EFFECT OF 2, 4-DICHLOROPHENOXYACETIC ACID ON PROTEOLYTIC ACTIVITY OF RED KIDNEY BEAN PLANTS (PHASEOLUS VULGARIS)

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Theodore Lynn Robstock

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AN ABSTRACT

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Recent investigations have shown that the treatment of red kidney bean plants with 2,4-dichlorophenoxyacetic acid results in a reduction of carbohydrates, increase in nitrogen and amino acids, changes in the vitamin content, and changes in the activity of a number of enzymes involved in carbohydrate metabolism (1-5). The purpose of this investigation was to determine if the treatment of red kidney bean plants with 2,4-dichlorophenoxyacetic acid also altered the proteolytic activity. Hemoglobin, glycylglycine, cystinyldiglycine, chloroacetyl-tyrosine, and cystinyldiglycyldiglycine were used as substrates for these determinations. Glycylglycine and chloroacetyl-tyrosine were prepared by the method of Fischer (6), cystinyldiglycine by the method of Loring and du Vigneaud (7), and cystinyldiglycyldiglycine by the procedure of Greenstein (8).

The red kidney bean plants were divided into leaf, stem, and root tissue. None of the tissues showed carboxypeptidase or aminopolypeptidase activity with chloroacetyl-tyrosine and cystinyldiglycyldiglycine substrates, respectively. Dipeptidase activity was detected in the three groups of tissue with glycylglycine and cystinyldiglycine substrates. Hemoglobin was also hydrolyzed by the red kidney bean plant tissues. The proteolytic activity in the leaf tissue of the treated plants was lower than in the non-treated, the activity in the non-treated stems was lower than the treated, whereas the activity of the treated root tissue was not significantly different from that of the non-treated roots.

Evidence is also presented which indicates that a large portion of the increase in nitrogen in the treated stem tissue is 80 percent ethanol insoluble and is probably protein.

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INTRODUCTION

Only within the last few years have growth-regulating compounds been used on a large scale. A group of English workers were the first to make use of such a compound to control weeds in oats (1). Since that time the demand for the manufacture of such chemicals has resulted in the development of a multimillion dollar chemical business. According to United States Tariff Commission figures 2,4-dichlorophenoxyacetic acid (2,4-D), which is one of a number of growth-regulating compounds, was manufactured at a rate of 917,000 pounds in 1945. By 1948 this compound was being produced at a rate of 27,512,000 pounds annually (2). These figures illustrate how rapidly the use of these compounds has expanded over a period of just a few years.

In the field of plant growth-regulators considerable confusion has arisen as a result of terminology. The term phytohormone or plant hormone has been used in at least two different senses. In its restricted sense a hormone is a substance produced in any part of the plant where it influences a specific physiological process, and in a broader sense growth-regulating compounds are generally called plant hormones (3). According to the first definition, the term phytohormone cannot be applied to any synthetic substance which has not been shown to be present in plants, although

the synthetic substance may have physiological activity very similar to that of a naturally occurring compound. For example, naphthaleneacetic acid could not be classified as a hormone; but indoleacetic acid could be classified as such by virtue of its being isolated from plant material. Van Overbeek has proposed that a phytohormone be defined as an organic substance, other than the recognized energy supplying substances, which regulates the physiological functions in plants (3). This definition includes synthetic as well as naturally occurring substances, vitamins as well as hormones, and growth inhibitors as well as growth promoters.

The applications of plant growth-regulators are many. Root initiation can be induced in cuttings of many species of plants by brief treatment with growth-regulators (4). By the treatment of some flowers with certain of these chemicals, parthenocarpic fruit development can be induced (5, 6). Some of the compounds are capable of inducing development of flower buds and so permit some control of the time of flowering (7). Other chemicals are capable of inhibiting bud development. Alpha-naphthaleneacetic acid, as well as 2,4-dichlorophenoxyacetic acid, is employed to prevent the preharvest drop of fruit (9, 10). The ripening of bananas is accelerated by exposure to a low concentration of 2,4-dichlorophenoxyacetic acid (11). Perhaps the most widely used of the growth-regulating compounds is the use of 2,4-dichlorophenoxyacetic acid, hereafter designated as 2,4-D, for the control of weeds.

Several investigations have been undertaken on the effect of the treatment of red kidney bean plants with 2,4-D on the metabolism of the plant. It has been found that there are striking changes in the carbohydrate, nitrogen, amino acid, and vitamin contents of the plants following treatment with 2,4-D (12, 13, 14). Neely and co-workers have demonstrated that the activity of several of the enzymes involved in carbohydrate metabolism are changed considerably as a result of the treatment of the plant with this compound (15, 16). In view of the striking changes in the protein (NX 6.25) and amino acid contents of bean plants following treatment with 2,4-D (12, 13), it appeared to be of interest to study the effect that treatment with this compound had upon the proteolytic enzyme activity of red kidney bean plants.

HISTORICAL REVIEW

The English workers Slade, Templeman, and Sexton (1) in August, 1940 observed that when alpha-naphthaleneacetic acid was applied to oats, weedy with yellow charlock, at the rate of 25 pounds per acre, the charlock was killed with little injury to the oats. To these workers goes the credit for the first use of a plant growth-regulator as a selective weed killer. To E. J. Kraus, of the University of Chicago, goes the credit for first suggesting the use of plant growth-regulators as herbicides in the United States. It is reported that Kraus suggested the idea as early as August, 1941 (17).

