experiments, it was found that the protein content of embryos from high and low protein wheat seeds did not differ significantly, nor did their weights differ (31). Aleurone and endosperm protein contents of low and high protein seeds did differ and were positively correlated with seedling dry weight. They were unable to detect any difference in seedling growth between composite seeds of low or high protein embryos grown on the same type of endosperm.

Ayers, et al., (2) showed that there was no major change in the protein patterns of higher protein seed, although quantitative differences of individual proteins were obvious. Perhaps most interesting was the finding that the nitrogen content ($\mu g\ N\ mg^{-1}$ protein) of the gliaden fraction was increased significantly by any method used to increase seed protein: urea applications, nitrate fertilizer, or applications of the substituted uracil herbicide, terbicil, at subtoxic levels. They also found that materials are removed more quickly and more completely from high protein seed than from low protein seed during seedling growth.

The authors suggest that the protein changes may result in a more

easily and quickly hydrolyzed storage protein or in a more active enzyme component in high protein seed.

This present study deals with the utilization of glutamic acid and glutamine in germinating wheat seeds and their influence on seedling growth.

ABSTRACT

The relationship of seedling vigor of Logan wheat (Triticum aestivum L.) with glutamine content of the endosperm proteins was studied by adding glutamine to wheat seeds. Glutamine added to wheat seeds by means of a glass fistula increased seedling dry weight.

Tracer studies in which L-glutamine-[^{14}C(U)] was injected directly into the seed showed that utilization of endosperm-derived glutamine was related both to endosperm protein content and to the nitrogen supply in the media. Similar studies with L-glutamic-[^{14}C(U)] acid showed that there was little difference in the use of this amino acid by seedlings grown from low or high protein seed either with or without a source of inorganic N.

Nitrate uptake from solution was found to be related primarily to the nitrate content of the media in the days immediately preceding the test, not to the protein content of the seed.

L-glutamine was metabolically most active in seedlings grown from low protein seeds when inorganic nitrogen was not supplied in the media. In seedlings grown from high protein seeds and when inorganic N was supplied in the media, catabolism of L-glutamine dropped off. There were no differences in the amounts of either L-glutamic acid or its amide incorporated into various plant fractions regardless of endosperm protein content or inorganic N supply.

INTRODUCTION

Early seedling growth and yield of crops may be related positively to seed size and protein content of the seed (1,40). Lowe and Ries (31) showed that the endosperm protein, not embryo protein, increased in high protein wheat and was related to seedling vigor. Using "composite seeds" produced by transplanting embryos from seeds of one protein level to endosperms of seeds with another protein content, they demonstrated that embryos from either high or low protein seed produced larger seedlings when grown on high protein endosperms. There was no difference between seedlings from high or low protein embryos grown on the same type of endosperm.

Lowe, et al., (29) working with a Mexican semi-dwarf wheat, Inia 66, demonstrated a high correlation between increases in all amino acids measured as $\mu moles \cdot g^{-1}$ of meal and seedling dry weight. Expressing the amount of amino acid on a mole percent basis, however, revealed that certain amino acids increased disproportionately in seed protein and that some of these were positively correlated with increases in seedling vigor. These amino acids were proline ($r = 0.57^{*}$, $n = 17$), phenylalanine ($r = 0.62^{**}$, $n = 17$) and glutamic acid including the amide ($r = 0.74^{***}$, $n = 17$). Because acid hydrolysis in 6 N HCl was used in the analysis of seed proteins, glutamic acid was considered to be glutamic acid plus glutamine. Glutamic acid content (mole percent) was also positively correlated with yield in field trials in Michigan ($r = 0.782^{**}$, $n = 7$).

Strbac, et al., (51) found that high protein seed produced either by

applications of herbicides at subtoxic rates or by nitrogen fertilizer contained more nitrogen per gram of meal and per gram of protein. The increase in percent nitrogen occurred primarily in the gliaden proteins. There was a decrease in the percent nitrogen of the albumin proteins (51).

Ayers, et al., (2) showed that despite the relative alterations of amino acids in seed proteins reported by Lowe, et al., (29) there was no qualitative shift in protein patterns of high protein seed, although quantitative changes of individual proteins were obvious. It is of interest, relative to the present study, that the nitrogen content (μg N mg^{-1} protein) of the gliaden fraction was increased by any of the methods used to increase seed protein: urea applications, nitrogen fertilizer, or application of the substituted uracil herbicide, terbacil, at subtoxic levels. Glutamic acid is perhaps most prevalent in the gliaden fraction where it has been reported to comprise 43.7% of the amino acids (3). Lewis and Berry (26), working with Datura stramonium L., found that glutamine was the primary N storage compound and ammonia "detoxifier" in leaf tissue. The level of N incorporation into glutamine was dependent on the level of nitrogen and degree of induction of nitrate reductase. Chemicals used at subtoxic levels to increase protein content in seeds increased nitrate reductase activity (41). Thus, the authors' speculation (2) that the increase in protein nitrogen content might be due to a substitution of glutamine for glutamic acid residues in the gliaden proteins is highly probable.

Beside the reported effects on seedling growth, high protein seeds' physiological behavior was measurably different. Both the rate of removal and completeness of removal of material in high protein seeds during seedling growth were greater (2). In this study the seedlings were grown

in vermiculite and distilled water for the first nine days. High protein seeds also imbibe water marginally faster than low protein seeds, they germinate faster and the seedlings they produce have a higher respiratory rate although the RQ remains constant (28).

The present study deals with the utilization patterns of the carbon skeletons of glutamic acid and glutamine in germinating wheat seeds and the influence of these amino acids on seedling growth.

MATERIALS AND METHODS

Standardization of Seed. Seed of a soft white winter wheat (Triticum aestivum L. cv. Logan) was produced in the greenhouse in 1972 using two rates of N fertilizer to obtain different seed protein contents. Seeds from each lot were individually weighed to 44 $\pm$ 2 mg and assayed for total protein by an automated Kjeldahl procedure (8). The seed from plants receiving the low rate of N fertilizer contained 144 mg $\cdot$ g^{-1} seed dry weight protein while that from plants receiving the high rate contained 160 mg $\cdot$ g^{-1} seed dry weight protein, or an 11% increase. These will hereafter be referred to as low and high protein seeds respectively. All seeds used in the ^{14}C-amino acid tracer studies and in the endosperm replacement study were weighed individually to 44 $\pm$ 2 mg.

Surface Sterilization and Preparation of the Seed. A 0.3% (w/v) suspension of Captan 80W (captan, Stauffer Chemical Co.) was autoclaved for 20 min. Lots of low and high protein seeds were soaked for 1 min in absolute ethanol, rinsed three times with sterile distilled water, soaked for 5 min in the Captan 80W suspension, and then dried on sterile filter paper in a petri dish for 45 min in a vacuum oven at 43 C at a pressure of less than 165 millibars absolute pressure. Using aseptic procedures in a sterile box, a 1.6 mm diameter hole (1/16th inch) was drilled in the side of each grain as shown in Figure 1.

Respiration Train for Tracer Studies. Culture tubes (25 x 200 mm) were used to construct a growth tube with two side arms for air circulation and one access tube topped with a silicone rubber septum for

Figure 1. A Wheat Seed with Glass Fistula Showing the Placement in the Side of the Seed.

