STUDIES ON THE MODE OF ACTION OF SUB-TOXIC LEVELS OF SIMAZINE IN INCREASING PLANT PROTEIN CONTENT

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ABSTRACT

STUDIES ON THE MODE OF ACTION OF SUB-TOXIC LEVELS OF SIMAZINE IN INCREASING PLANT PROTEIN CONTENT

By

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These studies were initiated to provide an understanding of the mechanism whereby sub-lethal concentrations of 2-chloro-4,6-bis(ethylamino)-s-triazine (simazine) increases the protein content of plants. Previous research has shown that simazine increases the protein content per plant only under certain environmental and nutritional conditions. It has also been postulated that nitrate assimilation and carbohydrate degradation may be involved in the mode of action.

No evidence was obtained for the direct interaction of 2-chloro-4-ethylamino-6-isopropylamino-s-triazine (atrazine) with proteins. Radioactive atrazine did not bind to proteins from oat (Avena sativa L.) shoots and roots, and ^{14}C-atrazine was not incorporated into proteins.

Barley (Hordeum vulgare L.) proved to be an excellent plant species for these studies, and the following conclusions are based on studies with 'Coho' barley. Simazine at 1×10^{-8} M consistently increased the water-soluble protein per plant

in short term experiments. Maximum increases in protein content and uniform plants were obtained if the plants were exposed to low temperatures and low nitrogen levels before treatment with simazine. The water-soluble carbohydrate content also accumulated under these conditions. Plants grown under conditions producing low carbohydrate did not respond to simazine.

In experiments where simazine increased protein content, the soluble carbohydrate content was decreased. This response approximated a weight for weight exchange. The reduction in soluble carbohydrates by simazine was not related to an increase in respiration or inhibition of photosnythesis. Carbohydrate degredation is postulated to be stimulated by an increase in nitrate uptake.

Studies with ^{14}C-leucine indicated that stimulations of protein synthesis by simazine preceded increases in nitrate uptake and protein content. Therefore, the effect of simazine on increases in nitrate assimilation and carbohydrate degradation may be secondary responses. ^{14}C-Leucine incorporation into protein was stimulated by simazine but not by 2-hydroxy-4,6-bis(ethylamino)-s-triazine (hydroxy-simazine) in in vitro experiments.

Atrazine has been shown to stimulate nucleic acid synthesis. It is hypothesized that this is also the primary action of simazine. The stimulatory effect of chromatin directed nucleic acid synthesis may result in an increase in

protein synthesis. This in turn should increase nitrogen uptake. However, nitrate nitrogen must be reduced before being incorporated into protein. Reduction of nitrate utilizes reduced nicotinamide adenine dinucleotide (NADH) as a co-factor. NADH is provided by carbohydrate breakdown. This may account for the reduction in soluble carbohydrates due to simazine. Simazine did not alter plant protein content under conditions not favorable for the accumulation of soluble carbohydrates (high temperatures and high nitrogen levels).

Simazine has been shown not to increase the protein content of plants utilizing ammonium as a source of nitrogen. This is not due to decreases in simazine uptake brought about by ammonium treatment. The assimilation of nitrogen from ammonium and nitrate is different, and nitrate has been shown to stimulate glycolysis. This provides NADH for nitrate reduction and may result in an increase in carbon compounds for amino acid synthesis. Under the conditions necessary for simazine to increase protein content, protein synthesis in ammonium grown plants may be limited by the lack of carbon skeletons.

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INTRODUCTION

Simazine (2-chloro-4,6-bis(ethylamino)-s-triazine) is one of the most widely used triazine herbicides. Since the introduction of this chemical in 1955, it has been used extensively as a soil sterilant for non-crop land, as a selective herbicide for many perennial horticultural crops, and as an aquatic herbicide.

Besides possessing herbicidal properties, simazine has been shown to influence the growth of many plants. In 1957, Bartley (5) reported that corn (Zea mays L.) plants treated with simazine as a herbicide was greener and produced higher yields than untreated plants. Since this observation, numerous articles have been published showing that plant growth is often increased by simazine applications (14,19,20, 35,53,54,57,64,76).

Improvements in plant composition have usually been obtained by plant breeding, use of fertilizers, or advances in cultural practices. There is little evidence demonstrating that chemicals (other than endogenous plant hormones) can consistently increase plant protein content. Increases in percent protein due to herbicide treatment are usually accompanied by a decrease in dry weight resulting in no net increase in total protein per plant.

Laboratory studies have shown that micromolar applications of simazine produce a significant increase in plant growth and total protein content (53,54,76). In field experiments with cereal grains, legumes, and forage crops substantial increases in protein content due to sub-herbicidal applications of simazine have been obtained (14,19,20 35,57,64). However, consistent increases in protein content have been difficult to achieve (1,56,75,77).

The objective of this research was to elucidate the mode of simazine action in increasing plant protein content. Resolution of this problem may aid in understanding the factors necessary to produce plant protein increases. Furthermore, the information may contribute to directed synthesis of more effective compounds.

LITERATURE REVIEW

Physical properties of simazine. Synthesis and testing of triazine compounds as herbicides began in 1952. Simazine was one of the first triazines tested to show biological activity. Simazine is synthesized by reacting cyanuric chloride with two equivalents of ethylamine in the presence of an acid acceptor (25). It is characterized by having a low vapor pressure (6×10^{-9} mm Hg at 20 C), low water solubility (2×10^{-5} M at 20 C), molecular weight of 201.7, and a melting point of 225-227 C (30). Photodecomposition by ultraviolet irradiation dechlorinates simazine, but under normal field conditions this is negligible (32).

Uptake and movement in plants. Simazine is readily absorbed by plant roots and translocated into leaves (13,43, 65). Simazine moves in the apoplast and tends to accumulate around the margins of broadleaf plants and at the tips of grass leaves (11).

Uptake and translocation appear to be via the transpiration stream. Sheets (65) reported that plants grown at 37 C contained twice as much simazine as plants grown at 26 C. Furthermore, plants grown at a constant temperature contained more simazine at a lower relative humidity.

Foliar uptake of simazine is restricted by the cuticle. However, once this barrier is damaged or broken, simazine readily enters the leaf (13). In an intact leaf, small amounts of simazine, 6% of that placed on the leaf surface, can penetrate the cuticle (51). These results are supported by the fact that sub-herbicidal effects of simazine can be observed due to foliar applications (71,82).

Mode of action. Simazine is a potent photosynthetic inhibitor. Exer (17) reported that 7 X 10^{-7} M simazine inhibited the Hill Reaction by 50% in isolated corn and spinach (Spinacia oleracea L.) chloroplasts. Moreland et al. (46) confirmed these findings by showing that a concentration of 5 X 10^{-6} M simazine reduced by 50% the ability of isolated barley (Hordeum vulgare L.) chloroplasts to oxidize water to molecular oxygen. Also, barley plants were kept alive and growing in the presence of lethal concentrations of simazine if they received glucose through severed tips of leaves.

Starch production in Coleus blumei Benth. is blocked by simazine in the light (22). However, starch-free coleus leaves are able to form starch in the presence of simazine when fed sucrose in the dark. Similarly, Ashton et al. (3) showed that 1.3 X 10^{-6} M simazine inhibited $^{14}CO_2$ fixation by 30% and 5 X 10^{-6} M completely blocked photosynthesis in beans (Phaseolus vulgaris L.).

A review by Gysin (25) concludes that the inhibitory effects on the Hill Reaction correspond with the herbicidal

activity of the triazines. Furthermore, 2-hydroxy-4,6-bis-(ethylamino)-s-triazine (hydroxy-simazine), which has been shown to be one of the first metabolites of simazine, does not interfere with the photochemical process (26). This compound is also inactive as a herbicide.

The exact mechanism by which chemicals inhibit the Hill Reaction is unknown. Moreland (45,47) concluded that the effect of herbicides on the Hill Reaction could be best explained by: (a) the lipophilic and hydrophilic groups of the molecule which assist the chemical in reaching the reactive sites; (b) the molecular configuration which enables the molecule to fit into the structure of the reactive sites within the chloroplast; and (c) the presence of specific groups which bind the herbicide molecule to the reactive sites within the chloroplasts.

There is a relationship between the structure of various triazines and herbicidal activity (26). Replacement of the chlorine by a methoxy group weakens the inhibitory effect of simazine as does replacement of one or both of the imino hydrogen atoms with a second alkyl group (47). The inhibitor potency may be related to the bonding ability of the imino hydrogens (23).

Photosynthesis is a crucial process for plant growth, and inhibition of this process would profoundly affect other plant processes. The Hill Reaction is the initial step which may control the course of the entire reaction. Thus, interference with the Hill Reaction would limit the successive

steps of the overall CO_2 fixing process.

Selective phytotoxicity and metabolism of simazine. Previous research on herbicides has shown that many factors may be involved in explaining resistance and susceptibility of plants to herbicides. Three common explanations are: differences in absorption and/or translocation of the herbicide in tolerant and susceptible plants; biochemical systems inhibited in the sensitive plants are not affected in the resistant plants; and resistant plants metabolize the herbicide to a less toxic moiety.

Ragab and McCollum (52), reported that selectivity to simazine was not due to differential absorption since both resistant corn and susceptible cucumber (Cucumis sativus L.) plants readily absorbed simazine. Similar work by Davis et al. (13) and Sheets (65) has shown that selectivity to simazine was not a consequence of difference in absorption and translocation.

The second possible explanation for herbicidal resistance of certain plants is that the herbicide inhibits some process in the susceptible plants but has no effect on the resistant plant. Previous observations indicate that the action of simazine involves the inhibition of the reactions required for photosynthesis (17,46,47). However, Moreland and Hill (47) found that tolerance is not due to biological inactivity of the herbicides in tolerant plants. Chloroplasts from corn were as susceptible to simazine as chloroplasts from sensitive plants.

Several investigators have observed a positive correlation between the extent of metabolism and the degree of resistance to simazine (10,28,44). Roth (59) first reported that simazine was metabolized in plants. He demonstrated that when simazine was added to freshly pressed corn juice it was metabolized to a non-biologically active compound. However, water-soluble extracts of wheat (Triticum aestivum L.), a susceptible plants, were unable to inactivate simazine. Later researchers established that certain plants are able to convert simazine to hydroxy-simazine, which was not phytotoxic (10,27,28,44). In corn the catalyst responsible for the conversion of simazine to hydroxy-simazine was later identified as 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one, or its glucoside (60).

Metabolism of s-triazines in biological systems has been reviewed by Shimabukuro et al. (68). S-triazines appear to be metabolized by hydrolysis, N-dealkylation, and glutathione conjugation. Research using inbred corn lines which differ in their tolerance to simazine indicated that the metabolism to hydroxy-simazine, catalyzed by the benzoxazinone compound, was not a basic factor in selectivity (2,48). Furthermore, some susceptible plants, wheat and rye (Secale cereale L.), contain benzoxazinone (27). Current research has demonstrated that alternate metabolic pathways exist that effectively metabolize simazine (66,68). Hydrolysis contributes to total detoxification but does not appear to be essential for tolerance (70).

Funderburk and Davis (21) reported that when corn, cotton (Gossypium hirsutum L.), and soybeans (Glycine max L.) were treated with alkyl-labeled simazine considerable amounts of $^{14}CO_2$ were detected. N-Dealkylation also occurred when plants were treated with hydroxy-simazine (31). In plants that contain benzoxazinone either N-dealkylation or hydrolysis may occur first to yield the same end products (67).

Shimabukuro (33) found that sorghum (Sorghum bicolor L.), which is highly tolerant, rapidly converted 2-chloro-4-(ethylamino)-6-(isopropyl)-s-triazine (atrazine) to water-soluble compounds. Sorghum leaf discs converted 62% of the absorbed atrazine to the water-soluble compounds in 7 hr (34). Later work identified the metabolites as S-(4-ethylamino-6-isopropylamino-s-triazinyl-2)-glutathione and s-γ-L-glutamyl-(4-ethylamino-6-isopropylamino-s-triazinyl-2)-L-cysteine (36). Glutathione-s-transferase catalyzed the initial conjugation of reduced glutathione with 2-chloro-s-triazine compounds (69). Glutathione-S-transferase is present in leaves of highly tolerant plants such as corn, sorghum, sugar cane (Saccharum officinarum L.), and Johnson-grass (Sorghum halepense L.). The activity of this enzyme was later shown to be a major factor in detoxification and selectivity of 2-chloro-s-triazine herbicides in higher plants (69,70).