Slade, Templeman, and Sexton (1) continued their work by searching for growth-regulators more active than alpha-naphthaleneacetic acid. Of the many compounds tested, they found that substituted phenoxyacetic and naphthoxyacetic acids showed the most promise for selective herbicidal activity. In 1941 these workers concluded that 2-methyl-4-chlorophenoxyacetic acid (Na salt) was one of the two most active of the compounds tested. Trials in the field in 1943 and 1944 confirmed their findings as to the merits of 2-methyl-4-chlorophenoxyacetic acid as a selective herbicide.

The other most active compound tested by Slade and co-workers, 2,4-dichlorophenoxyacetic acid, was further tested

by Nutman, Thorton, and Quastel (18). Blackman (19) in 1943 carried out experiments to compare 2-methyl-4-chlorophenoxyacetic acid with 2,4-dichlorophenoxyacetic acid and found the former to be more selective and less likely to injure crops than 2,4-D when applied to control broadleaf annual weeds growing with cereals.

The first investigators in the United States to demonstrate the physiological properties (cell elongation, morphogenesis, root development and parthenocarpy) of substituted phenoxy acids were Zimmerman and Hitchcock (20). Mitchell and Hamner (21) suggested the possibility of using 2,4-D and similar compounds with "Carbowax" as selective herbicides as a result of their search for suitable carriers for growth-regulating compounds. A short time later in 1944 Hamner and Tukey (17) conducted the first actual experiments designed to test the potentiality of the growth-regulators as herbicides. Bindweed (Convolvulus arvensis L.) was successfully killed by treatment with 2,4-D and 2,4,5-trichlorophenoxyacetic acid. Since that time numerous workers have utilized these and similar compounds to determine their herbicidal effects on other weeds.

Beal (22, 23) observed the telemorphic effects of the application of substituted phenoxy compounds on sweet pea, marigold and bean plants. The term "telemorphic" response was used to mean morphological responses induced or incited in a plant even at a point considerably distant from the point of application of the substance. These effects were

manifested by abnormal growth responses of the different organs of the plant treated with growth-regulators.

As a result of their investigations on the morphological and histological changes due to 2,4-D treatment in bindweed and sowthistle, Tukey et al (24) proposed four possible mechanisms contributing to the herbicidal action of 2,4-D: (1) chlorophyll depletion reducing food synthesis in tissues, (2) translocation of food interferred with by phloem proliferation, (3) food reserves depleted by increased respiratory activity, and (4) invasion of soil organisms due to disorganization and rupture of rhizonic and root cortex.

Burton (25) studied the formative effects of certain substituted chlorophenoxy compounds on bean leaves. It was found that treatment of young plants with 0.5 percent 2-chlorophenoxyacetic acid in carbowax inhibited the formation of intercellular spaces in the mesophyll of bean leaflets. Similar application of 0.5 percent 4-chlorophenoxyacetic acid in lanolin inhibited the activity of plate meristem in the laminae of bean leaflets. As a result veins were not distinct and their parenchyma became continuous although the vascular elements remained discrete. Chlorenchymatous tissue was confined to the margin. Similar treatment with 0.5 percent 2,4-D in either lanolin or carbowax as the carrier induced progressive modification of bean leaves. The earliest leaves were similar to those induced by treatment with 2-chlorophenoxyacetic acid. In later leaves the external form and internal structure resembled that formed in leaflets treated

with the 4-chloro compound.

Pridham has reported that seedlings of bean plants sprayed with 2,4-D during ripening of pods developed 2,4-D symptoms (viruslike crisp foliage, dwarfing of growth, and serration and fusion of leaflets) in juvenile and mature leaves (26).

A number of investigations have been undertaken designed to reveal the mechanism of the action of 2,4-D on plants. It was found by Mitchell (27) that within three weeks after having been sprayed with an aqueous solution containing 1,000 p.p.m. of 2,4-D, plants of morning glory were dead and had been essentially depleted of sugars, starch and dextrin. Soon after treatment sugars had increased above the amounts in control plants, and then decreased until nearly depleted.

Rasmussen (28) observed that the reducing sugar content of dandelion roots increased rapidly following application of 2,4-D, but later decreased towards the control levels. The sucrose declined slowly and the dextrin and levulin content decreased rapidly after treatment. There was found an increase in nitrogen soluble in 80 percent alcohol and also an increase in protein in the roots after treatment.

In experiments employing bindweed, Smith, Hamner and Carlson (29) found rapid increases in total sugars in all parts of the plant following 2,4-D treatment; but the increases were followed by decreases to the control levels after a few days. The starch-dextrin fraction decreased in all parts of treated plants. Total nitrogen decreased

steadily in the leaves and increased in both stems and underground parts. Further evidence of altered metabolism was found in the increased respiratory activities of the treated rhizome and root slices.

Workers at the Agriculture Experiment Station of the University of Idaho have also observed that 2,4-D treatment resulted in an increase of the protein content of seven varieties of wheat (30).