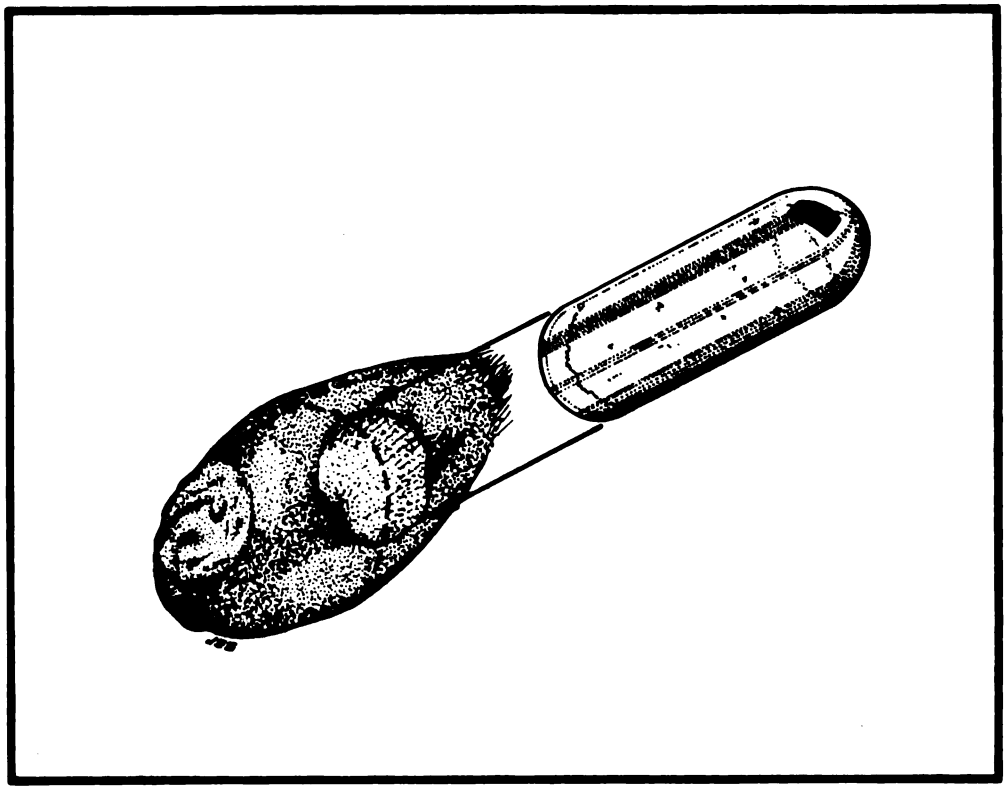

Figure 1

injecting nutrients (Fig. 2). Latex foam plugs were inserted into the side arms used for air circulation to scrub the air of microorganisms.

Compressed air was passed through a splitter comprised of 16, 1.2 m capillary tubes to the lower side arm of each growth tube. Exhaust gases from each tube were bubbled through a set of two CO_2 trap tubes each containing 5 ml of a solution of Methyl Cellosolve (ethylene glycol monomethyl ether, Sequanal Grade, Pierce Chemical Co., Rockford, Illinois) and monoethanolamine 2:1 (19). The flow rate was adjusted to about 10.5 $cm^3 \cdot min^{-1} \cdot tube^{-1}$.

The growth tubes were filled to a depth of 5 cm with Turface (Wyandotte Chemicals Corp., Wyandotte, Michigan) which was covered by a 1.5 cm layer of vermiculite.

Liquid Scintillation Counting. All ^{14}C samples were counted on a Hewlett-Packard Tri-Carb Scintillation Counter. Aliquots of 200 µl were counted in a fluor system consisting of 4.0 g PPO (2,5-diphenoxazole, Research Products International, Corp., Elk Grove Village, Illinois) and 50 mg dimethyl-POPOP (1,4-bis 2-(4-methyl-5-phenoxazolyl), RPI, Corp.) per liter of solvent system. The solvent system was toluene and Triton X-100 (Technical Grade, Rohm and Haas), 2:1 (38,54). Conversions to dpm's were made on the basis of a standard curve prepared by plotting external standard cpm's against efficiency for a series of acetone-quenched standards prepared from toluene-^{14}C Carbon-14 Standard (New England Nuclear).

Fractionation of Plant Material. Plants were homogenized with a mortar and pestle in 10 ml of 70% (v/v) ethanol with about 400 mg of 75-105 µm glass homogenizing beads at room temperature. The ethanolic suspension was boiled for 10 min in a water bath at 80-85 C, centrifuged,

Figure 2. Diagram of the Respiration Train Used for the ^{14}C Amino Acid Tracer Studies.

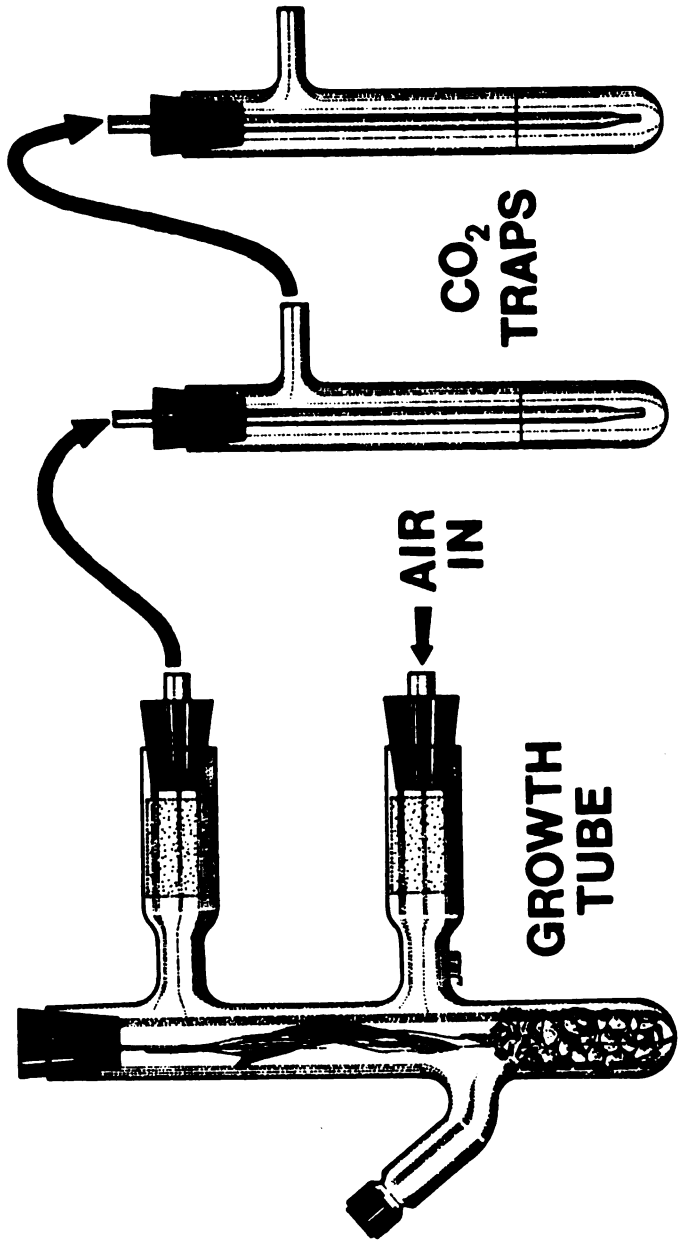

Figure 2

and the supernatant saved. The extraction was repeated twice with 5 ml aliquots of 70% ethanol and all three extracts were combined. The residue was dried and transferred to hydrolysis tubes.

The ethanolic extracts were evaporated on a vacuum evaporator. The residue was taken up in 1.5 ml of 0.01 N HCl. An aliquot of 0.5 ml was separated into neutral and alkaline fractions on Dowex 50W-X8, 100-200 mesh cation exchange column (1.0 cm diam X 7.5 cm, hydrogen form). The flow rate was adjusted to 1.0 ml · min^{-1}. The neutral fraction was eluted with 50 ml of slightly acidified water and the alkaline fraction with 50 ml of 14.5% NH_3 solution. The neutral aqueous fraction contains soluble carbohydrates, the alkaline fraction free amino acids.

The residue from the ethanol extraction was suspended in 5 ml of 6 N HCl in hydrolysis tubes. To remove gases from the system, each tube was frozen in dry ice and acetone, evacuated to 25 millibars absolute pressure, thawed, refrozen and reevacuated. Hydrolyses were carried out in these sealed tubes for 24 hr at 110 C after which the tubes were centrifuged and the supernatant decanted. The residue was washed twice with 5 ml of 0.01 N HCl which was added to the hydrolysate. The hydrolysate was evaporated on a rotary evaporator to dryness and the residue taken up in 0.01 N HCl. Aliquots of 0.5 ml were separated on a cation exchange column as previously described. The neutral fraction contains hydrolyzable carbohydrates and the alkaline fraction contains protein amino acids.

All eluates were evaporated on a flask evaporator to 10 ml for counting.