Sub-herbicidal effects of simazine on plant growth and metabolism. Several s-triazines are unique herbicides which possess the ability to influence nitrogen metabolism when

applied at sub-toxic rates. Field studies are often variable, however, simazine has increased the protein content of many crops. Generally, susceptible species respond more favorably to triazine applications than resistant species.

Bartley (5) was the first to report that the growth of corn was stimulated by simazine. He observed that corn was greener and taller when simazine was applied at the rate of 18 kg/ha. Ries et al. (55) showed that peach (Prunus persica L.) and apple (Pyrus malus L.) trees treated with simazine had a higher leaf nitrogen and more terminal growth than untreated trees. They were the first to suggest that simazine may influence nitrogen metabolism.

Fink and Fletchall (19) reported that simazine and atrazine increased the percent nitrogen content of corn foliage. However, this was accompanied by a decrease in dry weight. They also observed a threefold increase in the nitrate content of corn treated with rates of 2.5, 5, and 10 kg/ha of simazine and atrazine under low nitrogen fertility conditions. These authors concluded that atrazine or simazine may stimulate nitrogen uptake when the available soil nitrogen is low. Other researchers have reported nitrate accumulation in corn due to herbicidal applications of simazine or atrazine (15,50). Similar work on corn showed that the dry weight of 14-day old plants was reduced with rates of atrazine greater than 2 kg/ha (16). However, 1 kg/ha increased the percent nitrogen in corn grown for 14 and 27 days, but the nitrogen content per

plant was not increased. Gramlich and Davis (24) reported that corn treated with 2 kg/ha atrazine was larger and contained 60% more protein than the control.

Tweedy et al. (75) studied the effect of simazine on wheat and sorghum grown at various nitrogen levels. In one experiment, 1.12 kg/ha simazine increased the grain and crude protein content of sorghum grain when the plants were under nitrogen stress. Later experiments did not verify the earlier results, however, nitrogen appeared not to be deficient. Similar results were obtained with bermudagrass (Cynodon dactylon L.) (42).

Kay (35) found that wheatgrass (Agropyron intermedium Beauv.) yield was quadrupled by applications of 1.1 kg/ha atrazine in a 4-year study. Furthermore, Arabian grass (Schismus arabicus Nees.) and red-stem filaree (Erodium cicutorium L.) yields were increased six-fold and the protein content eight-fold. These increases are the largest ever attributed to the triazines. The author states that a portion of the increases were probably due to the complete control of weed competition in the herbicide treated plots. Applications of simazine to other forage grasses have also resulted in increases in yield and protein content (1).

Vergara et al. (77) found that the percent protein of rice (Oryza sativa L.) grain was increased by simazine applications, however, this was accompanied by decreases in yield resulting in less grain protein per hectare. Similar results

were obtained with cotton leaves treated with simazine (72).

Ries et al. (57) increased the yield and protein of several crops grown in Michigan and Costa Rica. Applications of less than 0.5 kg/ha simazine increased the crude protein per hectare of ryegrass (Lolium perenne L.), 52%; pea (Pisum sativum L.) seed, 41%; alfalfa (Medicago sativa L.) forage, 10%; bean seed, 45%; rice foliage, 33%; and oat groats, 12%. Later work by Schweizer and Ries (64) showed that seeds with higher protein content due to simazine application produced larger seedlings and higher yields.

Although it has been 15 years since the first report that simazine may act as a growth regulator, the mechanisms of action of these sub-toxic levels remain unsolved.

Early studies dealt with the effect of simazine on nitrogen metabolism of corn. Ries and Gast (54) hypothesized that environmental conditions influence the effect of simazine on nitrogen metabolism. The application of 2.5×10^{-6} M simazine to corn plants grown under low nitrogen conditions increased the nitrogen content of the leaves by 90%. In a second test neither simazine nor hydroxy-simazine altered the nitrogen content of corn. The difference in responses was attributed to more optimum environmental conditions in the second experiment, primarily higher temperatures. Increases in growth of corn due to simazine were also obtained by Freney (20). He reported that simazine applied at 3×10^{-7} M in solution cultures increased the yield of corn tops, uptake

of nitrogen, phosphorous, magnesium and potassium. Similar results were obtained by DeVries (14).

Further work on the nutritional and environmental parameters necessary for simazine to increase the nitrogen content of corn revealed that low concentrations of simazine increased the dry weight and nitrogen content only when the plants were grown at low concentrations of nitrate nitrogen and at sub-optimum temperatures (76). Simazine did not alter the growth of corn plants when nitrogen was supplied as the ammonium ion, or if nitrate was present at high concentrations. The nitrate reductase activity of leaf extracts from corn plants grown under sub-optimum conditions increased tenfold after exposure to 9.6×10^{-6} M simazine for 7 days. However, the nitrogen content of these plants was only 12% higher in the simazine treated plants.

Increases in protein content as a result of simazine treatment are not limited to resistant corn plants. Ries *et al*. (53) reported that increases up to 79% in water extractable proteins could be obtained in rye treated with low concentrations of simazine. These stimulations also occurred only when nitrate was utilized as the nitrogen source. Furthermore, the increase in nitrogen content was accompanied by a large increase in nitrate reductase activity and a slight increase in respiration. Gel electrophoresis indicated that there was no qualitative difference between proteins from treated versus untreated plants.

Wray et al. (81) concluded that simazine may be specific for nitrate assimilation in barley. It did not effect sulfate or phosphate uptake. The stimulations of nitrate uptake and the elevation of nitrate reductase was dependent upon the continuous application of simazine.

Recent work indicates that simazine may affect processes other than nitrogen assimilation. Singh et al. (71) reported that the activities of nitrate reductase, glutamic-pyruvic transaminase, α-amylase, starch phosphorylase, and adenosine triphosphatase were stimulated in bush bean (Phaseolus vulgaris L.) plants sprayed with 1×10^{-7} M simazine. This led the authors to conclude that simazine creates a metabolic condition favorable for greater use of carbohydrates needed for nitrate reduction and protein synthesis. Carbohydrate degrading and nitrogen assimilating enzymes have also been reported to be stimulated in peas and sweet corn by foliar applications of 1×10^{-5} M simazine (82).

A decrease in the starch and soluble sugar content has been observed in many plants treated with simazine (1,77). This is usually explained by either an increase in respiration or a decrease in photosynthesis.

The effect of simazine on respiration has not been clearly established. Ries et al. (53) reported a small stimulation of respiration of rye plants treated with low concentrations of simazine. However, Tweedy (74) observed that simazine did not influence respiration in excised barley roots. Similar

results were obtained with atrazine applied to excised bean embryos (4). Tieszen (73) has reported that simazine applications (1.5 to 6 X 10^{-7} M) resulted in no significant stimulatory or inhibitory effects on respiration of rye plants grown at various temperatures. However, photosynthesis was inhibited at high temperatures (27 C day, 17 C night) by concentrations of simazine greater than 1.5 X 10^{-7} M.

Recently Penner and Early (49) reported that atrazine may stimulate nucleic acid synthesis. A 40% increase in chromatin directed RNA synthesis was obtained by treating etiolated soybeans for 5 hr with 1 X 10^{-6} M atrazine. The presence of atrazine in the extraction medium was sufficient to produce a similar response. However, hydroxy-atrazine, a non-phytotoxic metabolite, was inactive on chromatin activity.

Summary of literature review. Simazine is readily absorbed through the roots and translocated to the leaves. Foliar uptake is small, causing little post-emergence activity. However, once in the plant it is a potent photosynthetic inhibitor.

Selectivity is due to the rate at which simazine is metabolized. Tolerant species, such as corn, sorghum, and some grasses metabolize simazine to hydroxy-simazine, which is non-phytotoxic. All plants examined so far metabolize simazine either by hydrolysis, N-dealkylation, or glutathione conjugation. Hydrolysis is limited to tolerant species as is glutathione conjugation. Glutathione-S-transferase catalyzes

the reaction between 2-chloro-s-triazine and reduced glutathione.

Non-phytotoxic concentrations of simazine has increased the growth and/or protein content of many plants. Laboratory studies with corn have shown that not only is growth increased but nitrate reductase is greatly stimulated. Maximum stimulation occurs when the plants are grown at suboptimum temperatures, low nitrate nitrogen levels and high light intensities.

Carbohydrate degrading enzymes are also increased by low applications of simazine indicating the possibility of increased sugar metabolism.

Recent work illustrates that triazines may stimulate nucleic acid synthesis. This stimulation in RNA synthesis appears to be due to an increase in DNA template availability.

Presently, it is not known how many of these observations are secondary responses. The most common explanation for the mode of action of simazine in increasing protein content centers around nitrate assimilation. The role of carbohydrate utilization is not clear. Research workers reporting stimulation of carbohydrate degrading enzymes by simazine have not reported increases in total protein synthesis per plant in the same tests. Furthermore, previous research has shown that simazine increases the protein content only under certain environmental conditions, however, these conditions are not all essential for carbohydrate breakdown. The effect of

atrazine on RNA synthesis also does not appear to be influenced by environmental or nutritional parameters.

The objective of this study was to provide a more thorough understanding of the metabolic changes caused by simazine which ultimately result in an increase in the protein content of plants.

MATERIALS AND METHODS

Plant growing procedures. Growing conditions were similar in all experiments with whole plants. 'Coho' barley or 'Gary' oat seeds were planted in 11 X 7 cm plastic cups (30 seeds/cup) filled with vermiculite. The plants were grown in a growth chamber providing 25,000 lumens/m^2 of light with a 16 hr day (20 C) and 8 hr night (15 C). In all barley experiments, the plants were transplanted after 6 days. The plants were carefully removed from the vermiculite and the roots washed with water. Four uniform plants were inserted in slits of a sponge disk. The disk was cut to fit a 180 ml plastic cup, covered with aluminum foil, and containing 130 ml of modified Hoagland's solution (Appendix A). Three mM nitrogen was used in the form of $Ca(NO_3)_2$ and KNO_3. In the experiment where nitrogen level was a variable, the plants were transplanted into solutions containing either 3 or 9 mM nitrogen.

The plants were grown under controlled conditions at 15 C day (16 hr) with 25,000 lumens/m^2 of light and 10 C night (8 hr). After 4 days, the plants were selected for uniformity, treated with simazine, and placed back in the growth chamber with 25,000 lumens/m^2 at 20 C day (16 hr) and 15 C night (8 hr). In the first barley experiment the plants

did not receive the temperature pre-treatment. Solutions were prepared by diluting a 2X Hoagland's solution with either water (control) or aliquots from concentrated simazine solutions prepared by serial dilution of a 2 X 10^{-5} M stock solution.

Harvesting and protein determinations. Harvesting consisted of separating the roots from the shoots and determining fresh weights. The shoots were cut into 5 mm sections and a 750 mg sample was used to determine water-soluble protein. The remainder was dried for dry weight and total protein determinations.

Water-soluble proteins were extracted by grinding the 750 mg sample in 5 ml of 0.I M potassium phosphate buffer (pH 6.8) in a pre-chilled mortar. The homogenate was centrifuged at 25,000 X g for 30 min. The pellet was washed with 2 ml of buffer and the supernatant solutions combined. Proteins were precipitated by adding 0.75 ml of 50% trichloroacetic acid (TCA) to 3 ml of supernatant solution. This suspension was heated at 80 C for 15 min and centrifuged at 5,000 X g for 30 min. The pellet was washed with 5% TCA and 95% ethanol and air dried. The protein was digested in 3 ml of concentrated H_2SO_4 by heating. After cooling, 4 ml of water was added and the protein content measured by an automatic micro-Kjeldahl method (18). The bovine serum albumin (BSA) standard curve was linear between 2.5 and 25.0 mg protein.

A 500 mg sample of leaves was dried at 43 C for 24 hr, weighed, and ground in a Wiley mill to pass through a 40 mesh screen. Total nitrogen was determined with a 40 mg sample by the automatic micro-Kjeldahl procedure, and protein estimated by multiplying the total nitrogen by 6.25.

Binding to plant extracts. Several triazine herbicides have been shown to react with the sulfhydryl moiety of glutathione and cysteine (36,70). Sulfhydryl groups are critical in many enzymatic reactions as well as being essential for protein structure. The following studies were conducted to investigate the interaction between proteins and atrazine.