Sell and co-workers have carried out extensive studies on changes which take place in red kidney bean plants after treatment with 2,4-D (12). Both total nitrogen and amino acids accumulated in greater quantities in the stems of plants after treatment. The amino acids calculated as percent of crude protein showed the greatest differences in the quantity of amino acids of the stem tissue in the case of aspartic acid, lysine, valine, methionine, and phenylalanine. In view of these results it was suggested that there is a possibility of the protein being different in the treated stems. The reducing and non-reducing sugars were depleted in the stems of the 2,4-D treated plants. A considerable reduction in carbohydrate reserves and a decrease in acid hydrolyzable polysaccharides also was observed. The decrease in carbohydrate content and increase in protein suggested to these workers that a large portion of the carbohydrate is utilized in protein synthesis. The amount of crude fiber decreased in the stems following treatment, and the amount of ether extract increased slightly in the treated

stem tissue.

Similar studies were carried out on the leaves and roots of red kidney bean plants by Weller, Luecke, Hanner and Sell (13). The leaves of the treated plants contained slightly lower percentages of nitrogen and most of the amino acids than did the control plants. The percentages of crude protein and the amino acids valine, histidine, and arginine were almost the same in the treated and untreated roots. The other amino acids were slightly less in the roots of the treated plants. It was suggested that the reduction of most of the amino acids in the root and leaf tissue may be due in part to the translocation of these substances to the stem tissue. A depletion of non-reducing sugars was observed in both the leaves and roots of the treated plants. The percentages of reducing sugars, starch, polysaccharides, crude fiber, ash, ether extract, unsaponifiable material, and fatty acids were practically the same for both the leaves and the roots of the treated and non-treated plants.

The vitamin content has also been found to change following treatment of red kidney bean plants with 2,4-D (14). The content of thiamine, riboflavin, nicotinic acid and carotene was observed to be less in the leaves of treated plants, whereas the content of pantothenic acid increased in the treated leaves. In the stems it was found that the content of thiamine, riboflavin, nicotinic acid and pantothenic acid increased in the treated plants, whereas the carotene content was lower in the treated stems.

The marked chemical changes in the treated tissue suggested that the enzyme systems might be involved. Neely, Ball, Hammer and Sell (15, 16) carried out studies on a number of the enzymes involved in carbohydrate metabolism. It was found by these workers that the alpha amylase activity was considerably lowered and the beta amylase activity slightly lowered in the treated stem tissue. In the leaves there was a very slight increase in the beta amylase activity in the treated plants. Invertase activity was not detected in either the treated or non-treated leaves and stems of the red kidney bean plants. Pectin methoxylase activity increased in both the stems and leaves of treated plants. Phosphorylase activity decreased in the stem and leaf tissue of the 2,4-D treated plants.

Hagen, Clagett, and Helgeson of the North Dakota Agriculture College have made a study on the effect of 2,4-D upon the activity of the caster bean lipase (31). In experiments in which olive oil was used as substrate and solvent extracted caster bean meal as the source of enzyme and various concentrations of the sodium salt of 2,4-D added, it was found that the lipase activity was inhibited by the presence of 2,4-D at a very low concentration.

EXPERIMENTAL

Materials and Methods

Samples of Tissue

Preparation of leaf, stem, and root tissue. Seeds of the red kidney bean, Phaseolus vulgaris, were selected for uniformity of size and planted in four-inch pots in the greenhouse. Each pot contained four plants which were treated when the first trifoliate leaves were expanding. Two replications of 100 plants each were used as a source of both treated and non-treated (control) plant material. One drop (0.05 ml.) of a 0.1 percent solution of 2,4-dichlorophenoxyacetic acid (2,4-D) was applied to the base of the blade of a primary leaf of each treated plant. The plants were harvested six days after treatment. At the time of harvest the stem tissue had proliferated considerably but there were no signs of necrosis. The material was dried in the dark at 30° C. and after drying segregated into leaf, stem, and root tissue with the hypocotyl, first internode, and leaf petioles being grouped together as stem tissue. The material was ground in a micro Wiley mill to pass through a 60-mesh screen and stored in the dark in stoppered glass jars.

Analytical Methods

Determination of proteolytic activity with a hemoglobin substrate. The procedure employed for the determination of proteolytic activity using a protein as the substrate, with a few minor alterations, was a modification of the Ayre-Anderson method as revised by Miller (32). A one-gram sample of the powdered plant material was placed in an eight-inch test tube along with 0.625 g. of Difco Bacto-Hemoglobin. The tube was rotated so as to thoroughly mix the plant material and the substrate. Twenty-five milliliters of 0.1 M. acetate buffer of pH 4.8, which had been previously brought to the temperature of the water bath, was added. After agitation and stirring with a glass rod until a uniform suspension had been obtained, a trace of toluene (0.5 ml.) was added. The tube was placed in an automatic shaking device contained in a constant temperature water bath held at 48° C. and incubated the desired length of time. For each determination two tubes of material were prepared in the same manner and placed in the water bath at the same time. After fifteen minutes of shaking, 5 ml. of 36 percent trichloroacetic acid was added to one of the tubes and the shaking continued for an additional five minutes. The contents of the tube were filtered through paper and the precipitated material washed with a small volume of distilled water. During the washing care was taken so that the total volume of the filtrate did not exceed 50 ml. The filtrate was diluted to 50 ml. and the

amino nitrogen determined on a 10 ml. aliquot in a Van Slyke amino nitrogen apparatus. The sample was shaken five minutes in the reaction chamber of the apparatus.