Samples from both alkaline fractions were chromatographed on Whatman Number 1 sheets using n-butanol-acetic acid-water (80:20:20, v/v/v) or

phenol-water (75:25, w/w). Glutamic acid or glutamine standards were spotted on each chromatogram and the corresponding band in the sample was cut out and counted.

Endosperm Replacement. Low protein 'Logan' wheat seeds were prepared as previously described. In one group of seeds the cavity was filled with endosperm drilled out of low protein seeds from the same lot. A mixture of 60% L-glutamine (Sigma, Grade III) in low protein endosperm was packed into each seed. This is approximately the normal amount of glutamine in an average grain of wheat of this size. Autoclaved paraffin was used to seal the cavities.

Single seeds were placed in 200 x 25 mm culture tubes on top of a 5 cm deep layer of Turface and covered by a 1.5 cm layer of vermiculite. Seedlings were grown in a growth chamber at 24 C in constant light. Seedlings were watered with distilled water. Whole plants, including the remains of the caryopsis, were harvested 14 days after planting.

Fresh weights were determined within one hour after harvest and dry weights were taken after the plants were dried for 3.5 hr at 43 C under a vacuum of 130 millibars absolute pressure.

^{14}C-L-Glutamine in Low and High Protein Seeds. Low and high protein 'Logan' wheat seeds were prepared as previously described. Diluted L-glutamine-[^{14}C(U)] (0.016 mg · ml^{-1}, New England Nuclear) was filtered through a 22 um Micropore filter. Five ul (4.46 X 10^5 dpm) was injected into the cavity in each seed. All seeds were dried for 30 min at 43 C in a vacuum oven at 165 millibars absolute pressure. The holes were then packed with aseptically-obtained low or high protein endosperm and capped with autoclaved paraffin.

Seeds were planted just below the vermiculite layer in the autoclaved

growth tubes. Sterile distilled water was injected through the access tube into each growth tube. Ten days after planting, half-strength Hoagland's solution with 3 mM N as nitrate (17) was added to each tube to bring the water level to just below the seed. The growth tubes were arranged as a randomized complete block in a growth chamber which was kept at 21 C with constant light.

Plants were harvested 15 days after planting, dried to constant weight at 43 C and weighed.

Tracer Studies with and without an Inorganic Nitrogen Supply. Low and high protein 'Logan' wheat seeds were prepared as previously described. L-glutamic-[^{14}C(U)]-acid or L-glutamine-[^{14}C(U)] (0.054 mg · ml^{-1} and 0.062 mg · ml^{-1}, New England Nuclear) was filtered through a 22 µm Micropore filter. Five µl (1.11 · 10^6 dpm) was injected into the cavity in each seed. All seeds were dried for 30 min at 43 C in a vacuum oven at 165 millibars absolute pressure.

A "fistula" filled with the exact weight of aseptically-obtained low or high protein endosperm that had been drilled out of each seed was plugged into each cavity and sealed in place with autoclaved paraffin. The "fistula" was a short length (about 5 mm) of 100 µl capillary tube, sealed at one end.

Seeds were planted just below the vermiculite layer in the autoclaved growth tubes. Half-strength Hoagland's nutrient solution with either 0 or 3 mM N as nitrate was injected through the access tube into each growth tube and replenished as necessary throughout the course of the experiments.

Plants were grown in a growth chamber set to provide 16 hr of light with a temperature of 21 C and 8 hr of darkness with a temperature of 16 C.

Plants were harvested 9 (glutamic acid) or 12 (glutamine) days after planting, dried to constant weight at 43 C and weighed.

Nitrate Uptake Study. About 100 unsized seeds of low or high protein 'Logan' wheat were planted in vermiculite or soil in 10 X 15 cm styrofoam trays and germinated. Six days after planting, plants of uniform size within each treatment were selected and placed, four to a cup, in Hoagland's nutrient solutions with 4 mM N as nitrate (17). The plants were suspended from sponges which covered the 150 ml sample cups.

Nitrate levels in the solution were determined after 3 days by a bacterial assay (32).

Statistical Procedure. Randomized complete block designs with 4 or 5 replicates were used in all experiments. Results were evaluated by means of an analysis of variance and the F-test. Tracer studies in which the effect of inorganic nitrogen was studied were analyzed as 2 X 2 factorials. Where appropriate, the least significant difference (LSD) was also calculated. Levels of significance are indicated by asterisks as follows: $p = 0.05$*, $p = 0.01$**, and $p = 0.001$***.

RESULTS AND DISCUSSION

The replacement of endosperm by endosperm enriched with glutamine produced seedlings that were larger at harvest in terms of both fresh and dry weights (Table I). The ratio of fresh weight to dry weight did not differ from that of the control. This is obviously a real weight gain in cellular structural material in the seedling, not merely an increase in the amount of water taken up in the seedling.

Glutamine, at least when it is doubled in the seed, can cause the increased vigor reported in high protein seed. Other studies done in the greenhouse using a fistula in a manner similar to that described for the tracer studies were conducted by Ries and Wert (unpublished data). In these studies glutamine at 1.64 mg · $seed^{-1}$ increased the dry weight of seedlings grown from either low (144 mg · g^{-1} protein) or high (160 mg · g^{-1} protein) protein 'Logan' wheat. Addition of 1.64 mg · $seed^{-1}$ of glutamine to 'Mindy' wheat (140 mg · g^{-1} protein) increased both the dry weight and the weight of protein per plant. A purified gliaden protein fraction added to 'Bluebird' wheat at the rate of 3 mg · $seed^{-1}$ increased the dry weight of the seedlings by 31% over control seedlings that received 3 mg of endosperm each. Glutamic acid or glutamine constitutes over 40% of gliaden amino acids by weight (3).

The results of the first L-glutamine-[$^{14}C(U)$] tracer study show that glutamine does not follow the overall respiratory pattern in high protein seed as reported by Lopez and Grabe (28). In the first 6 days after planting, seedlings growing from low protein seeds evolve nearly twice as

Table 1. Dry Weight of 14 Day Old Wheat with Glutamine-enriched Endosperm.

The F-ratio for fresh weights is significant at p = 0.01**; the F-ratio for dry weight is significant at p = 0.05*. The ratios of fresh weight to dry weight did not differ.

	ROOTS + SHOOTS (mg · plant^{-1})		FRESH WT · DRY WT^{-1}
TREATMENT	FRESH WT	DRY WT	RATIO
Control	239.6	46.2	5.19
L-Glu enriched	310.9	56.5	5.50

much $^{14}CO_2$ as seedlings grown from high protein seed (Fig. 3). After the addition of a half-strength Hoagland's solution with 3 mM N, the pattern was reversed at 12 and 15 days. The stimulation in overall $^{14}CO_2$ evolution is probably caused by a general metabolic increase brought on by the addition of the nutrient solution. This general metabolic increase may also account in part for the increased $^{14}CO_2$ evolution from the seedlings grown from the high protein seed at this point (Fig. 4). When the evolution of $^{14}CO_2$ is calculated on the basis of the final dry weight of the plants in an attempt to remove respiratory rate as a factor, there is no longer a significant difference on day 12; the sample taken on day 15 still shows the difference.

No differences between seedlings grown from low or high protein seed were found in the amounts of glutamine carbon incorporated into any plant fraction on an absolute basis (Table II).

The unexpected influence of nitrogen on the utilization of glutamine at this point in the development of the seedling led to studies on the effect of nitrogen when supplied at the initiation of the test. In the first sample, the evolution of $^{14}CO_2$ from the seedlings grown from low protein seeds without nitrogen was better than double that of either the seedlings grown from low protein seeds with nitrogen or the seedlings grown from high protein seeds without nitrogen (Fig. 5). The higher $^{14}CO_2$ evolution from the seedlings grown from high protein seeds supplied with nitrogen (Fig. 5) was again apparently caused by a higher total seedling metabolic rate because it was not significantly higher when calculated on the basis of the final dry weight of the plants (Fig. 6).

A similar pattern persists to the sixth day with regard to the seedlings grown from low and high protein seed and not supplied with nitrogen.