For in vivo studies, 10 day old oat plants were treated with 100 ml of nutrient solution containing either 1 X 10^{-7} or 1 X 10^{-8} M ^{14}C-atrazine (ring-labeled, 6.1 μC/mg). After 4 days, the shoots were harvested and the water-soluble proteins extracted. Five 3 ml aliquots of the supernatant solution were placed in dialysis tubing (1 cm diameter) supported in test tubes containing 3 ml 0.1 M potassium phosphate buffer (pH 6.8). The test tubes were shaken for 24 hr in a cold room (4 C). Binding was determined by adding 1 ml of solution, from the inside and outside of the dialysis bag, to 15 ml of scintillation solution (4 g BBOT, 1000 ml toluene, and 400 ml Triton X-100). The counts were corrected for quenching by using a curve prepared by adding aliquots of supernatant solution, extracted from non-radioactive plants, to a standard amount of radioactivity.

The proteins in the original supernatant solution were precipitated, centrifuged, and washed 4 times with 5% TCA. The pellet was dissolved in a minimum amount of 0.1 M potassium phosphate buffer (pH 6.8). Aliquots of the solution were radioassayed and corrected for quenching.

In vitro binding studies were also conducted with proteins from oat roots and shoots. Oat plants were grown for 3 weeks in vermiculite. After 1 week, 100 ml of 50% Hoagland's solution was added every 2 days. The plants were divided into roots and shoots and the soluble proteins extracted. The proteins in the supernatant solution were precipitated with 100% saturated $(NH_4)_2SO_4$. The suspension was centrifuged at 5,000 X g for 30 min and the pellet dissolved in buffer. Excess salts were removed by dialyzing the solution for 48 hr against four changes of buffer. Protein concentrations were determined by the method of Lowry *et al.* (38).

Four 3 ml aliquots, from shoot extracts, containing either 3.9 or 7.8 mg protein/ml buffer were placed in dialysis bags. The proteins were dialyzed against 3 ml of buffer containing 1×10^{-6}, 1×10^{-7}, or 5×10^{-8} M ^{14}C-atrazine, and the amount of bound ^{14}C-atrazine was determined.

Binding was also studied by using the gel filtration technique described by Cuatrecasas *et al.* (12). The gel was prepared by suspending 20 g of Sephadex (G-25) in 500 ml of boiling distilled water. The gel was maintained at 90 C for 1 hr and after cooling, washed three times with distilled

water. The gel was cooled to 4 C prior to packing the column (1.5 X 30 cm). The column was equilibrated for 30 hr with 500 ml of 0.05 M potassium phosphate buffer (pH 7.0) containing 1 X 10^{-7} M ^{3}H-atrazine (ethyl-labeled, 140 μC/mg). Head pressure was maintained with a Mariotte flask. A 0.5 ml aliquot containing 3.2 mg of root or shoot protein/ml of buffer, obtained as in the previous experiments, was added to the column and eluted with the ^{3}H-atrazine-buffer solution. The flow rate was adjusted to 0.8 ml/hr and fractions were collected hourly for 36 hr. Protein peaks were identified by determining the absorbance at 280 mμ with a spectrophotometer. Fifty microliters of the eluant was used to determine radioactivity.

Studies with Lemna gibba L. Research with oats and rye was plagued by unaccountable variation. Although there was a tendency for simazine to increase the protein content, this small increase (10-20%) was usually masked by variations in plant growth. To reduce the experimental error Lemna gibba was tried as the test species. Rapid growth of uniform colonal material is possible, and the large numbers of plants taken in each sample tends to decrease the biological variation in an experiment (33,34).

Lemna gibba were grown aseptically in 125 ml flasks with 50 ml of medium (Appendix B) containing various concentrations of simazine. Simazine concentrations were made by adding aliquots from solutions prepared by serial dilutions of a

2 X 10^{-5} M stock. The initial experiment was conducted with and without 1% sucrose in the medium. Later experiments were conducted in the absence of sucrose.

Two plants, with four fronds each, were added to each flask under a sterile hood. The plants were grown with a 16 hr day (23 C) of 12,600 lumens/m^2 of light and 8 hr night (20 C).

The initial experiment was terminated after 10 days. The second experiment was harvested after 5, 10, and 15 days. In the third test, the plants were grown for 12 days and the medium was changed on the sixth day.

The plants were harvested by decanting the medium, rinsing the plants with distilled water, and placing them in aluminum weighing cups in a drying oven (43 C) for 24 hr. After drying, the plants were weighed, total nitrogen determined, and protein estimated by multiplying the total nitrogen by 6.25.

Barley growth studies. Simazine has been shown to increase the protein content of cereal crops in the laboratory and field (53,57). Experiments with oats as the test plant showed that small increases in protein content could be obtained, but these increases were not sufficient for short-term physiological studies. Rye seedlings proved impractical because they lodged under the conditions used in these studies.

In the first experiment, the barley plants were transplanted directly into 130 ml of Hoagland's solution (3 mM nitrate nitrogen) with various concentrations of simazine. The nutrient solutions were changed every 3 days and after 10 days the plants were harvested. Fresh weight, dry weight, soluble protein, and total protein were determined.

In the second experiment the effect of nitrogen level and temperature on the response of barley to simazine was studied. The plants were transplanted into solutions containing 3 and 9 mM nitrate nitrogen and placed in growth chambers with a 15 C day (25,000 lumens/m^2) and 10 C night. After pre-treatment, four replications of plants treated with 3 and 9 mM nitrogen were harvested. This served as a zero time. The remaining plants were grown at two temperature regimes; one at 25 C day (16 hr), 20 C night, and the other at 20 C day (16 hr) and 15 C night (8 hr). The light intensity was 25,000 lumens/m^2.

The plants were harvested after 5 and 10 days, as previously described, and soluble carbohydrates were determined on aliquots from the supernatant after precipitation of the water-soluble proteins. Soluble carbohydrates were determined as described by Hodge and Hofreiter (29).

The effect of simazine on water-soluble proteins in short term experiments was studied by harvesting plants 3 and 5 days after treatment. Total free amino acids were extracted by boiling 500 mg of fresh shoots in 5 ml of 70% ethanol.

The extraction was repeated three times and the supernatant solutions combined, evaporated to dryness, and redissolved in 10% isopropanol. The free amino acid content was determined by the ninhydrin colorometric method of Rosen (58), and expressed as μg of amino nitrogen per plant.

Effect of simazine on respiration. The influence of simazine on respiration was studied using both etiolated and non-etiolated seedlings. Barley seeds were surface sterilized by soaking them in 1% sodium hypochlorite for 15 min. Twenty seeds were planted in 10 g of sterile vermiculite in a 500 ml flask. The vermiculite was saturated with 50 ml of water (control), 1×10^{-8}, 1×10^{-7}, and 1×10^{-5} M simazine. A vial containing 4 ml of 2.0 N NaOH, CO_2 free, was hung from a rubber stopper having an inlet and outlet tube. The inlet tube was connected to an inner tube inflated with compressed air. The flasks were equilibrated and the outlet tube clamped shut. The CO_2 given off by the seeds was trapped in the NaOH with the inner tube maintaining a constant pressure. The amount of CO_2 respired was measured by titrating the NaOH with 0.11 N HCl to a pH of 7.0. A flask containing only vermiculite was used as a blank. The experiment was conducted for 15 days in a dark growth chamber at 25 C.

Barley plants were harvested after the pre-treatment and dry weight, soluble protein and soluble carbohydrate content were determined. The remaining plants were treated with 0 and 1×10^{-8} simazine and during the dark period of the third day

of treatment the plants were placed in sealed 2 liter jars. Ambient air, bubbled through water, was forced into the jars and exited through outlet tubes. The flow rate passing through each jar was calibrated precisely between the range of 1.0-1.4 cc/min. The system was equilibrated over night and a 5 cc sample was taken from the outlet tube of each sample. A 1 cc aliquot of the sample was analyzed for CO_2 with a gas-chromatography procedure (8).

After samples for CO_2 analysis were taken, the plants were freeze-dried, weighed, and 100 mg leaves ground in 5 ml of 0.1 M potassium phosphate buffer (pH 6.8). The soluble carbohydrate content was determined by taking an aliquot from the supernatant solution after the proteins were precipitated for analysis.

Effect of simazine on photosynthesis. Photosynthesis was determined by measuring $^{14}CO_2$ fixation. Barley plants were treated for 1.5 and 3 days with 0, 1×10^{-7}, and 1×10^{-8} M simazine. At the end of the treatment period, the plants were placed in an air-tight, clear, plastic box (45 X 45 X 90 cm) covered with black plastic. The $^{14}CO_2$ source was evolved from $Ba^{14}CO_3$ (1 mC/39.5 mg) by reacting 1 mg $Ba^{14}CO_3$ with 4 ml of 50% lactic acid in a 125 ml flask fitted with two tubes. The $^{14}CO_2$ was forced into the chamber by running one tube into the chamber and forcing the $^{14}CO_2$ out with the other tube. The black plastic was removed from the box to allow $^{14}CO_2$ fixation for 30 min. During the fixation period the air was

kept turbulent by placing a small fan in the chamber. Fixation was conducted under fluorescent light in addition to natural sunlight providing a total of 36,000 lumens/m^2. At the end of the fixation period, the plants were freeze-dried, weighed, and ground through a 40 mesh screen. A 50 mg sample was combusted and the evolved $^{14}CO_2$ trapped in 10 ml of ethanol-ethanolamine (2:1 v/v). A 1 ml aliquot was radioassayed and corrected for quenching.

Influence of nitrogen form on simazine uptake. Barley plants were grown in Hoagland's solution containing 5 X 10^{-8} M ^{14}C-simazine (ring-labeled, 4.9 μC/mg), 0.1 mM potassium phosphate buffer (pH 6.5), and 3 mM nitrate or ammonium nitrogen. Ammonium was added as $(NH_4)_2SO_4$ and nitrate nitrogen as $Ca(NO_3)_2$ and KNO_3. The pH of both solutions was adjusted to 6.5 with KOH. The plants were harvested and the pH of the nutrient solution determined after 1, 2 and 3 days. The roots were rinsed, separated and dried. After drying, the shoots and roots were ground to pass through a 40 mesh screen and 50 mg combusted. The evolved $^{14}CO_2$ was radioassayed as previously described.

Effect of simazine on ^{14}C-leucine incorporation and nitrate uptake. ^{14}C-Leucine incorporation, protein content and nitrate uptake were determined after 0, 12, 24, and 48 hr of treatment with 0 and 1 X 10^{-8} M simazine. ^{14}C-Leucine incorporation was measured by placing 10-6 mm barley leaf

segments in a 25 flask which contained 3 ml of solution composed of 0.15 μC ^{14}C-leucine (uniformly labeled, 2.35 mC/mg), 0.01 M potassium phosphate buffer (pH 6.0), and 60 μg chloramphenicol. Chloramphenicol at this concentration did not effect ^{14}C-leucine incorporation. The flasks were placed in a water bath shaker maintained at 25 C. After 4 hr, the solution was decanted and the segments rinsed three times with water. The segments were freeze-dried, weighed and the soluble proteins extracted. The proteins were precipitated with 10% TCA (final concentration), collected on a Millipore filter (0.45 μ), and washed with 5% TCA and 95% ethanol. Filters were air dried and broken into small sections in a liquid scintillation vial. The filters were dispersed in 15 ml of scintillation fluor, previously described, and allowed to set for 2 days in the liquid scintillation spectrometer. The samples were radioassayed and corrected for quenching.

Nitrate uptake was measured by determining the loss of nitrate from the nutrient solution. The nutrient solution was brought back to its original volume with distilled water, stirred, and an aliquot taken for nitrate analysis by the method of Lowe and Hamilton (37).

The effect of simazine on ^{14}C-leucine incorporation was further studied by adding simazine to the mixture containing ^{14}C-leucine. Ten-day old barley leaves were cut into 6 mm section. Twenty segments were floated on the 3 ml mixture, previously described, containing 0, 1 X 10^{-7}, and 1 X 10^{-5} M

simazine. Simazine stocks were prepared in ethanol and 30 µl of stock was added to the mixture to give the desired concentration. The control also contained 30 µl of ethanol. The amount of ^{14}C-leucine incorporated into protein was determined after 2, 4, 8, and 12 hr. An aliquot of the initial filtrate was counted to see if the increase in incorporation was due to an increase in ^{14}C-leucine uptake. This experiment was repeated with the additional treatment of 1 X 10^{-5} M hydroxysimazine. The experiment was terminated after 8 and 12 hr of incubation. To check for bacteria growth an aliquot of the medium was added to potato starch agar plates.