Five hours after taking the first sample the remaining tube was treated in the same manner and the amino nitrogen determined on a 10 ml. aliquot. The difference between the two determinations was taken to represent the increase in amino nitrogen as a result of the action of the proteolytic enzymes in the plant material on the hemoglobin substrate. Treated and non-treated tissue were run at the same time for each determination. The results of the determinations were expressed on the basis of the increase in milligrams of amino nitrogen per plant per five hours of hydrolysis.

Determination of proteolytic activity using synthetic substrates. For these determinations 0.750 g. of the appropriate powdered plant material was suspended for four hours in 10 ml. of an aqueous 20 percent glycerol solution. At thirty minute intervals the tubes containing the suspension were tipped several times in order to resuspend any material which may have settled to the bottom of the tubes. After four hours the suspension was centrifuged and the supernatant liquid filtered. The clear filtrate served as the source of enzyme. Longer extraction periods did not result in a measurable increase in enzymatic activity.

The synthetic substrates used for the determinations consisted of one percent solutions of glycylglycine, chloro-

acetyl-L-tyrosine, L-cystinyldiglycine, and L-cystinyldiglycyldiglycine.

The procedure employed using glycylglycine as a substrate was one by Blagoveshchenskii and Melamed (33). A series of three tubes were necessary for each determination. The first tube contained 3 ml. of a one percent solution of glycylglycine in 0.067 M. phosphate buffer pH 7.4 and 2 ml. of the glycerol extract. The second tube contained 3 ml. of 0.067 M. phosphate buffer pH 7.4 and 2 ml. of the glycerol extract. The last tube contained 3 ml. of a one percent solution of glycylglycine in 0.067 M. phosphate buffer pH 7.4 and 2 ml. of a 20 percent glycerol solution. Two-tenths milliliter of toluene was added to each tube to inhibit bacterial growth and the tubes were incubated in a constant temperature water bath at 30° C. for 48 hours. At the end of this time, the solutions were removed from the water bath and diluted to a volume of 10 ml. An aliquot of the diluted solution was analyzed for amino nitrogen in a Van Slyke amino nitrogen apparatus. The sum of the amino nitrogen determinations from the two blank tubes was subtracted from the value of the amino nitrogen obtained from the tube containing both the plant extract and the substrate.

A similar procedure was employed with the other synthetic substrates. The peptides were dissolved in water, the pH adjusted to 7 with dilute ammonium hydroxide, and diluted with distilled water so as to give a one percent solution. Three tubes were prepared as in the method using

glycylglycine for each substrate. After 48 hours of incubation in the water bath at 30° C., the tubes were removed and the amino nitrogen determined on all of the tubes with the exception of those containing cystinyldiglycyldiglycine.

In the case of the determinations with cystinyldiglycyldiglycine as the substrate the solutions were filtered in order to collect any cystine that had precipitated in the course of the reaction. The precipitate was dissolved off of the filter with 6 N. hydrochloric acid and the nitrogen determined by a semi-micro Kjeldahl. The tubes containing the plant extract only and the substrate only were treated in a like manner. In all of the determinations treated and non-treated tissue were used at the same time and the results were expressed as milligrams of amino nitrogen per plant.

Synthesis of Peptides

L-Cystinyldiglycine

Benzyl chloroformate (34). In a three-necked flask fitted with a rubber stopper carrying an exit tube and a delivery tube extending to the bottom was placed 250 g. of dry toluene. The tubes were equipped with stopcocks so that the reaction flask could be disconnected. The flask containing the toluene was weighed and cooled in an ice bath. Phosgene was bubbled into the toluene until 55 g. had been absorbed. After the required amount of phosgene had been absorbed, the connection to the phosgene tank was replaced by a separatory funnel, and with gentle shaking, 54 g. of

C. P. benzyl alcohol was added rapidly through the separatory funnel. The flask was allowed to stand in the ice bath for one-half hour and at room temperature for an additional two hours. The solution was then concentrated under reduced pressure at a temperature below 60° C. to remove the major portion of the toluene, hydrochloric acid, and phosgene.

Dicarbobenzoxy-L-cystine (35). Seven and two-tenths grams of L-cystine were dissolved in 60 ml. of 1 N. sodium hydroxide contained in a 500 ml. three-necked flask fitted with a mechanical stirrer, dropping funnel, and cooled in an ice bath. Twenty-four grams of the benzyl chloroformate prepared above were added dropwise to the vigorously stirred solution while 120 ml. of 1 N. sodium hydroxide were added dropwise at the same time over a period of 20 to 25 minutes. The mixture was stirred for an additional 30 minutes and extracted with ethyl ether. The cooled, aqueous phase was acidified to the appearance of the blue color of Congo red with 6 N. hydrochloric acid. The white amorphous precipitate was filtered and washed with small portions of cold water until the washings were free of acid. The dry filter cake was dissolved in 200 ml. of ethyl acetate and filtered. The ethyl acetate was removed under reduced pressure and the residue taken up in 200 ml. of chloroform. Dicarbobenzoxy-L-cystine separated as fine needles, m.p. 122-124° C.