Figure 3. $^{14}CO_2$ Evolution from Seedlings Grown from Low and High Protein Seeds Supplied with L-Glutamine-[$^{14}C(U)$] in the Endosperm.

Each point is a mean of 4 samples calculated as the percent of the amount of ^{14}C not yet evolved as $^{14}CO_2$. The F-ratios of days 3, 6, 12 and 15 were significant at p = 0.05* according to analyses of variance.

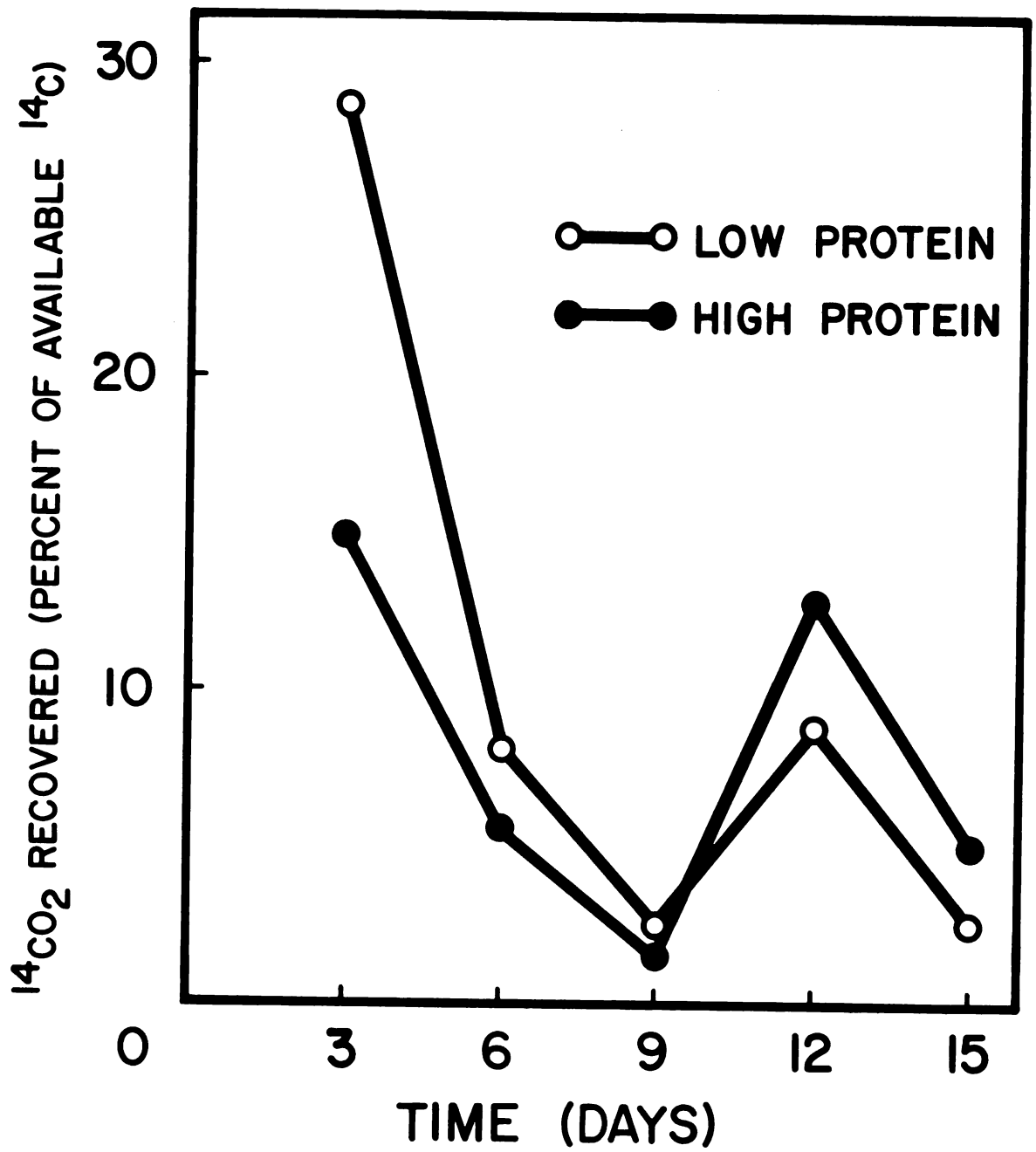
30
20
10
0
3
6
9
12
15
LOW PROTEIN
HIGH PROTEIN
$^{14}CO_2$ RECOVERED (PERCENT OF AVAILABLE ^{14}C)
TIME (DAYS)

Figure 3

Figure 4. $^{14}CO_2$ Recovered per mg Final Dry Weight from Seedlings Grown from Low and High Protein Seeds Supplied with L-Glutamine-[$^{14}C(U)$] in the Endosperm.

Each point is a mean of 4 samples. The F-ratios of days 3, 6 and 15 were significant at p = 0.01**, p = 0.05* and p = 0.05* respectively according to analyses of variance.

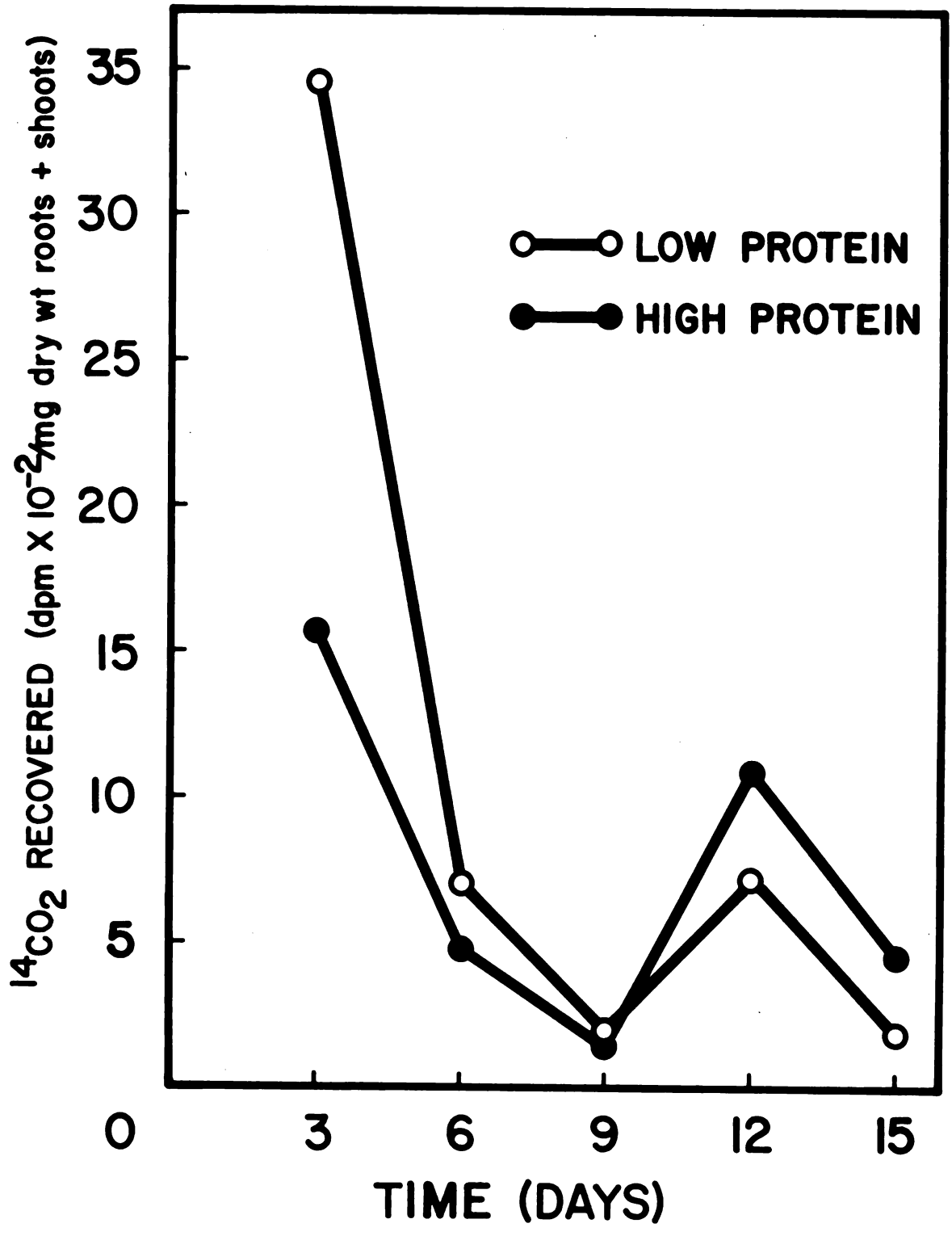
$^{14}CO_2$ RECOVERED (dpm X 10^{-2}/mg dry wt roots + shoots)
35
30
25
20
15
10
5
0
3
6
9
12
15
TIME (DAYS)
LOW PROTEIN
HIGH PROTEIN

Figure 4

Table II. ^{14}C Activity of Several Fractions of Seedlings Grown from Low and High Protein Wheat Seed Supplied with L-Glutamine-[$^{14}C(U)$] in the Endosperm.