Experimental design and statistical analysis. A randomized complete block with at least four replications was employed in all of the simple and factorial experiments. The data were subjected to analyses of variance and whenever a significant F value was obtained for main effects or interactions, the means were compared to Tukey's ω procedure.

A split plot statistical design was used for the experiment with nitrogen levels and temperatures. The first split was for temperature, the second for harvest time, and the third for nitrogen level.

Radioactive simazine and atrazine were provided by Geigy Chemical Corporation, Ardsley, New York. The chemicals were checked for purity by thin-layer chromatography and were at least 98% parent compounds. ^{14}C-Leucine (98% purity) was purchased from Amersham/Searle Corp., Arlington Heights, Illinois. All other chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Binding to plant extracts. Equilibrium dialysis between the water-soluble fraction of ^{14}C-atrazine treated oat plants and buffer indicated that small amounts of atrazine may be bound to macromolecules (Table 1). The binding ratio (^{14}C-inside/^{14}C-outside) did not increase when the ligand concentration was decreased. This denoted that there was either a limited number of available binding sites or the small differences were due to experimental error.

There was no significant amount of radioactivity associated with the protein after repeated washings with 5% TCA. This demonstrated that ring-labeled ^{14}C-atrazine was not incorporated into proteins as has been reported for chain-labeled ^{14}C-atrazine (63).

In vitro studies with proteins extracted from oat plants indicated that there was no atrazine-protein interaction (Table 2). The binding ratio was approximately 1.0 regardless of protein or atrazine concentration. Experiments with gel filtration and ^{3}H-atrazine confirmed the results from the dialysis experiments (Figure 1). Also, proteins from oat roots did not bind to atrazine (Figure 2).

Studies with Lemna gibba L. Simazine did not consistently increase the growth and/or protein content of oat and rye

Table 1. In vivo binding of ^{14}C-atrazine to the soluble fractions of oat shoots.[a]

Atrazine concn	^{14}C-atrazine inside	^{14}C-atrazine outside	Ratio
(M)	(dpm/ml)		
5×10^{-8}	156	134	1.16
1×10^{-7}	247	210	1.17

[a]F value for difference between treatments not significant.

Table 2. Dialysis equilibrium ratios between ^{14}C-atrazine and protein extracted from oat shoots.[a]

Protein	Atrazine concn	^{14}C-atrazine inside	^{14}C-atrazine outside	Ratio
(mg/ml)	(M)	(dpm/ml)		
	5×10^{-8}	71	74	.96
3.9	1×10^{-7}	149	138	1.08
	1×10^{-6}	1391	1391	1.02
	5×10^{-8}	66	72	.92
7.8	1×10^{-7}	156	142	1.10
	1×10^{-6}	1405	1429	.98

[a]F value for difference between treatment not significant.

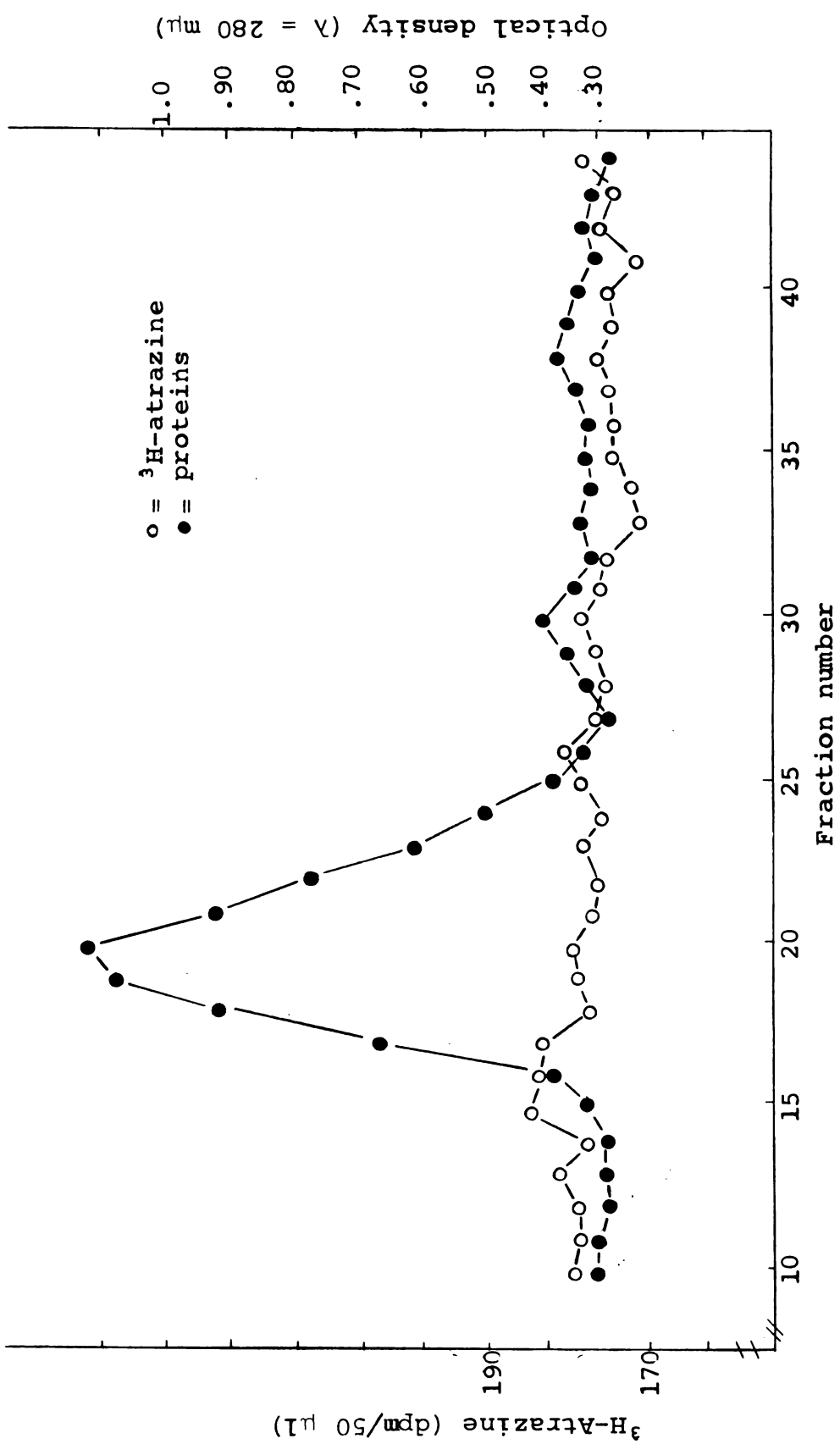

Figure 1. Eluation pattern of protein from oat shoots and ^{3}H-atrazine. A 0.5 ml sample containing 1.6 mg of protein was added to a 1.5 X 30 cm Sephadex (G-25) column saturated with ^{3}H-atrazine, the protein was eluated with 1 X 10^{-7} M ^{3}H-atrazine at a flow rate of 0.8 ml/hr for 36 hr.

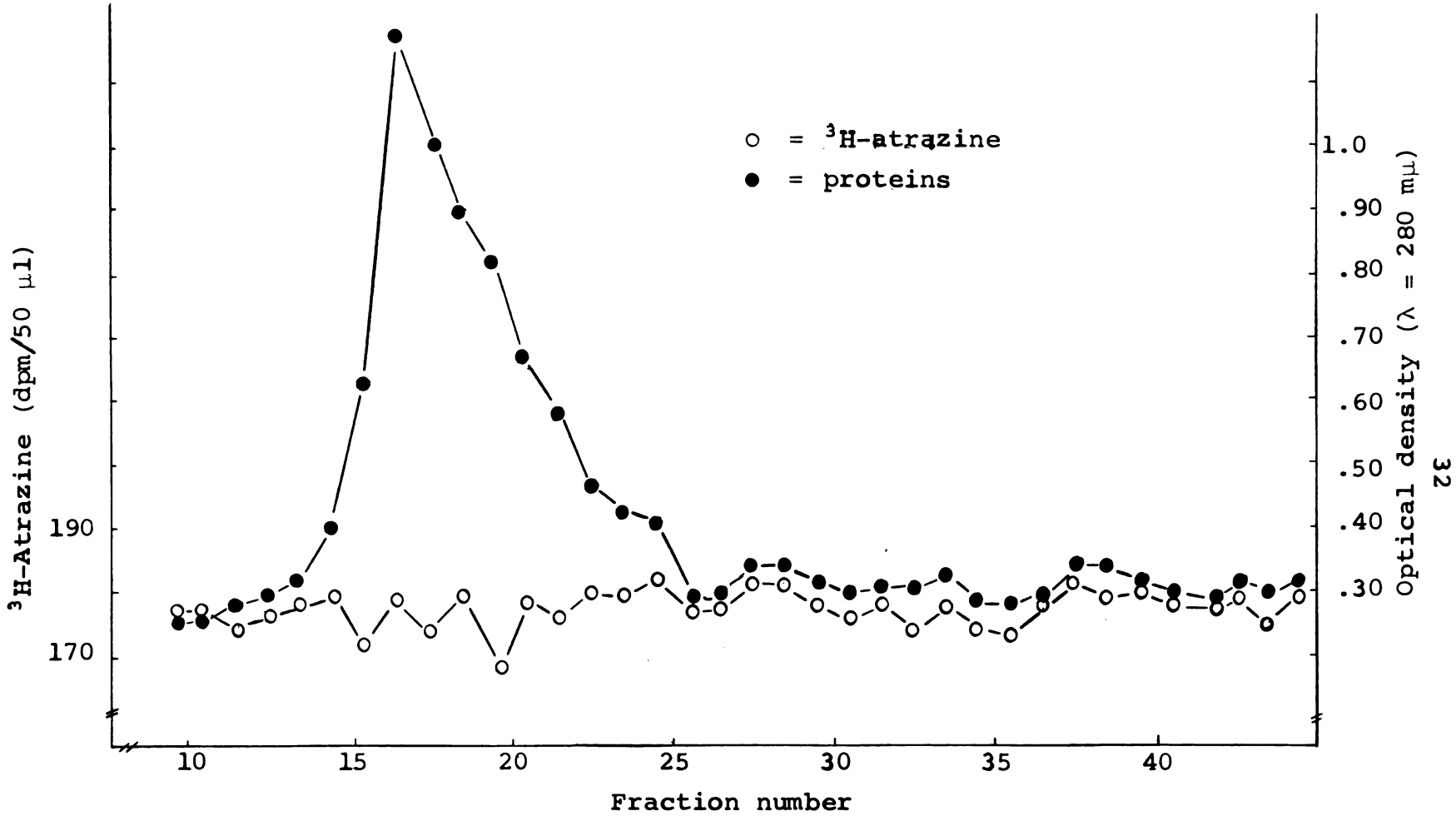

Figure 2. Eluation pattern of protein from oat roots and ^{3}H-atrazine. A 0.5 ml sample containing 1.6 mg of protein was added to a 1.5 X 30 cm Sephadex (G-25) column saturated with ^{3}H-atrazine. The protein was eluated with 1 X 10^{-7} M ^{3}H-atrazine at a flow rate of 0.8 ml/hr for 36 hr.

plants grown either in nutrient cultures or vermiculite. Plants treated with simazine tended to contain more protein; however, these results were inconclusive due to variation in plant growth. In order to minimize variation, studies using Lemna gibba as the test plant were initiated.

The first experiment was conducted with and without sucrose in the growing medium. After 10 days, 1 X 10^{-8} M simazine increased the protein content 15% in plants grown on the sucrose deficient media (Table 3). There was no stimulatory response to simazine in the presence of sucrose.

The second experiment was conducted in the absence of sucrose. Neither growth nor protein content were increased by simazine after 5, 10, and 15 days of treatment (Table 4). This experiment was repeated and no favorable response to simazine was observed (Table 5). Further studies were conducted with varying light intensities, nutrient levels, and temperature regimes. Since simazine did not consistently alter plant growth at any environmental or nutriental condition, experiments with Lemna gibba were terminated.