Dicarbobenzoxy-L-cystinyldiglycine (36,37). Ten grams of finely pulverized dicarbobenzoxy-L-cystine suspended in 60 ml. of anhydrous ethyl ether in a 125 ml. glass stoppered

Erlenmeyer flask was cooled in an ice-salt bath. Eight and two-tenths grams of finely pulverized phosphorous pentachloride were added and the suspension shaken vigorously. After several minutes the material went into solution. The stopper was removed momentarily at frequent intervals in order to release any pressure which may have built up inside the flask. After 15 to 20 minutes with shaking and cooling, the acid chloride of dicarbobenzoxy-L-cystine precipitated. An additional 10 to 15 ml. of dry ethyl ether was added and the material filtered on a dry filter. After washing the precipitate with a small volume of dry ethyl ether, the product was added in small portions along with 38 ml. of 1 N. sodium hydroxide to 4.4 g. of glycine dissolved in 60 ml. of 1 N. sodium hydroxide cooled in an ice-salt bath. During the addition of the acid chloride and for one-half hour afterward, the solution was stirred vigorously. The cooled alkaline solution was acidified with hydrochloric acid and the crude precipitate of dicarbobenzoxy-L-cystinyldiglycine filtered. The amorphous precipitate was washed several times with cold water until the washings were neutral or only faintly acid to Congo red. The moist solid was dissolved in 150 ml. of boiling dioxane and the hot solution filtered. The filtrate was evaporated under reduced pressure to a volume of 75 ml. and on standing fine needles of dicarbobenzoxy-L-cystinyldiglycine appeared. These were filtered off and washed with ether. After extraction with ethyl acetate and crystallization the melting

point was 175-178° C.

L-cystinyldiglycine (37). One and seven-tenths grams of sodium cut in small pieces was added gradually to 6.2 g. of dicarbobenzoxy-L-cystinyldiglycine dissolved in 50 ml. of liquid ammonia contained in a 250 ml. three-necked flask fitted with a mercury-sealed stirring device. The solution was cooled in a trichloroethylene-solid carbon dioxide bath and stirred gently. The completion of the reaction was shown by the appearance of the blue color due to free sodium. After the ammonia had been allowed to evaporate, the flask was evacuated on the water pump in order to remove the remaining ammonia; and the residue remaining was dissolved in a small volume of water. The flask was again evacuated for a short time and the cooled solution made neutral with hydriodic acid. The reduced peptide was oxidized by aerating the solution until the sodium nitro-prusside test was negative. The solution was made slightly acid to litmus with hydriodic acid, concentrated in vacuo until crystals of sodium iodide began to separate, and the oxidized peptide precipitated by the addition of several volumes of ethanol. The compound was obtained halogen free by repeated solution of the amorphous product in a small volume of water and precipitation with ethanol. The amorphous compound was then dissolved in a small volume of water (15 ml.), enough ethanol added to give the solution a cloudy appearance, and the mixture allowed to stand overnight in the refrigerator. The peptide first appeared as a syrup but gradually solid-

ified. The peptide was filtered, enough ethanol was added to give a cloudy appearance, and the cooling repeated. The melting point of the crystalline product was 204-206° C. (decomposition).

Anal. Calcd. for $C_{10}H_{18}O_6N_4S_2 \cdot 2H_2O$: N, 14.36. Found: N, 14.29.

L-Cystinyldiglycyldiglycine

Dicarbobenzoxy-L-cystinyldiglycyldiglycine (38). Twenty grams of glycine anhydride was dissolved in 100 ml. of 2 N. sodium hydroxide and the solution allowed to stand for one-half hour at room temperature. An equal volume of water was added and the solution chilled. With vigorous stirring, dicarbobenzoxy-L-cystinyldichloride, prepared from 23.2 g. of dicarbobenzoxy-L-cystine as previously described, was added in small portions along with the alternate addition of a total volume of 80 ml. portions of 1 N. sodium hydroxide. The addition required about an hour and the stirring was continued for an additional half hour. The solution was filtered and the cooled filtrate treated with 5 N. hydrochloric acid to the appearance of the blue color of Congo red. The compound appeared as a gelatinous mass which was immediately filtered off and washed with cold water. The product was dissolved in 150 ml. of hot dioxane and evaporated to 50 ml. under reduced pressure. On the addition of the dioxane solution to a double layer of ethyl ether and water, an oil separated which rapidly crystallized into needles on standing in the refrigerator. The material

was filtered, washed with cold water, ether, and dried. After extraction with hot ethyl acetate, the product, a white solid, melted at 207-209° C.