No differences were found using the analysis of variance.

ENDOSPERM PROTEIN (mg · g^{-1} dry wt)	^{14}C RECOVERD (dpm · plant^{-1})				ROOTS + SHOOT DRY WT (mg · plant^{-1})
	AQUEOUS FRACTION	HYDROLYZABLE CARBOHYDRATE	FREE AMINO ACIDS	PROTEIN AMINO ACIDS	
144 (LOW)	13481	8719	5841	12703	36.8
160 (HIGH)	17569	8690	6722	11456	42.6

Figure 5. $^{14}CO_2$ Evolution from Seedlings Grown from Low or High Protein Seeds Supplied with L-Glutamine-[$^{14}C(U)$] and Grown with and without a Nitrate Supply.

Each point is a mean of 4 samples calculated as the percent of the amount of ^{14}C not yet evolved as $^{14}CO_2$. The F-ratios for the protein X nitrogen interactions were significant at $p = 0.001$***, $p = 0.01$** and $p = 0.05$* for days 3, 6 and 9 respectively according to analyses of variance.

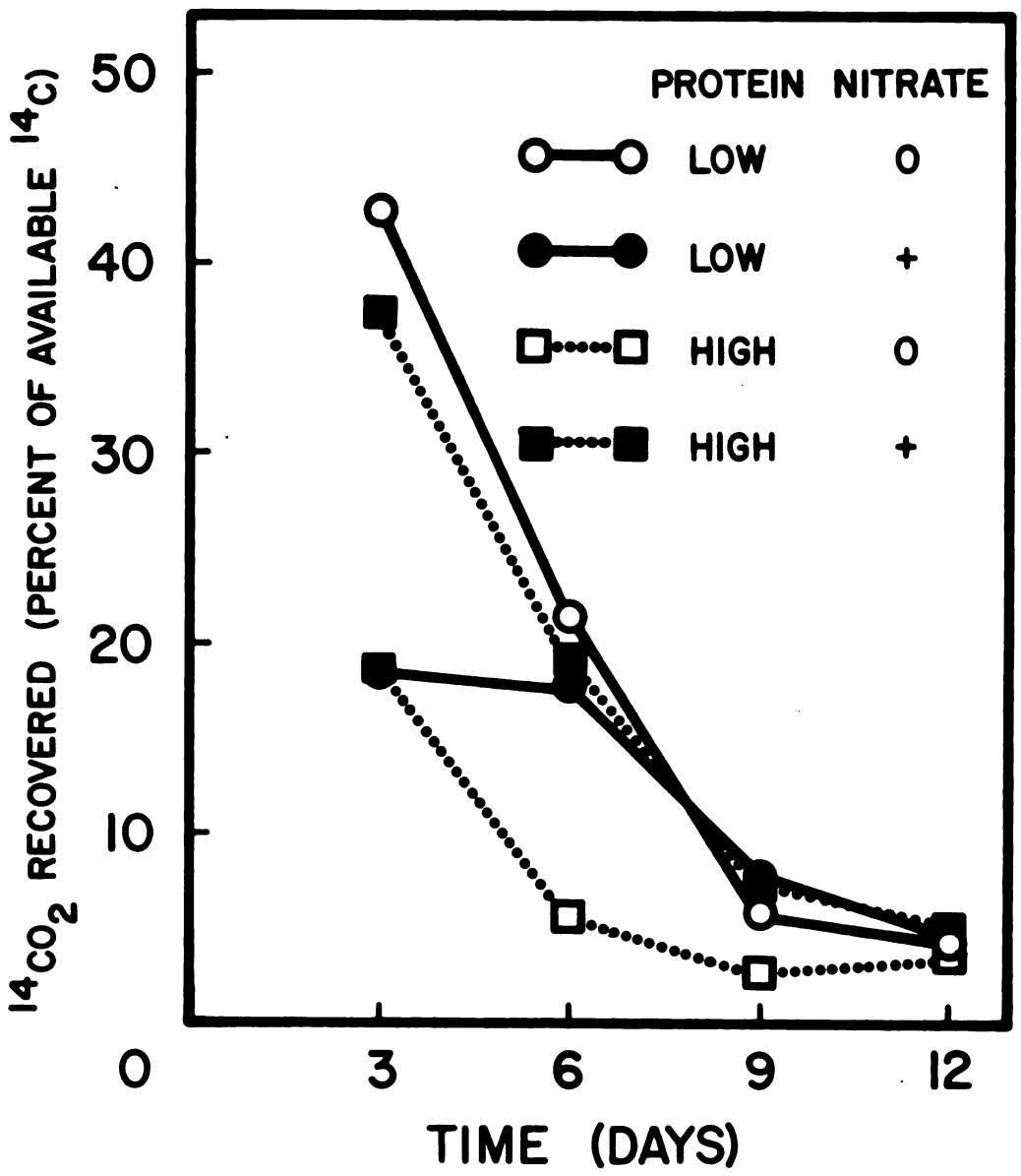
PROTEIN NITRATE
LOW 0
LOW +
HIGH 0
HIGH +
$^{14}CO_2$ RECOVERED (PERCENT OF AVAILABLE ^{14}C)
50
40
30
20
10
0
3
6
9
12
TIME (DAYS)

Figure 5

Figure 6. $^{14}CO_2$ Recovered per mg Final Dry Weight from Seedlings Grown from Low or High Protein Seeds Supplied with L-Glutamine-[$^{14}C(U)$] and Grown with and without a Nitrate Supply. Each point is a mean of 4 samples. The F-ratios for the effect of protein were significant at p = 0.05* and p = 0.01** for days 3 and 6 respectively.