Growth studies with barley. Barley plants were grown in nutrient cultures and preliminary experiments indicated a favorable response to simazine. In the first experiment, the plants were transplanted directly into nutrient solutions (3 mM nitrogen) containing various concentrations of simazine. There was a 37% increase in the water extractable protein content when treated for 10 days with 5 X 10^{-8} M simazine (Table 6). The total protein content of simazine treated

Table 3. Effect of simazine on growth and total protein content of Lemna gibba L. grown in nutrient solutions with and without sucrose for 10 days.[a]

Simazine concn	Dry wt		Protein	
	- sucrose	+ sucrose	- sucrose	+ sucrose
(M)	(mg/culture)		(mg/culture)	
0	20.9 b	41.1 a	7.6 b	13.7 a
5×10^{-9}	23.7 c	40.2 a	8.5 bc	14.1 a
1×10^{-8}	22.0 bc	39.6 a	8.8 c	13.5 a
5×10^{-8}	21.6 b	41.8 a	7.8 b	14.2 a
1×10^{-7}	21.0 b	39.8 a	7.6 b	13.1 a
5×10^{-7}	13.5 a	21.0 b	4.9 a	7.2 b

[a]Means followed by unlike letters are significantly different at the 5% level.

Table 4. Effect of simazine on growth and protein content of Lemna gibba L. grown for 5, 10, and 15 days on a sucrose deficient medium.[a]

Simazine concn	Dry wt			Total protein		
	Days after treatment			Days after treatment		
	5	10	15	5	10	15
(M)	(mg/culture)			(mg/culture)		
0	10.7 a	18.6 a	34.2 a	3.9 a	7.2 a	14.2 a
1×10^{-10}	10.7 a	18.4 a	35.6 a	3.9 a	7.8 a	14.6 a
1×10^{-9}	10.3 a	18.9 a	34.7 a	3.6 a	7.9 a	14.4 a
1×10^{-8}	10.4 a	21.1 a	33.2 a	3.7 a	7.5 a	14.8 a
1×10^{-7}	9.4 a	20.6 a	35.1 a	3.6 a	7.0 a	14.9 a
1×10^{-6}	2.3 b	2.5 b	2.7 b	1.1 b	1.3 b	1.2 b

[a]Means followed by unlike letters are significantly different at the 5% level.

Table 5. Effect of decreasing concentrations of simazine on Lemna gibba grown for 12 days in a sucrose deficient medium.[a]

Simazine concn	Day wt	Protein
(M)	(mg/culture)	(mg/culture)
0	27.6	9.7
1×10^{-10}	27.8	10.2
5×10^{-10}	27.5	10.3
1×10^{-9}	26.7	10.4
5×10^{-9}	28.8	10.8
1×10^{-8}	26.1	9.7
1×10^{-7}	26.0	9.5

[a]F value for difference between treatments not significant.

Table 6. Growth and protein content of barley shoots grown for 10 days in nutrient solution containing various concentrations of simazine.[a]

Simazine concn	Fresh wt	Dry wt	Water extractable protein	Total protein
(M)	(mg/plant)	(mg/plant)	(mg/plant)	(mg/plant)
0	1006 b	178 b	9.2 b	32.6 a
1×10^{-9}	1094 b	184 b	9.4 b	31.7 a
5×10^{-9}	994 b	165 b	8.9 b	33.8 a
1×10^{-8}	1263 d	204 c	11.2 bc	34.1 a
5×10^{-8}	1192 cd	169 b	12.6 c	35.2 a
1×10^{-7}	1131 c	173 b	11.1 bc	32.7 a
5×10^{-7}	721 a	101 a	5.1 a	16.7 b

[a]Means followed by unlike letters are significantly different at the 5% level.

plants tended to be higher; however, there was no statistical difference between treatments. Previous research has shown that the water soluble protein fraction is a better indicator of the simazine response (57). In future experiments, only the differences in water extractable proteins will be discussed.

Consistent increases in protein were not obtained in later experiments when the plants were treated immediately after transplanting. More uniform plants were obtained if they were placed at a low temperature (15 C day, 10 C night) for 4 days after transplanting. All future barley experiments were subjected to this pre-treatment and selected for uniformity before treatment.

Nutritional and environmental parameters have been shown to influence the response of corn to simazine (54,76). Corn readily metabolizes simazine and the parameters necessary for simazine to increase protein content are conditions that decrease metabolism. The effects of temperature and nitrogen nutrition were studied using barley, a susceptible plant.

Simazine, 1 X 10^{-8} M, increased the water-soluble protein content only when the plants were grown at 3 mM nitrogen with a low temperature (Figure 3). Furthermore, simazine reduced the soluble carbohydrate content under these conditions. There was no effect from simazine when the plants received 9 mM nitrogen at the low temperature. Similarly, simazine did not alter the protein or carbohydrate content at the high temperature regardless of nitrogen level.

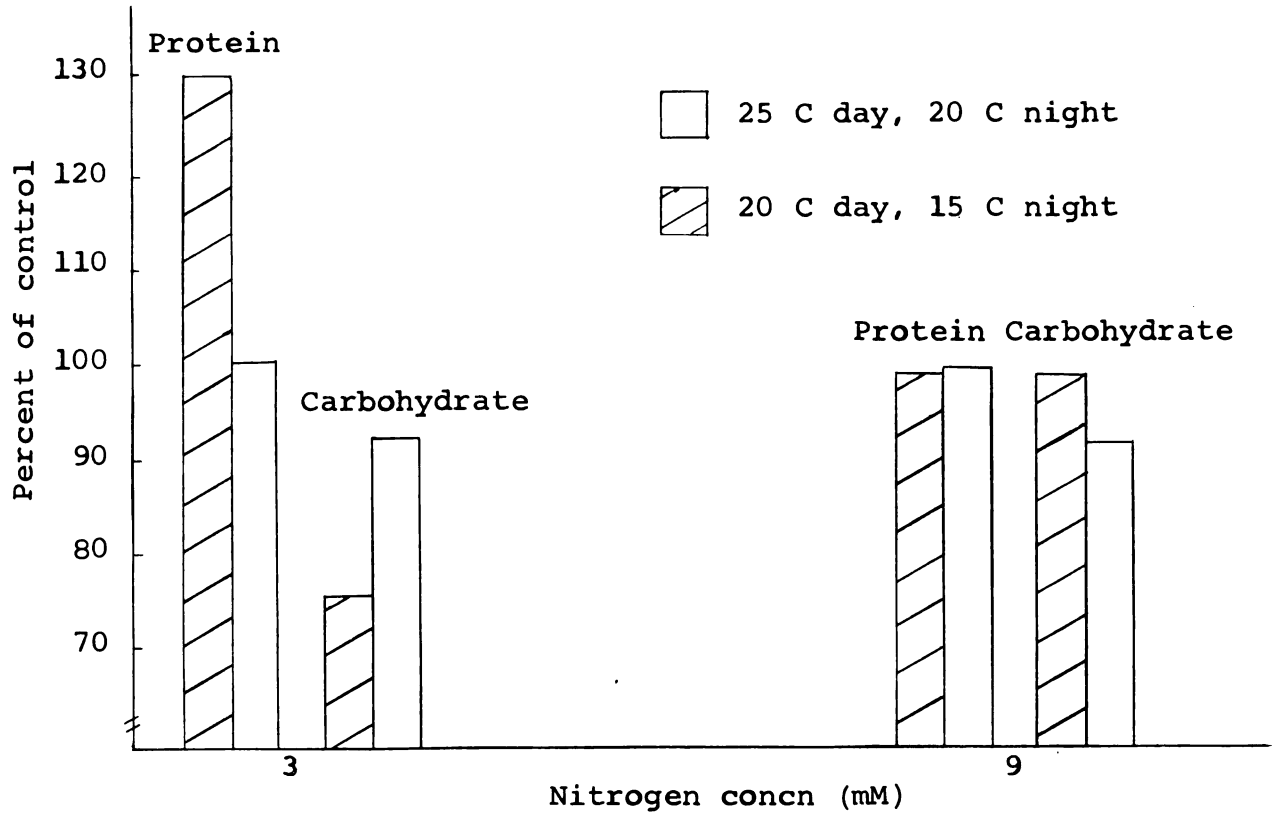

Figure 3. Influence of temperature and nitrogen level on the response of barley plants to 1 X 10^{-8} M simazine. Means of 5 and 10 day harvested were combined. F value for interaction of simazine x temperature x nitrogen level was significant at the 1% level for both protein and carbohydrate.

The lack of response to simazine at the high nitrogen level may be due to the carbohydrate status of the plants before treatment. The plants receiving 3 mM nitrogen during the pre-treatment contained 4.5 mg soluble carbohydrates per plant as compared to 2.4 mg/plant for the plants grown with 9 mM nitrogen (Table 7). The soluble carbohydrate content of the control was reduced to 2.3 mg/plant when grown at 20 C day and 15 C night with 3 mM nitrogen. Whereas, 1 X 10^{-8} M simazine reduced the carbohydrate content to 1.7 mg/plant. If the plants were grown at this temperature under 9 mM nitrogen, the carbohydrate content was reduced to 1.6 mg/plant at both 0 and 1 X 10^{-8} M simazine.

The carbohydrate content of the plants grown under 3 nM nitrogen at the high temperature (25 C day, 20 C night) was reduced from 4.5 to 1.4 mg/plant regardless of chemical treatment. This decrease is probably due to an increase in respiration. The failure of carbohydrates to accumulate at the 9 mM nitrogen level may be due to nitrate-stimulated glycolysis (9). The reduction of carbohydrates by simazine at the low temperature and low nitrogen level may be due to either simazine-stimulated respiration or an increase in nitrate uptake.

The previous experiment indicated that increases in protein content could be observed in short-term experiments. The next experiment was harvested after 3 and 5 days of treatment and included rates above and below 1 X 10^{-8} M.

Table 7. The effect of simazine on the growth, protein content, and water-soluble carbohydrate content in barley plants grown at two nitrogen levels, and two temperatures.[b]

Treatment			Dry wt		Total protein		Water-soluble proteins[a] (leaves)	Water-soluble carbohydrates[a] (leaves)
N level	Temp	Simazine	shoot	root				
(mM)		(M)	(mg/plant)		(mg/g)	(mg/plant)	(mg/plant)	(mg/plant)
Zero time		-	34	17	171	5.8	2.8	4.5
3	20-15 C	0	101	26	156	15.8	7.8	2.3
		10^{-8}	105	26	166	17.4	10.1	1.7
3	25-20 C	0	114	31	218	24.9	12.3	1.4
		10^{-8}	111	31	230	25.5	12.4	1.3
Zero time			40	20	193	7.7	3.9	2.4
9	20-15 C	0	118	31	220	26.0	12.8	1.5
		10^{-8}	118	32	221	26.1	12.5	1.6
9	25-20 C	0	125	38	258	32.2	13.1	1.1
		10^{-8}	121	39	264	31.9	12.9	1.0

[a]F value for interaction of simazine x nitrogen x temperature was significant at the 1% level for water-soluble protein and carbohydrates.

[b]Data are averages of 5 and 10 day harvest.

The water extractable protein content was increased by 33% by treating with 1 X 10^{-8} M simazine for 3 days (Figure 4). A small increase was observed at the 5 X 10^{-9} M rate, however, 1 X 10^{-7} M decreased the protein content after 5 days of treatment. The total free amino acid content was increased at both 1 X 10^{-7} and 1 X 10^{-8} M simazine (Figure 5). However, the increase due to 1 X 10^{-7} M appeared to be a herbicidal effect since there was a reduction in protein content at this rate. Amino acid accumulation is a frequent observation when photosynthesis is inhibited.[1]

Effect of simazine on respiration. The decrease in carbohydrate content could be explained if simazine directly influences respiration. Ries et al. (53) were able to obtain such an effect with rye plants that had been treated for 25 days. This data is often used to explain increases in activity of starch and sugar degrading enzymes (71,82). However, 1 X 10^{-5}, 1 X 10^{-7}, and 1 X 10^{-8} M simazine did not alter the respiration rate of barley plants grown in the dark for 15 days (Table 8). Tweedy (74) did not observe any effect of simazine on respiration in excised barley roots. These data demonstrate that simazine probably has no direct effect on respiration.

In the next experiment, respiration of whole plants was determined after being treated for 3 days. Simazine, 1 X 10^{-8} M, increased the protein and decreased the carbohydrate

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[1]S. K. Ries, unpublished data.