L-cystinyldiglycyldiglycine (38). Twelve and six-tenths grams of disarbobenzoxy-L-cystinyldiglycyldiglycine was dissolved in 250 ml. of liquid ammonia. The three-necked 500 ml. round bottom flask containing the solution was fitted with a mercury-sealed stirrer and cooled in a trichloroethylene-dry ice mixture. Metallic sodium was added in small pieces until a blue color appeared in the solution. After the ammonia had been allowed to evaporate, the flask was evacuated on the water pump for several hours. The residue was dissolved in enough 0.5 N. sulfuric acid to bring the reaction just acid to litmus and treated with a slight excess of Hopkin's mercuric sulfate reagent. After standing several hours, the mercury salt was centrifuged off and washed five times with distilled water, suspended in water, and treated with hydrogen sulfide gas. The filtrate after aeration was again treated with the mercuric sulfate reagent, centrifuged, and washed. After removal of the mercury with hydrogen sulfide, the filtrate was aerated and treated with saturated barium hydroxide solution until weakly alkaline. The barium sulfate was centrifuged off and the supernatant solution treated with a rapid current of air. When the oxidation was completed as shown by a negative sodium nitroprusside test, the solution was shaken with norit for one-half hour; and the clear, colorless

filtrate was treated with enough dilute sulfuric acid to exactly remove the barium hydroxide. After removal of the barium sulfate by centrifugation, the supernatant solution was evaporated to a small volume (15 ml.) under reduced pressure and treated with a large excess of ethanol. A colorless oil separated which rapidly crystallized in the refrigerator. After the crystallization was repeated three times, the product was dried in a vacuum desiccator over concentrated sulfuric acid. The melting point of the white crystalline product was 96° C. (decomposition).

Anal. Calcd. for $C_{14}H_{24}O_4N_6S_2 \cdot 2\ H_2O$: N, 16.7. Found: N, 16.7.

Glycylglycine

Glycine anhydride (39). A mixture containing 70 g. of glycine and 350 ml. of ethylene glycol was heated with continuous stirring for 50 minutes at a temperature of 174° to 176° C.; and the dark, brown mixture was cooled overnight in the refrigerator. The suspension was centrifuged and the brown, crystalline mass washed with a small volume of absolute methanol until the washings were nearly colorless. The brown crystals were dissolved in 220 ml. of boiling water and the solution cooled overnight. The crystals were filtered off, washed with methanol, and air dried. The product was dissolved in 250 ml. of boiling water, decolorized with decolorizing carbon, and the hot solution filtered. The crystals, which formed upon standing overnight in the

refrigerator, were filtered off, washed in turn with ice-water, 50 percent methanol, and absolute methanol. The product was pure white crystals of 2,5-diketopiperazine.

Glycylglycine (40). One gram of pulverized glycine anhydride was shaken with 10 ml. of 1 N. sodium hydroxide at room temperature. The glycine anhydride went quickly into solution; and after 15 to 20 minutes, the solution was neutralized with an equivalent amount of 1 N. hydrochloric acid. The solution was evaporated under reduced pressure to a small volume and cooled. The glycylglycine appeared as a colorless crystalline mass. Melting point 209° C.

Anal. Calcd. for $C_4H_6O_3N_2 \cdot H_2O$: N, 18.66. Found: N, 18.07.

Chloroacetyl-L-Tyrosine (41)

Ten grams of L-tyrosine was suspended in 70 ml. of absolute ethanol and hydrogen chloride gas bubbled through the suspension until solution was complete. One hundred and forty milliliters of absolute ethanol was added and the solution refluxed on the steam bath for 24 hours. After this time the solution was evaporated to dryness under reduced pressure.

Ten grams of the crude hydrochloride of the ethyl ester of tyrosine was covered with 100 ml. of chloroform. After cooling to 0° C., 41 ml. of 1 N. sodium hydroxide was added with shaking. The free ester quickly went into solution in the chloroform. Five grams of chloroacetyl chloride, dissolved in 50 ml. of chloroform, was added in small portions to the cool mixture. After about one-half

of the acid chloride had been added, 20 ml. of sodium carbonate solution (4.7 g. of the anhydrous salt) was added alternately with shaking to the cooled mixture. At the close of the reaction, the chloroform phase was separated and dried with 2 g. of anhydrous sodium sulfate. Most of the chloroform was removed on the steam bath and the chloroacetyl-L-tyrosine ester crystallized from the solution upon the addition of a small volume of petroleum ether.

Ten grams of the ethyl ester of chloroacetyl-L-tyrosine was dissolved in 70 ml. of 1 N. sodium hydroxide. After 15 minutes the solution was neutralized with hydrochloric acid and in a short time the chloroacetyl-L-tyrosine began to crystallize. The crystallization was completed on standing overnight in the refrigerator. Melting point 153° to 154° C.

Anal. Calcd. for $C_{11}H_{12}O_4NCl$: N, 5.44. Found: N, 5.65.

RESULTS AND DISCUSSION

The results of the determinations of proteolytic activity are summarized in Tables 1 to 4.

The proteolytic activity with a hemoglobin substrate is shown in Table 1. The activity in the 2,4-D treated stem tissue is almost a third more than in the non-treated tissue. Determinations with the leaf tissue show the reverse trend. In this case, the leaves of the non-treated plants show almost double the proteolytic activity of the treated leaves. Root tissue shows a very slight decrease in the activity of treated tissue. The results as determined by the method of Van Slyke after five hours' hydrolysis at 43° C. are expressed as the increase in milligrams of amino nitrogen per plant. In experiments in which the temperature of the reaction and the pH of the buffer were varied, it was found that the maximum hydrolysis for five hours took place at 48° C. and pH 4.8. In order to be certain that all of the increase in amino nitrogen was a result of enzymatic hydrolysis of the hemoglobin substrate, determinations were carried out on separate samples containing the substrate only and the plant material only. In neither of these cases was there a significant increase in amino nitrogen in the course of five hours under the con-

TABLE 1

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE PROTEOLYTIC ACTIVITY OF THE STEM, LEAF AND ROOT TISSUE OF THE RED KIDNEY BEAN PLANT USING A HEMOGLOBIN SUBSTRATE*

Tissue	Non-treated replicate		Treated replicate	
	1	2	1	2
Stems	1.73	1.89	2.60	2.48
Leaves	1.62	1.93	0.96	0.94
Roots	1.02	1.05	0.83	0.94

*Expressed as the increase in milligrams of amino nitrogen per plant for five hours of reaction at 43° C. and pH 4.8.

ditions of the experiment, which indicates that the increase in amino nitrogen in the determinations was a result of the enzymatic hydrolysis of the hemoglobin substrate.