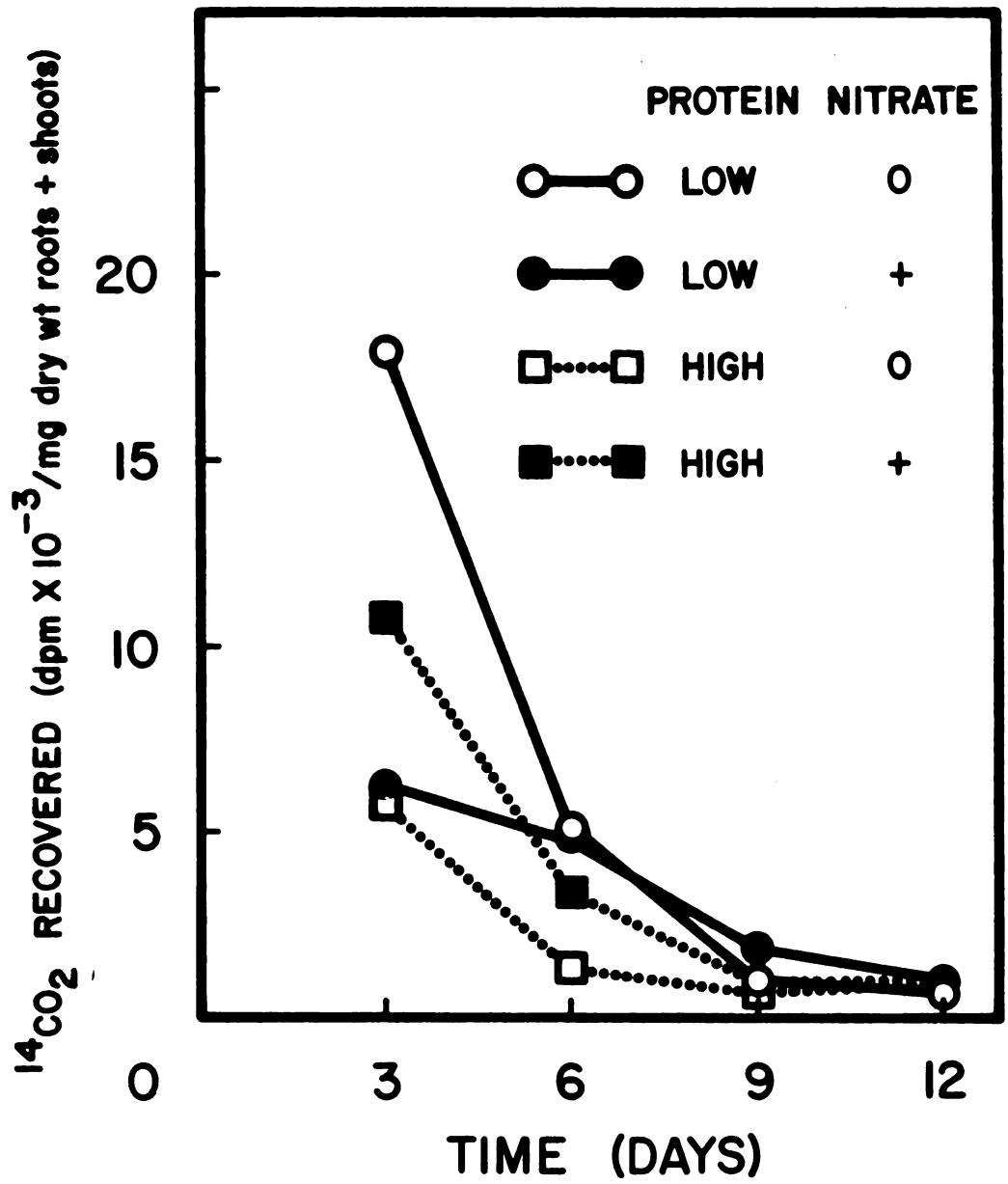
PROTEIN NITRATE
LOW 0
LOW +
HIGH 0
HIGH +
$^{14}CO_2$ RECOVERED (dpm X 10^{-3}/mg dry wt roots + shoots)
0
5
10
15
20
0
3
6
9
12
TIME (DAYS)

Figure 6

The nitrogen in the other two treatments may well have been depleted by this point. This would account for the rise in the $^{14}CO_2$ evolution of the seedlings grown from low protein seeds with nitrogen.

It seems likely, when the utilization patterns in the seedlings grown from high protein seed in these two experiments are compared, that there are metabolic increases that are not adequately reflected in calculating on the basis of final plant dry weight. This seems to be the most logical explanation for the rise in $^{14}CO_2$ output of the seedling grown from high protein seed with nitrogen. Both high protein and nitrate are known to increase overall respiration during germination (28,10) and respiration peaks between the third and sixth days of germination (11,10).

As in the previous experiment there were no differences among the treatments with regard to the amount of label recovered from the several plant fractions. The dry weights of the plants did differ, however. There was no difference in the weights of the seedling grown from low protein seed that received nitrogen and those grown from high protein seed that did not receive nitrogen. The addition of this small amount of nitrogen did not increase the growth of the seedlings grown from high protein seed (Table III).

In view of the large differences in glutamine utilization, it is surprising that only by calculating on the basis of final dry weight of the plants can any significant differences in utilization of the L-glutamic-[^{14}C(U)] acid be found. These differences are similar to the patterns observed with glutamine (Fig. 8). It may be more important that there is no significant increase in $^{14}CO_2$ evolution in seedlings supplied with nitrate and in the seedlings grown from high protein seed due to the expected general increase in metabolic rates (Fig. 7).

Table III. ^{14}C Activity in Several Fractions of Wheat Seedlings Supplied with L-Glutamine-[$^{14}C(U)$] in the Endosperm.

No differences in ^{14}C accumulation in a fraction were induced by any treatment combination according to analyses of variance. The F-ratios for effects of protein and nitrogen on dry weight were significant at p = 0.001*** and p = 0.01** respectively. Dry weight means followed by different letters differ significantly at the p = 0.05 level according to the LSD test.

ENDOSPERM PROTEIN (mg · g^{-1} dry wt)	INITIAL HOAGLAND'S NITRATE CONCN (mM)	^{14}C RECOVERED (dpm · $plant^{-1}$)				ROOTS + SHOOT DRY WT (mg · $plant^{-1}$)
		AQUEOUS FRACTION	HYDROLYZABLE CARBOHYDRATE	FREE AMINO ACIDS	PROTEIN AMINO ACIDS	
144 (LOW)	0	25324	17250	9035	20436	26.9 a
	3	20574	13214	10510	21746	33.4 b
160 (HIGH)	0	23824	12387	9198	18306	36.4 bc
	3	8915	10132	9848	23708	38.6 c

Figure 7. $^{14}CO_2$ Evolution from Seedlings Grown from Low or High Protein Seeds Supplied with L-Glutamic-[$^{14}C(U)$] Acid and Grown with and without a Nitrate Supply.

Each point is a mean of 4 samples calculated as the percent of the amount of ^{14}C not yet evolved as $^{14}CO_2$. The F-ratio for the effect of protein is significant at p = 0.05* on day 6.

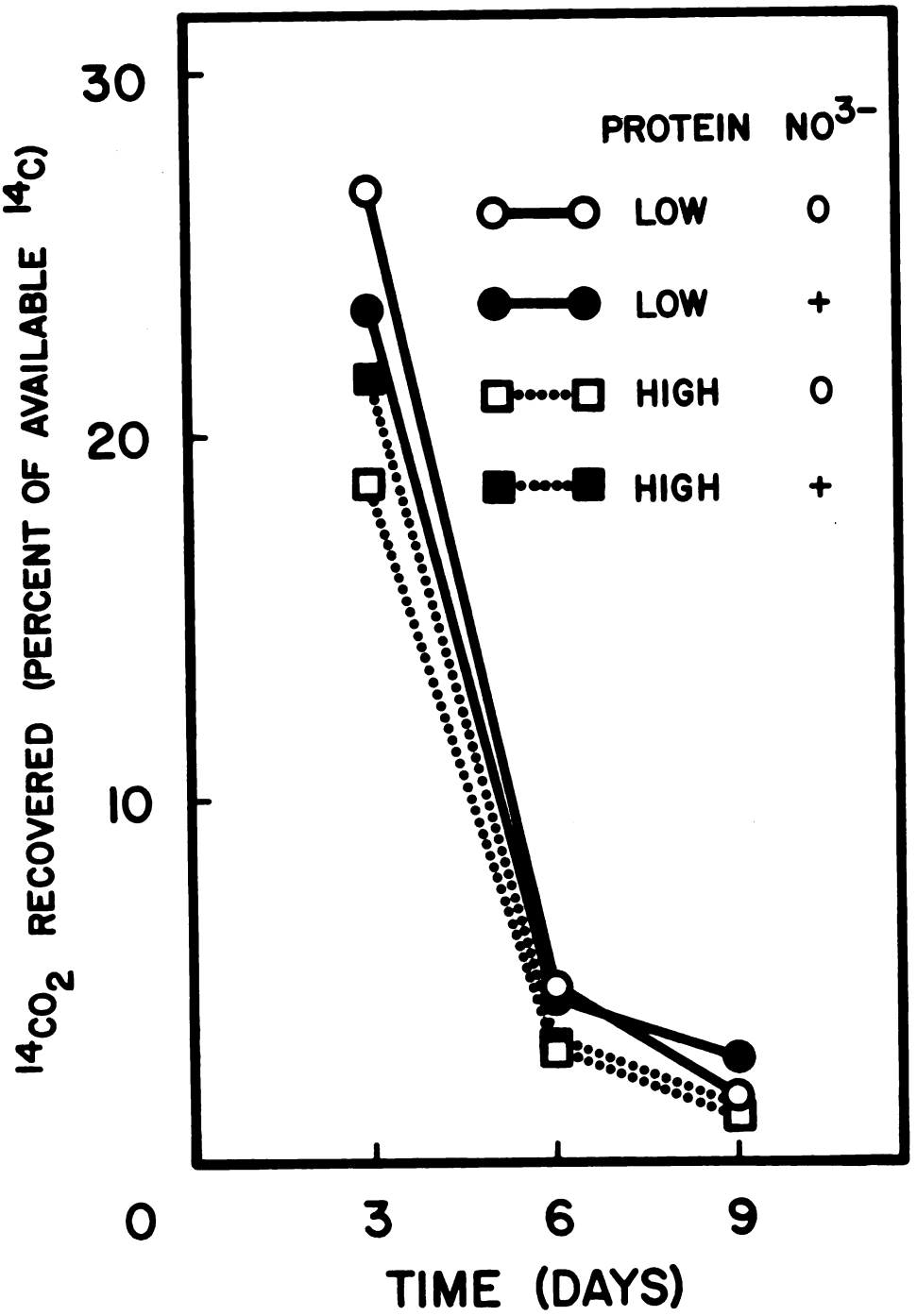
30
20
10
0
3
6
9
TIME (DAYS)
$^{14}CO_2$ RECOVERED (PERCENT OF AVAILABLE ^{14}C)
PROTEIN
NO^{3-}
LOW 0
LOW +
HIGH 0
HIGH +