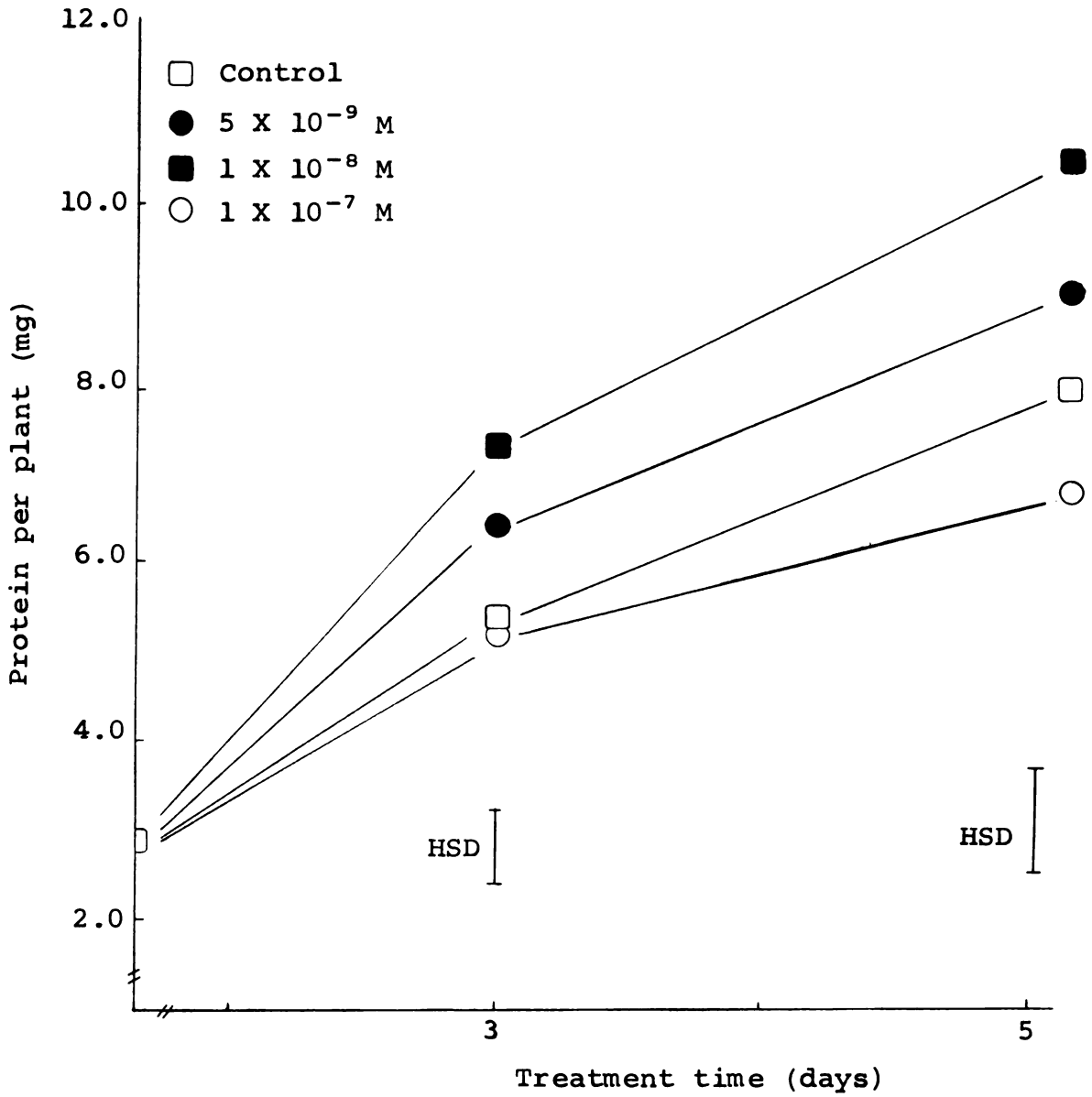

Figure 4. Water-soluble protein content of plants treated when 10 days old with various concentrations of simazine for 3 and 5 days.

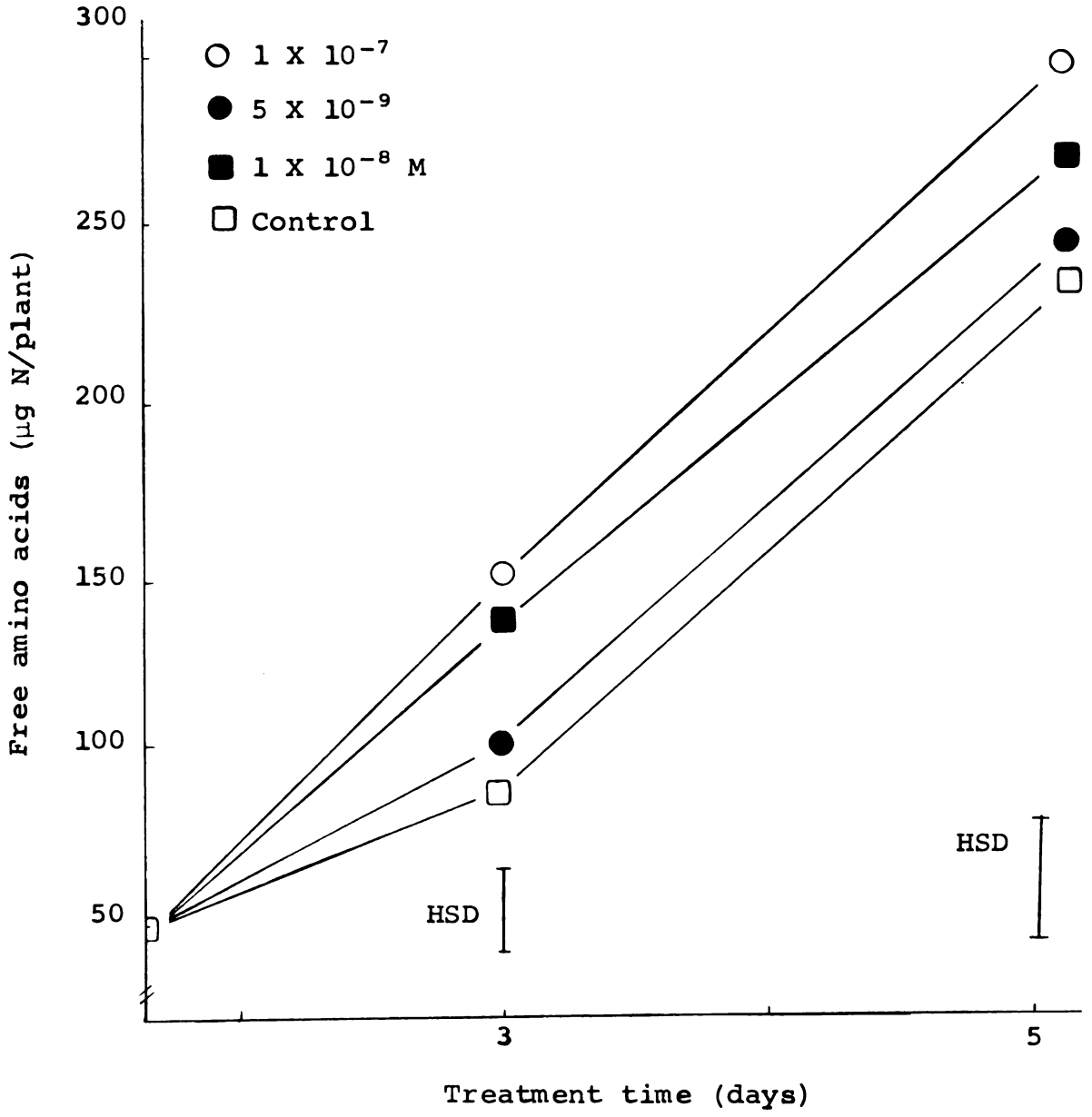

Figure 5. Free amino acid content of barley shoots from plants grown in nutrient cultures for 3 and 5 days containing various concentrations of simazine.

Table 8. Respiration of barley plants grown in the dark for 15 days and treated with various concentrations of simazine.[a]

Simazine concn	CO_2 evolution
(M)	(ml CO_2/20 seedlings)
0	90
1×10^{-8}	92
1×10^{-7}	87
1×10^{-5}	94

[a]F value for difference between treatments not significant.

Table 9. Effect of simazine on respiration, soluble carbohydrate, and water-soluble protein content of barley plants treated when 10 days old for 3 days.[a]

Simazine concn	Dry wt	Carbohydrate	Protein	CO_2 evolution
(M)		(mg/plant)		CO2/100 mg dry wt/hr
Zero time	35	5.5	3.0	-------
0	69 a	2.4 a	5.8 a	64.5 a
1×10^{-8}	72 a	1.4 b	7.0 b	66.3 a

[a]Means followed by unlike letters are significantly different at the 1% level.

content without influencing respiration (Table 9, on the preceding page). The inverse relationship between proteins and carbohydrates approximated a weight for weight effect. After the low temperature pre-treatment, the protein and carbohydrate content was 3.0 and 5.5 mg/plant, respectively. During the next 3 days, the control increased 2.8 mg protein/plant and decreased 3.1 mg carbohydrates/plant. Simazine treated plants increased 4.0 mg protein/plant and decreased 4.1 mg carbohydrates/plant. This data supports the hypothesis that simazine creates a metabolic condition in which carbohydrates are used for protein synthesis (71). Undoubtedly respiration would be stimulated in this conversion, however no differences were noticeable at the end of 3 days.

Effect of simazine on photosynthesis. The decrease in carbohydrate content of simazine treated plants is not due to inhibition of photosynthesis. There was no stimulatory or inhibitory effects of 1×10^{-8} M simazine on $^{14}CO_2$ fixation after 1.5 and 3 days of treatment (Table 10). However, 1×10^{-7} M decreased photosynthesis by 30% after 3 days of treatment.

Effect of simazine on ^{14}C-leucine incorporation. Earlier experiments demonstrated that within 3 days of treatment 1×10^{-8} M simazine increased the water extractable protein content at the expense of carbohydrates. Also,

Table 10. $^{14}CO_2$ fixation of barley plants grown for 1.5 and 3 days in nutrient solution containing various concentrations of simazine.

Simazine concn	$^{14}CO_2$ fixation[a] Days after treatment 1.5	3
(M)	dpm/100 mg dry wt/ 30 min x 10^{-3}	
0	246 a	337 a
1 X 10^{-8}	251 a	334 a
1 X 10^{-7}	241 a	236 b

[a]Means followed by unlike letters are significantly different at the 5% level.

Table 11. A comparison of the effect of simazine with that of hydroxy-simazine on ^{14}C-leucine incorporation into protein of barley leaf segments after 8 and 12 hours of incubation.

Treatment	^{14}C-Leucine incorporation Incubation time (hr) 8	12
	dpm/10 mg dry wt	
Control	13,787 a	18,751 a
1 X 10^{-7} M simazine	12,699 a	19,131 a
1 X 10^{-5} M simazine	16,363 b	24,684 b
1 X 10^{-5} M hydroxy-simazine	14,030 a	19,430 a

[a]Means followed by unlike letters are significantly different at the 5% level.

stimulations of nitrate uptake have been observed within 3 days (80). The following experiments were designed to investigate whether nitrate uptake is a primary effect or if the increase in nitrate uptake is in response to an increase in protein synthesis.

Simazine, 1 X 10^{-8} M, did not increase the protein content or nitrate uptake until 48 hr (Figures 6 and 7). However, a 25% increase in ^{14}C-leucine incorporation was observed after 24 hr (Figure 8). This experiment was repeated with ^{14}C-isoleucine and similar results were obtained. Protein synthesis was determined using only the primary leaf. Protein synthesis appeared to be decreasing after 24 hr. However, this may be due to the development of new leaves since it is known that protein synthesis in leaf tissue decreases with age (39).