Table 2 shows the results of an experiment in which the proteolytic activity was determined on the stem tissue employing synthetic peptide substrates. With glycylglycine the treated stems have more than double the activity of the non-treated material. The same trend is observed with L-cystinyldiglycine. When the substrates were either L-cystinyldiglycyldiglycine or chloroacetyl-L-tyrosine, no evidence of enzymatic hydrolysis of these compounds was noted.

The results of the determination of proteolytic activity with leaf tissue and synthetic substrates are summarized in Table 3. The treated leaves show less than half the activity of the non-treated leaf tissue with glycylglycine. With cystinyldiglycine the activity of the non-

TABLE 2

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE PROTEOLYTIC ACTIVITY OF THE LEAF TISSUE OF THE RED KIDNEY BEAN PLANT USING SYNTHETIC SUBSTRATES*

Substrate	Non-treated replicate		Treated replicate	
	1	2	1	2
Glycylglycine	0.22	0.22	0.50	0.45
L-Cystinyldiglycine	0.54	0.68	1.62	1.18
L-Cystinyldiglycyldiglycine	0.00	0.00	0.00	0.00
Chloroacetyl-L-tyrosine	0.00	0.00	0.00	0.00

*Expressed as the increase in milligrams of amino nitrogen per plant for 48 hours of reaction at 30° C.

TABLE 3

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE PROTEOLYTIC ACTIVITY OF THE LEAF TISSUE OF THE RED KIDNEY BEAN PLANT USING SYNTHETIC SUBSTRATES*

Substrate	Non-treated replicate		Treated replicate	
	1	2	1	2
Glycylglycine	0.20	0.21	0.13	0.13
L-Cystinyldiglycine	0.37	0.28	0.13	0.10
L-Cystinyldiglycyldiglycine	0.00	0.00	0.00	0.00
Chloroacetyl-L-tyrosine	0.00	0.00	0.00	0.00

*Expressed as the increase in milligrams of amino nitrogen per plant for 48 hours of reaction at 30° C.

treated leaves is more than double the activity of the 2,4-D treated material. As was the case with the stem tissue, the leaves did not show any hydrolytic activity on the substrates cystinyldiglycyldiglycine or chloroacetyl tyrosine.

Table 4 shows that the non-treated root tissue has slightly, but probably not significantly, more activity than the treated tissue when the substrates are glycylglycine and cystinyldiglycine. Again as was observed from the results of experiments with stem and leaf tissue, the root tissue was not able to bring about the hydrolysis of cystinyldiglycyldiglycine and chloroacetyl tyrosine.

TABLE 4

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE PROTEOLYTIC ACTIVITY OF THE ROOT TISSUE OF THE RED KIDNEY BEAN PLANT USING SYNTHETIC SUBSTRATES*

Substrate	Non-treated replicate		Treated replicate	
	1	2	1	2
Glycylglycine	0.30	0.20	0.20	0.15
L-Cystinyldiglycine	0.70	0.82	0.63	0.53
L-Cystinyldiglycyldiglycine	0.00	0.00	0.00	0.00
Chloroacetyl-L-tyrosine	0.00	0.00	0.00	0.00

*Expressed as the increase in milligrams of amino nitrogen per plant for 48 hours of reaction at 30° C.

Specific substrates for aminopolypeptidase, carboxypeptidase and dipeptidase have frequently been, respectively, leucyldiglycine, chloroacetyltyrosine and leucylglycine. The

methods for estimating the hydrolysis of these compounds have often times employed a titration procedure. In the event of the determination of aminopolypeptidase activity in a biological system in which a dipeptidase is also present, leucyldiglycine will not be a very satisfactory substrate since the glycylglycine, which is split off by the aminopolypeptidase, will be further split by the dipeptidase. As a result, a titration procedure will not give a true indication of aminopolypeptidase activity but a combination of aminopolypeptidase and dipeptidase activity. Greenstein (38) suggests as a substrate for aminopolypeptidase a tripeptide containing a very insoluble amino acid in the acyl position. The amino acid can be filtered off and determined separately from the other products of hydrolysis. Experiments have been reported by Greenstein (38) which show that cystinyldiglycyldiglycine is a satisfactory substrate for determining aminopolypeptidase activity in the presence of carboxypeptidase and dipeptidase.

The determinations of amino nitrogen in this work were done in a Van Slyke amino nitrogen apparatus. A titration procedure was not convenient to use because of the presence of colored substances in the plant extracts which made the end point of the titration difficult to detect.