Figure 7

Figure 8. $^{14}CO_2$ Recovered per mg Final Dry Weight from Seedlings Grown from Low and High Protein Seeds Supplied with L-Glutamic-[$^{14}C(U)$] Acid and Grown with and without a Nitrate Supply.

Each point is a mean of 4 samples. The F-ratios for the effect of protein were significant at p = 0.05* and p = 0.01** for 3 and 6 days respectively.

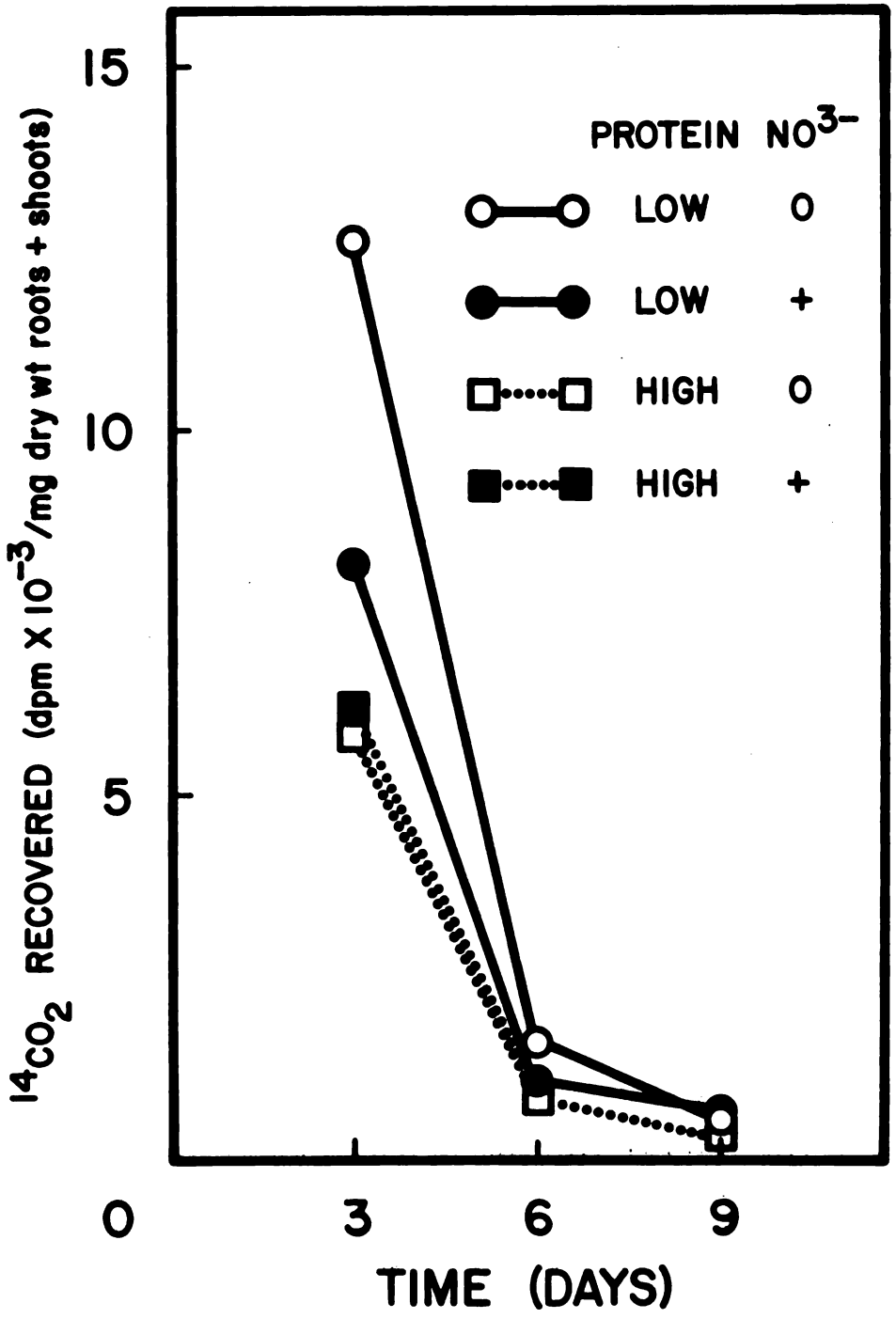
15
10
5
0
3
6
9
TIME (DAYS)
$^{14}CO_2$ RECOVERED (dpm X 10^{-3}/mg dry wt roots + shoots)
PROTEIN NO^{3-}
LOW 0
LOW +
HIGH 0
HIGH +

Figure 8

Again, no differences among treatments were found in the distribution of the label (Table IV). The final dry weight pattern was the same as in the previous experiment.

Glutamine and glutamic acid are involved in transaminations of some 16 or more of the most common amino acids. Glutamine is involved in both pyrimidine and purine biosynthesis (58) including involvement in the pathways leading to the production of both NAD and ATP. Glutamic acid, glutamine and proline are all primary nitrogen sources for synthesis of other amino acids during germination (11). Furthermore, glutamine seems to be the primary medium for nitrogen translocation (25,27). Glutamine may even donate its amide N for amino acid synthesis (6,24). It has also been suggested that the major pathway for nitrogen assimilation in higher plants may not be via glutamate dehydrogenase but via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway where glutamine is the major N acceptor (35). In addition, although asparagine is the major free amino acid in dry wheat seeds and in more mature parts of the plant, glutamine is the amino acid in highest concentrations where cells are growing and dividing most rapidly (57) and is metabolically the most active amino acid under normal conditions in several species: Hordeum vulgare (34,60,61), other Gramineae (4,53,59), and Lupinus angustifolius, Vicia atropurpurea, and Cucurbita pepo (56).

Clearly glutamine has a unique function in protein, pyrimidine and purine synthesis. The question remaining is the source of the glutamine that participates in these syntheses. Glutamine may be synthesized de novo in seedlings or be derived from endosperm proteins.

The period of most rapid protein synthesis in the embryo and seedling is between about the second and sixth days of germination. Respiration

Table IV. ^{14}C Activity in Several Fractions of Wheat Seedlings Supplied with L-Glutamic-[$^{14}C(U)$] Acid in the endosperm.

No differences in ^{14}C accumulation in a fraction were induced by any treatment combination according to analyses of variance. The F-ratios for effects of protein and nitrogen on dry weight were significant at p = 0.01** and p = 0.05* respectively. Dry weight means followed by different letters differ significantly at the p = 0.05 level according to the LSD test.