The effect of simazine on ^{14}C-leucine incorporation was further investigated by using leaf segments from untreated plants. The results showed that by floating leaf segments on solutions containing 1 X 10^{-5} M simazine, a 33% increase in ^{14}C-leucine incorporation could be obtained within 12 hr (Figure 9). This experiment was repeated and 1 X 10^{-5} M hydroxy-simazine was added to the mixture. Hydroxy-simazine did not influence protein synthesis whereas simazine at the same concentration increased ^{14}C-leucine incorporation by 30% (Table 11). Simazine did not alter ^{14}C-leucine uptake and only approximately 20% of that taken up was incorporated

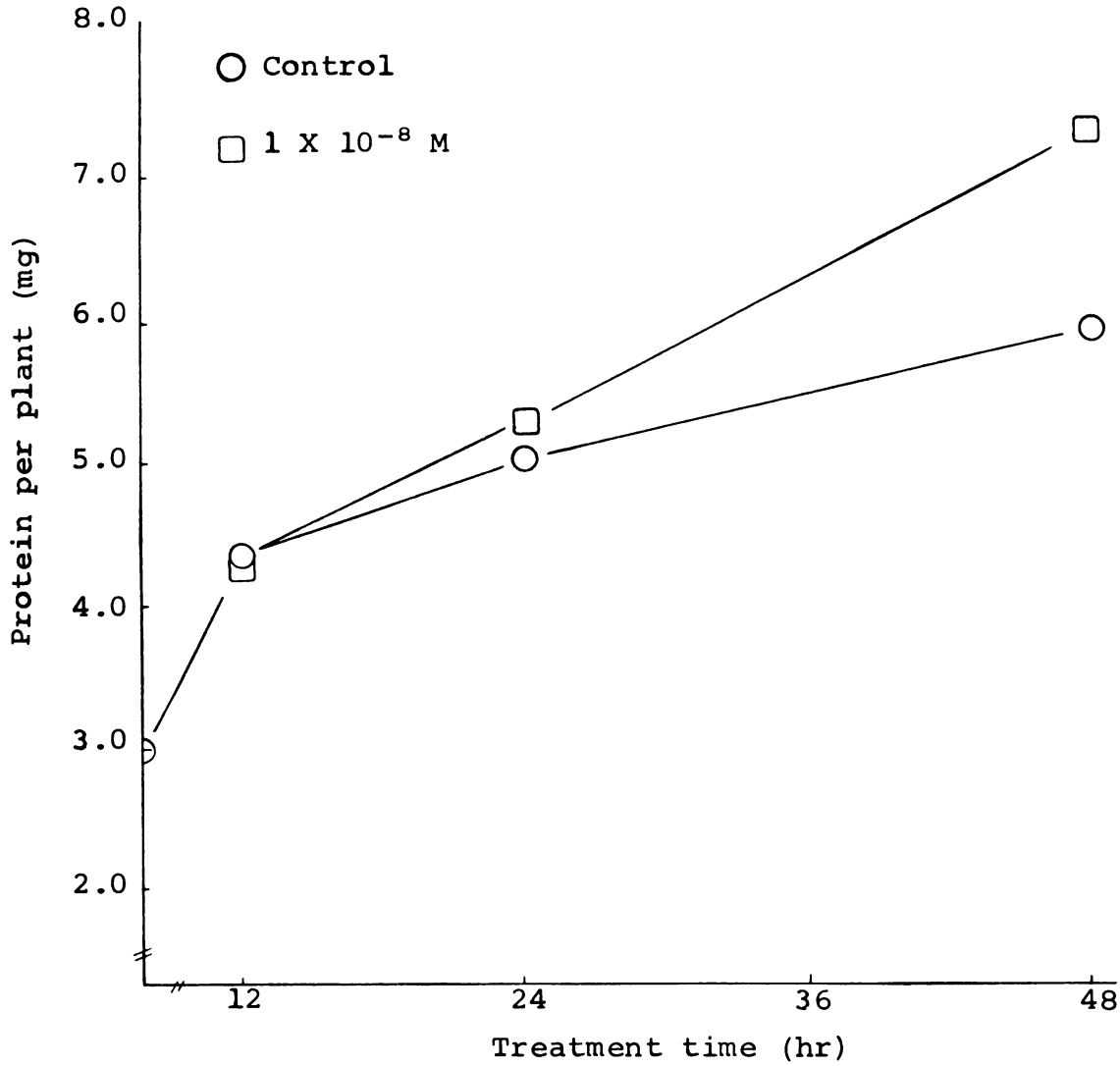

Figure 6. Effect of 0 and 1 X 10^{-8} M simazine on the water-soluble protein content of barley shoots from 10 day old barley plants grown for 12, 24, and 48 hr in nutrient cultures.

F value for treatments at the 48 hr harvest was significant at the 1% level.

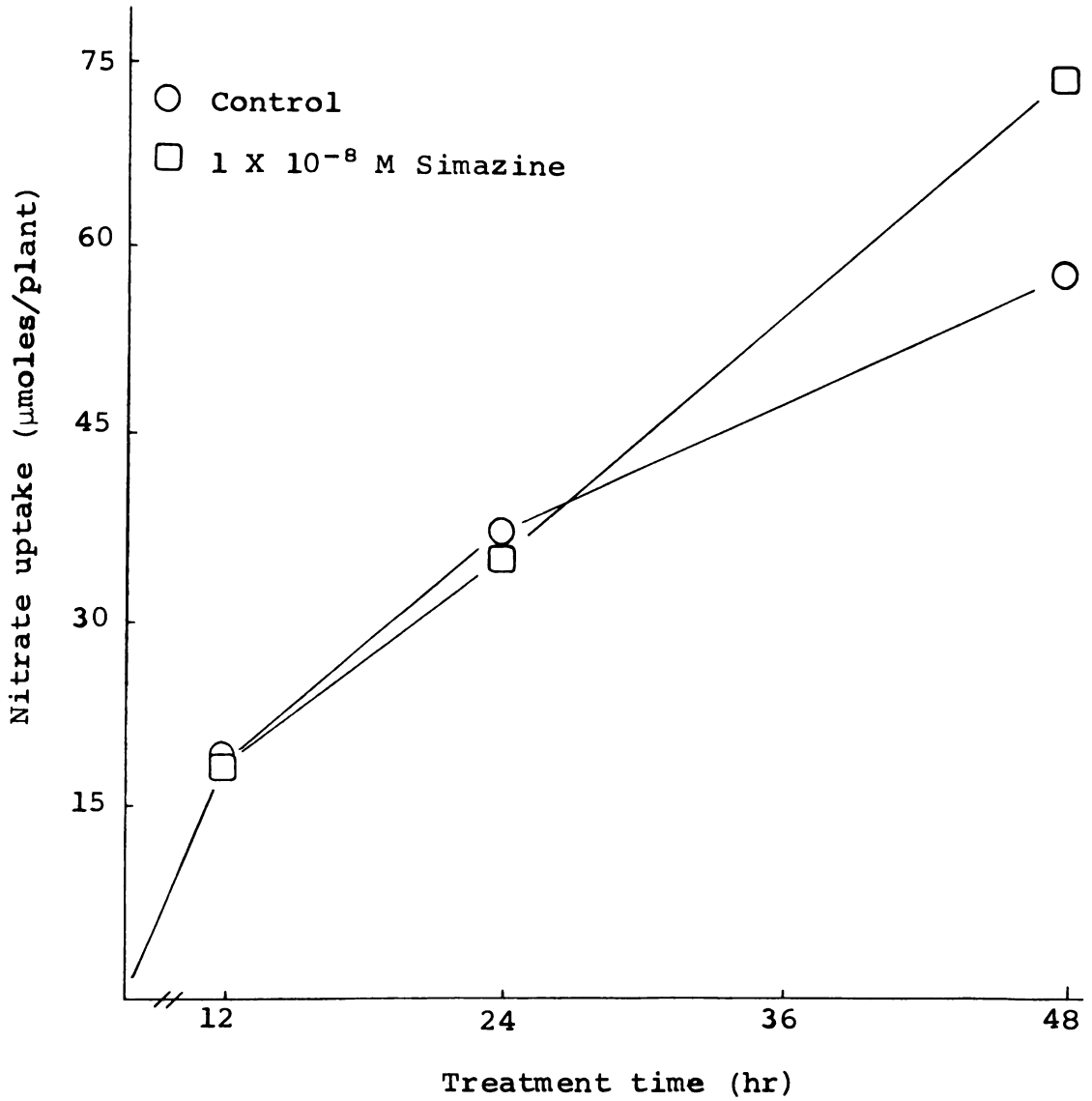

Figure 7. Influence of 0 and 1 X 10^{-8} M simazine on nitrate uptake of 10 day old barley plants grown in nutrient cultures containing 3 mM nitrate nitrogen for 12, 24, and 48 hr.

F value for treatments at the 48 hr harvest was significant at the 5% level.

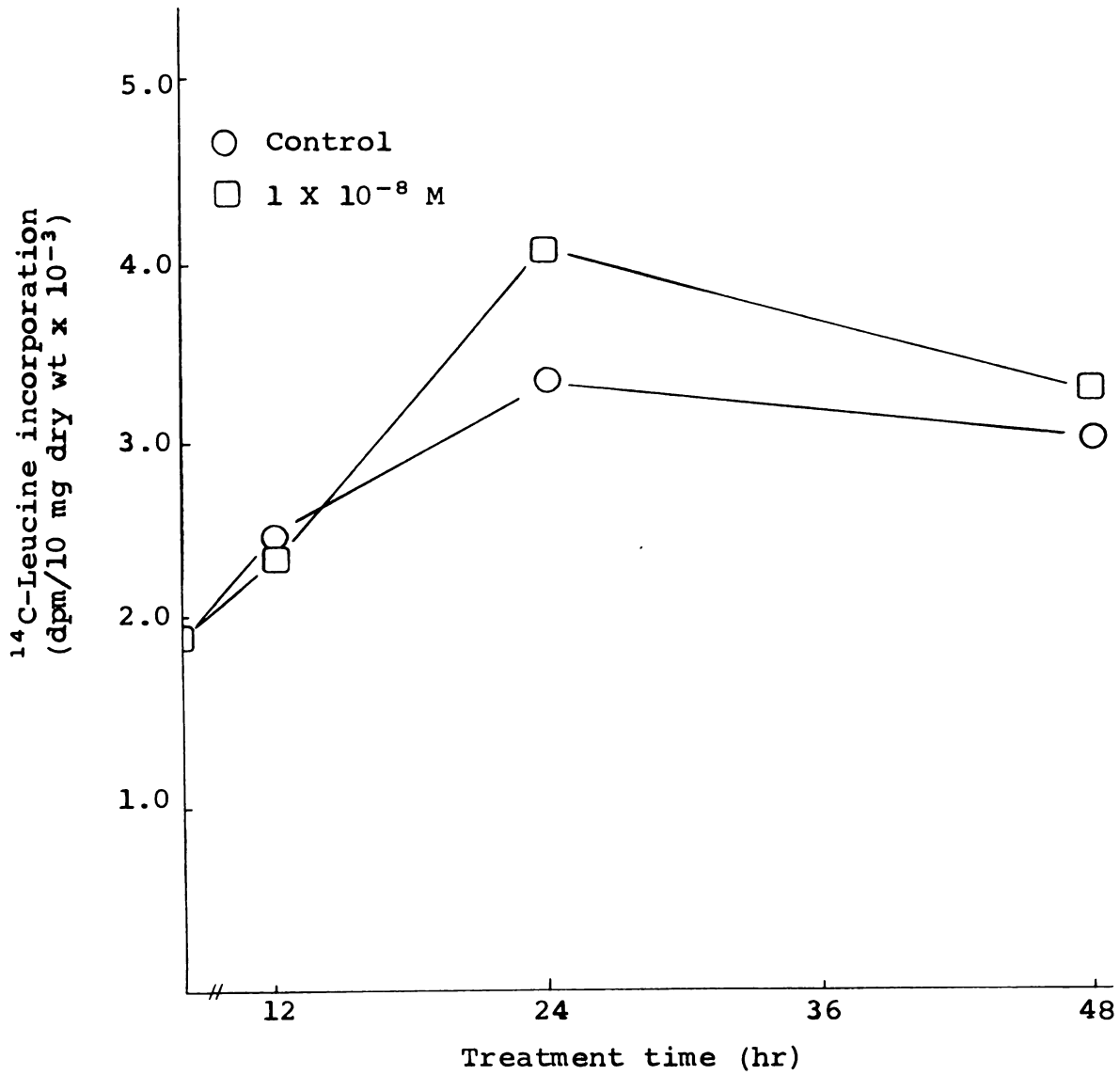

Figure 8. Effect of treating barley plants with 0 and 1 X 10^{-8} M simazine for 12, 24, and 48 hr on ^{14}C-leucine incorporation into protein.

F value for treatments at the 24 hr harvest was significant at the 5% level.