It has been reported that the amino acids glycine and cystine give nitrogen values with the Van Slyke method of analysis which are slightly higher than the theoretical (42). In the case of cystine in the determinations reported in

this work, the error is eliminated when the amino nitrogen value of the blanks is subtracted from the value of the reaction tube. The major portion of the error due to glycine will also be eliminated for the same reason. The small error remaining will not be large enough to significantly alter the results.

Glycylglycine and cystinyldiglycine are substrates used for the detection of dipeptidase activity. Chloroacetyltyrosine is a specific substrate for carboxypeptidase activity and cystinyldiglycyldiglycine is specific for aminopolypeptidase activity (38). Evidently there were no enzymes present in the immature bean plants which were able to exhibit carboxypeptidase or aminopolypeptidase activity; or if there were such enzymes present, they were not able to hydrolyze the substrates employed in these experiments for their determination. Dipeptidase activity was indicated in both treated and non-treated bean tissue. The proteolytic activity determined with either hemoglobin, glycylglycine or cystinyldiglycine as the substrates showed the same trend in the 2,4-D treated tissue and of approximately the same relative difference.

The decreased proteolytic activity in the 2,4-D treated leaf tissue was to be expected since there was evidence of an inhibition of the growth of the leaves of the treated plants. Although the total over-all dry weight of the treated and non-treated plants was about the same, the weight of the leaves of the treated plants was less than the weight

of the non-treated leaves. Since the weight of the stem tissue of the treated plants was greater than the weight of the stems of the non-treated plants, the total weight of the two groups of plants remained approximately the same. Weller, Luecke, Hamner and Sell have shown that the leaves of 2,4-D treated plants contained lower percentages of protein (NX 6.25) and amino acids than did the leaves of the non-treated plants (13). These conditions might be expected to result in lower proteolytic activity in the leaves of the treated plants.

The results of the determinations of proteolytic activity in the stem tissue show that the activity is increased in the treated plants. This increased proteolytic activity indicates the possibility of several abnormal conditions occurring in the treated stem tissue. If it is assumed that the proteins of the plant are in a state of steady breakdown and resynthesis, the increased proteolytic activity could be taken to indicate that there is an accumulation of protein in the stems of the treated plants. Sell, Luecke, Taylor and Hamner have shown that there is a considerable reduction in the carbohydrate content of the stems of treated red kidney bean plants together with an increase in nitrogen and amino acids and suggested at this time that the reduced carbohydrate content is due to a utilization of these compounds for the synthesis of protein (12). The possibility exists also that the metabolism of carbohydrates is altered in some way and as a result the protein can not

be further utilized and tends to accumulate.

The possibility arises also that the increased proteolytic activity results in an accumulation of amino acids and other protein degradation products in the treated stem tissue. In an attempt to clarify these possibilities, an experiment was undertaken on the stem tissue to separate and determine the protein and non-protein nitrogen of the 2,4-D treated and non-treated tissue. The plant material was extracted with 80 percent ethanol and the nitrogen was determined in the ethanol extract. The nitrogen remaining in the residue was taken to be due primarily to protein. The nitrogen soluble in the 80 percent ethanol was assumed to be primarily due to amino acids and other soluble products of protein degradation. The results of this experiment are summarized in Table 5.

TABLE 5

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE NITROGEN CONTENT OF THE STEMS OF RED KIDNEY BEAN PLANTS*

(Expressed on a dry weight basis)

Constituent	Non-treated replicate		Treated replicate	
	1	2	1	2
	Percent			
Total nitrogen	3.02	3.24	4.61	5.00
Soluble nitrogen (80 percent ethanol)	1.44	1.55	1.82	2.10
Insoluble nitrogen	1.58	1.69	2.79	2.90

*All nitrogen determined by Kjeldahl method

The results of this experiment indicate that the bulk of the increased nitrogen content of the treated stems appears to be located in the protein fraction, although there is an indication that a small portion of the increase in nitrogen is due to amino acids and other nitrogenous products. The character of the protein in the treated stem tissue compared to the non-treated tissue is not known at the present time. The work of Sell, Luecke, Taylor and Hamner (12) on the amino acid composition of the treated and non-treated stem tissue indicates that the composition of the protein in the treated tissue is different from that of the protein in the non-treated tissue.

The differences in the proteolytic activity between the treated and non-treated root tissue was not large enough to be of significance.

SUMMARY

1. The proteolytic activity of the leaf, stem and root tissue of red kidney bean plants treated with 2,4-dichlorophenoxyacetic acid and non-treated plants was determined using hemoglobin, glycylglycine, L-cystinyldiglycine, L-cystinyldiglycyldiglycine and chloroacetyl-L-tyrosine as the substrates.

2. With hemoglobin, glycylglycine and L-cystinyldiglycine as the substrates, the proteolytic activity of the non-treated leaves was almost double that of the treated leaves. The activity of the treated stems was almost double the activity of the non-treated stems and the proteolytic activity of the treated roots was slightly less than the non-treated roots.

3. Leaf, stem and root tissue did not hydrolyze chloroacetyl-L-tyrosine and L-cystinyldiglycyldiglycine.

4. When the nitrogen components of the treated and non-treated stem tissue were partially fractionated with 80 percent ethanol, the major portion of the increased nitrogen content of the treated stems was found in the insoluble (protein) fraction.

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