ENDOSPERM PROTEIN (mg · g^{-1} dry wt)	INITIAL HOAGLAND'S NITRATE CONCN (mM)	^{14}C RECOVERED (dpm · plant^{-1})				ROOTS + SHOOT DRY WT (mg · plant^{-1})
		AQUEOUS FRACTION	HYDROLYZABLE CARBOHYDRATE	FREE AMINO ACIDS	PROTEIN AMINO ACIDS	
144 (LOW)	0	22095	3404	15174	8141	25.6 a
	3	20459	2722	14448	6972	32.1 b
160 (HIGH)	0	15012	2139	13004	5010	35.6 bc
	3	15452	2052	14635	5236	38.0 c

and protein increase rates both peak at about day 3 and are highly correlated. Transfer of nitrogen out of the endosperm is virtually complete by the eighth day of germination (11). In view of this and the facts that amino acids derived from endosperm proteins are the major source of the CO_2 evolved (11) and that the seedling, not the endosperm, gives off most of the CO_2 (10), the patterns of $^{14}CO_2$ evolution in the tracer experiments should clearly indicate differences in utilization, especially in the samples taken after 3 and 6 days.

The elevated $^{14}CO_2$ evolution in the glutamine experiments for seedlings grown from high protein seed supplied with nitrogen versus that of those seedlings not supplied with nitrogen is consistent with the expected increase in metabolism. It is the elevation of $^{14}CO_2$ evolution in seedlings grown from low protein seeds not supplied with nitrogen versus either seedlings grown from low protein seed supplied with nitrogen or seedlings grown from high protein seeds not supplied with nitrogen that is unexpected and that provides the major clues to the utilization of glutamine derived from endosperm proteins.

According to Paech (37), when wheat is supplied with N more protein remains in the endosperm despite the fact that these seedlings have more protein. Fowden, et al., (12) found that free amino acids are not normally present in higher plant cells in concentrations even remotely approaching the amounts required for incorporation into protein molecules. Steward and Bidwell (50) report cases of C from glucose entering protein faster than C from exogenously supplied amino acid. Amino acids derived from endosperm protein would logically act as an exogenous amino acid supply as far as the cells of the embryo are concerned.

The patterns observed in these data would be expected if glutamine

derived from endosperm proteins served mainly as an emergency source of N for protein synthesis in the germinating seedling. Endosperm amino acids in general may only be used if amino acids cannot be synthesized de novo in the embryo because of the lack of an external inorganic N supply. Seedlings grown from low protein seeds may rely heavily on endosperm-derived glutamine N if there is no external inorganic N supply. When N is supplied, the utilization of endosperm-derived glutamine drops off. In seedlings grown from high protein seed with no external N supply, a larger supply of amino acids derived from the endosperm or a larger pool of glutamine could depress or appear to depress the utilization of endosperm-derived glutamine relative to utilization in seedlings grown from low protein seed.

Sims, et al., (49) reported that higher plant cells use nitrate in preference to exogenously supplied amino acids. There was a limited decrease in nitrate uptake when amino acids were available, however. This apparent preference for inorganic N and the normally low amount of free amino acid in plant cells led Fowden, et al., (12) to propose that amino acids not synthesized within a particular cell, enter separate pools, perhaps in vacuoles. These amino acids, endosperm-derived amino acids for instance, would not under normal circumstances be used mainly for protein synthesis but would be stored until their N could be used and their C respired away. In the case of glutamine, the main function of which is donating N, it would be withdrawn only as a last resort if no inorganic N was available. It could also be called on to donate N to endosperm-derived carbohydrates for amino acid synthesis when the amino acid pool is deficient and no source of inorganic N is available. This would be the case with the seedlings growing from low protein seeds not

supplied with N.

These data are also consistent with the finding that two distinct glutamate dehydrogenase isoenzymes exist, one soluble and primarily catabolic and one associated with primarily anabolic cytoplasmic inclusions (14,15,39). Only de novo synthesized glutamate/glutamine would under normal conditions be incorporated into seedling protein, endosperm-derived glutamate/glutamine functioning mainly as emergency N donors.

This apparent utilization of inorganic N in preference to stored N is consistent also with the dry weights of the plants grown from low and high protein seed. Nitrogen supplied to a low protein seed results in a plant of the same weight as the one grown from high protein seed with no nitrogen supply. Supplying N to high protein seeds does not result in a further increase, however (Tables III and IV). Lopez and Grabe reported this same effect in field trials where dry matter production of plants grown from high protein seed was higher only in nitrogen-poor soil (28). This apparent upper limit to N utilization is also illustrated by Jackson, et al., (18). They found that the higher the concentration of N that seedlings were grown on, the less they took up from solution later until finally there was no net uptake, nitrate was excreted from the roots. It is especially interesting that this nitrate was not the same as was taken up from solution and that there was no measurable difference in nitrate concentration in the roots.

In the nitrate uptake study with low and high protein seed, the protein content of the endosperm did not influence the uptake pattern (Table V). Seedlings germinated and grown for 6 days in vermiculite and distilled water took up more nitrate from solution than those germinated in soil, regardless of protein content of the endosperm.

Table V. Nitrate Uptake in Wheat Seedlings Grown from Low and High Protein Seed.

The plants were grown either in vermiculite or in soil for 6 days after planting, then transplanted to a half-strength Hoagland's solution with 4 mM nitrate nitrogen. Uptake was measured after 3 days. The F-ratio for the effect of the original media was significant at $p = 0.01^{**}$.

GERMINATION MEDIA	SEED PROTEIN ($mg \cdot g^{-1}$)	NITRATE UPTAKE RATIOS
Vermiculite	144 (LOW)	1.66
	160 (HIGH)	1.65
Soil	144 (LOW)	1.01
	160 (HIGH)	1.00

This raises one final question. Have breeding programs selected for primary reliance on the inorganic N supplied by N fertilizers? Would the utilization of endosperm-derived glutamine N by wild varieties be the same? In view of the fact that no special proteins high in glutamate/glutamine are elaborated by the developing seed (9) and that inorganic N is used preferentially when available, is the accumulation of more protein especially high in glutamine merely a method for detoxifying the ammonia resulting from fertilizer application or from the increased nitrate uptake induced by subtoxic herbicide applications that are used to produce high protein seed? Production of high-glutamine seeds would certainly be an easy path for the plant to take, especially with glutamine synthetase being available in the chloroplasts of the tissues supplying materials to the seed (16,36). What would normally have been stored as starch is converted to amino acid instead to eliminate excess ammonia. Lopez and Grabe (28) and Salunkhe, Wu and Singh (47) both report seed protein increases are accompanied by starch decreases.

Utilization of this protein reserve appears to be simply a last resort valuable only under stress conditions.

APPENDIX

APPENDIX

RECOMMENDATIONS AND COMMENTS ON THE METHOD

The technique developed for these experiments falls short of fulfilling its original design intentions on two counts. First, the purpose of the access tube was to be able to change nutrient solutions while easily maintaining asepsis. Although it is easy to add solutions, there is no convenient way to remove solutions before adding fresh ones without disturbing the plant or opening the growth tube. If the access tube were to be installed at the bottom of the growth tube, perhaps with a small filter or screen at the base of the growth tube, solutions could easily be added or removed without disturbing the plant or the aseptic environment.

Secondly, the technique was designed to test the utilization of tracer compounds that were injected directly into the seed. Using this technique, there is little room for doubt that their use patterns are any different from the same compounds normally present in the seed. The question can easily be raised when seeds are grown in a medium containing the test compound as to whether uptake via roots or by absorption into the seed will affect the use.

But admittedly, drilling a hole in the side of the seed has a detrimental effect on seedling growth. Therefore, the control used should always be (and in these experiments was) a seed whose fistula contains only the amount of endosperm removed from the seed when the hole is drilled. In addition, substantial amounts of the compound injected into the seed leach out again. The latter is probably the result of a combination of two things: the fact that any liquid chemical injected is

readily soluble in any moisture the seed absorbs and the presence of the hole, even though it supposedly has been sealed. The paraffin seal on the seeds in the experiments reported in this paper was always intact at the end of the experiment at least insofar as holding the fistula in place was concerned. Whether it provided a water-tight seal would be rather difficult to determine.

Though it is not a perfect duplication of real conditions in the seed, it seems that it is still a more reliable method to determine utilization than techniques in which the compound never was present inside of the seed.

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