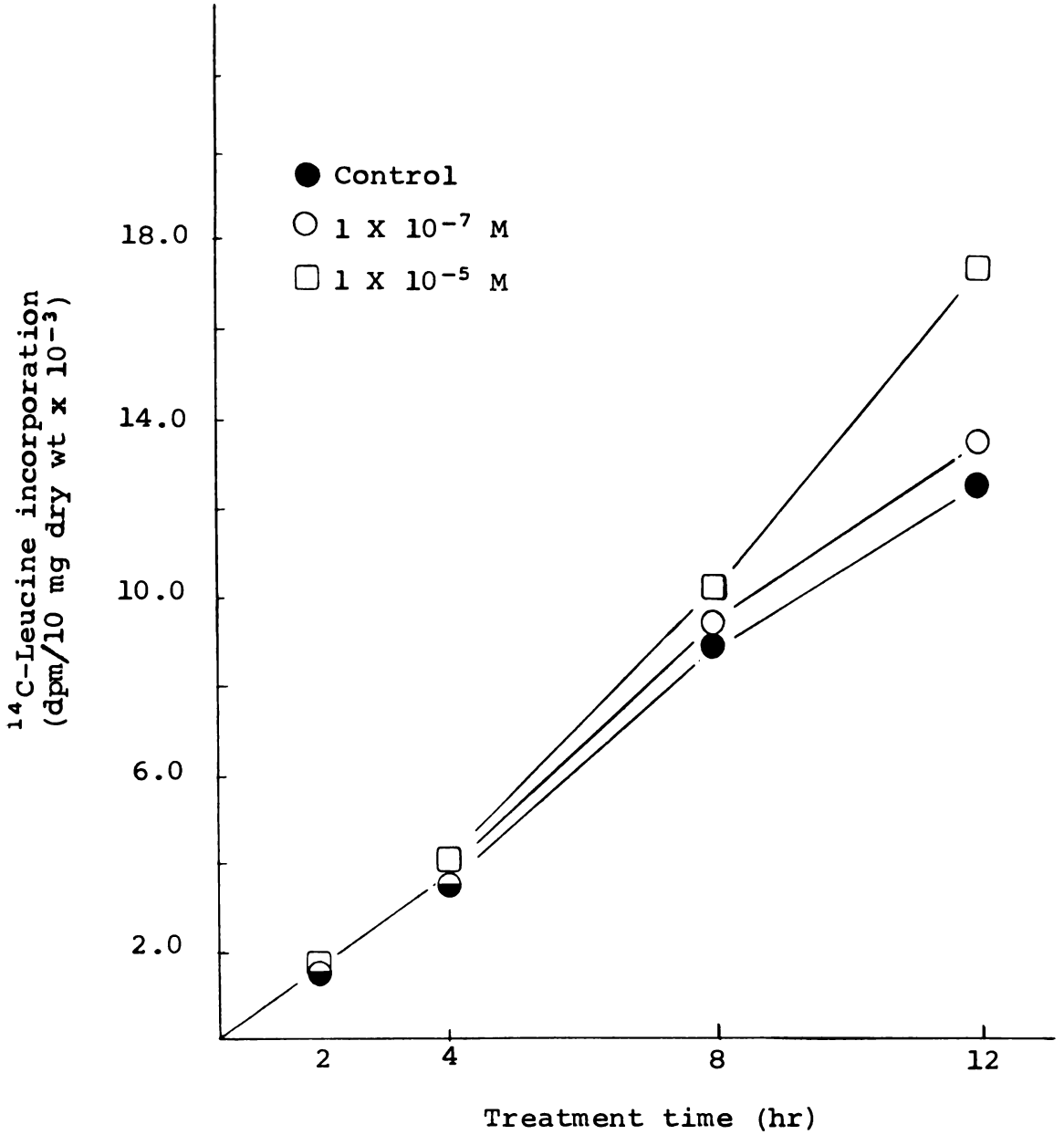

Figure 9. ^{14}C-Leucine incorporation into protein of barley leaf segments floated on solutions containing various concentrations of simazine for 2, 4, 8, and 12 hr.

F value for treatments at the 12 hr harvest was significant at the 5% level.

into protein. The aliquots of the ^{14}C-leucine solution spotted on potato starch agar plates indicated there was no bacterial growth within 5 days.

It appears that one of the initial actions of simazine may be on protein synthesis. However, the above responses are not dependent upon temperature or nutrition indicating that other processes must also be altered to result in an increase in protein content.

Proposed mode of action. During the past 10 years many modes of action of sub-toxic levels of triazines have been proposed. Ries et al. (53) postulated that simazine acts upon nitrate assimilation. This was supported when it was shown that simazine increased nitrate uptake but not sulfate and phosphate (81). These latter results may have been incorrectly interpreted. Uptake of these ions was determined by measuring the radioactivity in the soluble fraction. However, at the rate used, 4×10^{-7} M simazine, nitrate is known to accumulate, whereas sulfate and phosphate may have been metabolized into water insoluble compounds. Furthermore, these authors based their conclusions on large increases in nitrate reductase and it has been shown that there is no direct correlation between nitrate reductase activity and protein content (62,83,84). The data on carbohydrate degrading enzymes is also plagued by the lack of protein data (71,82).

None of the existing hypotheses can successfully explain all of the responses known to be brought about by low concentrations of simazine. A well constructed hypothesis must be able to explain the following:

1. Simazine may increase nitrate reductase activity eight-fold but this results in only a 30% increase in protein content.

2. Simazine increases protein only at sub-optimum temperatures and nitrogen levels.

3. Increases in protein content due to simazine occur only when the plants are grown under nitrate nitrogen and not with ammonium nitrogen.

4. Simazine increases protein content when rye plants are grown under a light intensity of 25,000 but not at 6,000 lumens/m^2.

5. Simazine stimulates ^{14}C-leucine incorporation before increasing nitrate uptake.

6. Increases in ^{14}C-leucine incorporation are not dependent upon the conditions necessary for simazine to increase protein.

7. Simazine decreases soluble carbohydrates without directly affecting respiration or decreasing photosynthesis.

8. Atrazine stimulates RNA synthesis in isolated chromatin from etiolated soybean plants.

The failure of simazine to increase protein content under low light intensities and the necessity of sub-optimum temperatures and nitrogen levels can be explained in terms of carbohydrate accumulation. In these tests simazine did not increase the protein content when the carbohydrate content was not high prior to treatment. The reduction in carbohydrate content is in response to an increase in nitrate uptake. Carbohydrate degradation is linked to nitrate reduction.

The increase in nitrate uptake is brought about by a stimulation in protein synthesis. The increase in protein synthesis may be due to an increase in RNA synthesis brought about by an increase in DNA template availability.

However, these facts do not explain why responses to simazine are observed only when nitrate is the source of nitrogen. McReynolds and Tweedy (41) observed that simazine uptake was reduced by 30-50% when plants were grown on ammonium nitrogen as compared to nitrate nitrogen. These experiments were conducted in vermiculite. However, the uptake of simazine is not decreased by ammonium nitrogen in nutrient cultures even though the pH drops drastically (Table 12). Also, simazine does not increase the protein content in vermiculite grown plants treated with ammonium nitrogen when excess simazine is added to compensate for the decrease in uptake (53). Thus, the lack of a response to simazine when ammonium is the nitrogen source may not be explained by a decrease in simazine uptake.

The differential response of plants to simazine when ammonium and nitrate are the nitrogen sources may be due to the contrasting effects on metabolism brought about by these two nitrogen sources. Nitrate must be reduced to ammonium before incorporation into amino acids. The initial reduction of nitrate to nitrite is catalyzed by nitrate reductase which has a specific requirement for nicotinamide adenine dinucleotide (NADH) as an electron donor (6,40,61). There is

Table 12. Effect of nitrate and ammonium on uptake of simazine in barley plants grown in nutrient solution and 0.1 mM phosphate buffer.

Nitrogen source	Time (days)	pH	Dry wt[a] roots	Dry wt[a] leaves	Simazine uptake[a] roots	Simazine uptake[a] leaves
			(mg/plant)		(dpm/plant)	
NO_3^-	1	6.5	16 a	46 a	180 ac	837 a
NH_4^+	1	5.6	17 a	48 a	162 a	792 a
NO_3^-	2	6.8	20 b	61 b	115 b	1507 b
NH_4^+	2	3.7	22 b	64 b	154 ab	1512 b
NO_3^-	3	7.2	31 c	75 c	203 c	2852 c
NH_4^+	3	3.1	34 c	77 c	212 c	2812 c

[a]Means followed by unlike letters are significantly different at the 5% level.

considerable evidence to suggest that respiratory metabolism supplies the electron donors for nitrate reduction (7,9,40). Beevers and Hageman (7) concluded that 3-phosphoglyceraldehyde and cytoplasmically located NAD-dependent phosphoglyceraldehyde dehydrogenase were the electron generating systems for nitrate reductase in both light and dark.

Weissman (79) investigated the effect of ammonium and nitrate nitrogen on the level of pyridine nucleotide in soybeans and sunflower (Helianthus annuus L.) roots. Although the total nucleotide concentration was the same with both nitrogen sources, nitrate treated plants had an increased proportion of total oxidized plus reduced nicotinamide adenine dinucleotide phosphate (NADP(H)). Roots supplied with nitrate maintained a high level of NADPH, provided by nitrate stimulated glucose-6-phosphate dehydrogenase activity (78). The increased production of NADPH stimulated NADPH-dependent glumatic acid dehydrogenase in nitrate treated plants. In contrast, glutamic acid dehydrogenase of roots exposed to ammonium was totally NADH-dependent. Furthermore, roots treated with nitrate contained a low NAD:NADH ratio despite active NADH utilization via nitrate reductase and glutamic acid dehydrogenase. The author explains this low ratio as a result of NAD conversion to NADH by nitrate stimulated glycolysis.

Previous experiments have demonstrated that simazine stimulated nitrate uptake. However, there was an increase in protein content only under conditions where carbohydrates

could be utilized. It appears that the carbohydrates facilitated nitrate reduction. A by-product of this conversion may be an increase in carbon compounds for amino acid synthesis. At the sub-optimum conditions required for the simazine response, the carbon source may limit protein synthesis. Ammonium nitrogen would not increase the carbon source whereas nitrate would, due to carbohydrate breakdown.

The initial effect of simazine is hypothesized to be an increase in nucleic acid synthesis. This results in an increase in protein synthesis. The increase in protein synthesis stimulates nitrate uptake. However, the nitrate can only be reduced if sufficient carbohydrates are present in order to provide NADH. The increase in glucose catabolism results in an increase in α-ketoglutaric acid. The results of this proposed mechanism would be an increase in nitrogen assimilation at the expense of carbohydrates. If carbohydrates are not available (high temperature and high nitrogen levels) no response to simazine should be observed. Experimental evidence on plant composition, environmental and nutritional parameters, and enzymatic activities support the proposed hypothesis.

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APPENDICES

APPENDIX A

HOAGLAND'S SOLUTION[a]

Chemical	Stock solution (g/l)	Final solution (ml stock/l H_2O)
$CaCl_2 \cdot 2H_2O$	147	1.5
K_2SO_4	87	1.0
$MgSO_4 \cdot 7H_2O$	98.6	2.5
KH_2PO_4	27.2	2.5
Fe (Chelate 12%)	16.6	2.5
Minor elements:		1.25
$ZnSO_4 \cdot 7H_2O$	0.088	
H_3BO_4	1.144	
$MnCl_2 \cdot 4H_2O$	0.724	
$CuSO_4 \cdot 5H_2O$	0.032	
$H_2MO_4 \cdot H_2O$	0.008	
KOH	56	adjust pH to 6.3

Equal quantities of 1.0 M stock solutions of KNO_3 and $Ca(NO_3)_2 \cdot 4H_2O$ for the following NO_3 concentrations:

	2 mM	3 mM	4 mM	8 mM	16 mM
ml stock/l H_2O:	0.66	0.99	1.33	2.66	5.32

[a]Modified from Hoagland, D. R. and D. I. Arnon. 1938. The water-culture method for growing plants without soil. Univ. Calif. Agri. Exp. Sta. Circ. 347 pp.

APPENDIX B

HILLMAN'S MEDIUM[a]

Chemical	Final concentration mg/l
KH_2PO_4	680
KNO_3	1515
$Ca(NO_3)_2 \cdot 4H_2O$	1180
$MgSO_4 \cdot 7H_2O$	492
H_3BO_3	2.86
$ZnSO_4 \cdot 7H_2O$	0.22
$Na_2MoO_4 \cdot 2H_2O$	0.12
$CuSO_4 \cdot 5H_2O$	0.08
$MnCl_2 \cdot 4H_2O$	3.62
Fe (Chelate 12%)	5.40
pH 4.6	

[a]Hillman, William S. 1961. Experimental control of flowering in Lemna. III. A relationship between medium composition and the opposite photopheriodic responses of L. perpusilla 6746 and L. gibba G-3, Am. J. Bot. 48:413-419.