EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON PROTEOLYTIC ACTIVITY OF RED KIDNEY BEAN PLANTS

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Recent work has shown that treatment of plants with 2,4-dichlorophenoxyacetic acid (2,4-D) results in a reduction of carbohydrate and an accumulation of nitrogen (2, 10, 13, 14, 15). SELL et al. (14) and WELLER et al. (16) have observed differences in carbohydrate, nitrogen, and amino acid content that occur primarily in the proliferated stem tissue of the red kidney bean plants. LUECKE et al. (8) have also noticed changes in the content of several vitamins after treatment with 2,4-D. FELBER (3) and NEELY et al. (11, 12) have shown lower activity for peroxidase, alpha and beta amylase, and phosphorylase, and higher activity for pectin methoxylase in the treated red kidney bean plants. Suggestions have been made that the decrease in carbohydrate and the increase in amino acids and nitrogen content in the stem tissue were due to the conversion in part of the carbohydrates into proteins (14). If this postulate is valid then the proteolytic activity might be increased in the treated plants. This communication is a report of the results of an investigation on the effect of 2,4-D on the proteolytic activity of the red kidney bean plants.

Samples of leaf, stem, and root tissue of the red kidney bean plant, Phaseolus vulgaris, were prepared according to the procedure previously described by NEELY et al. (12). The proteolytic activity, using hemoglobin as the substrate, was determined on a sample of one gram of the powdered plant material by using MILLER'S (9) revision of the Ayre-Anderson method. Hemoglobin was employed as the substrate since it is a readily available protein and is soluble in solutions at a pH utilized in the analysis. The temperature of the medium was 48°C and a 0.1 M acetate buffer of pH 4.8 was utilized. The amino nitrogen was determined in a Van Slyke amino nitrogen apparatus. For the other proteolytic activity determinations, 0.750 gm. of the appropriate powdered plant material was suspended in 10 ml. of an aqueous 20% glycerol solution for four hours. At 30 minute intervals the reaction tubes were inverted several times to resuspend any settled material at the bottom of the tubes. After four hours the suspension was centrifuged, filtered, and the clear filtrate used as the enzyme preparation. Longer extraction periods did not increase enzymatic activity.

The synthetic substrates consisted of 1% solutions of glycylglycine, L-cystinylglycine, L-cystinylglycylglycine and chloroacetyl-L-tyrosine. The glycylglycine and chloroacetyltirosine were prepared according to

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FISCHER (4), the L-cystinyl diglycine by the method of LORING and DU VIGNEAUD (7), and the L-cystinyl diglycyl diglycine by the procedure of GREENSTEIN (5).

A modification of the method described by BLAGOWESTSCHENSKI and MELAMED (1) was employed for glycylglycine. For the other synthetic substrates, the peptides were dissolved in distilled water, the pH adjusted to 7 with dilute ammonium hydroxide and distilled water added to give a 1% solution. The hydrolysis was carried out and the amino nitrogen of the substrates, with the exception of L-cystinyl diglycyl diglycine, was determined in a Van Slyke apparatus. The extent of hydrolysis of the latter compound was determined by filtering the reaction mixture and determining the nitrogen in the residue by a semi-micro Kjeldahl procedure.

The results of the various methods with synthetic substrates, hemoglobin, and with different portions of the plant tissue are given in tables I and II. The data in table I were obtained after a study of the effect of temperature and pH indicated maximum hydrolysis during a reaction period of five hours occurred at 48° C and pH 4.8. To be certain that the increase in amino nitrogen was a result of enzymatic hydrolysis of the hemoglobin, separate determinations were made on the substrate and on the plant material. Neither of these determinations indicated a significant increase in amino nitrogen, thus the increase in the amino nitrogen was due to the enzymatic hydrolysis of the hemoglobin substrate. The activity in the stem tissue of the 2,4-D treated plants was almost a third more than in the non-treated stem tissue. A reverse trend was noted in the leaf tissue. The leaves of the non-treated plants showed almost double the proteolytic activity of the treated leaves. The root tissue of the treated plant showed a very slight decrease in activity.

In table II are shown the results in which the proteolytic activity was determined on synthetic peptide substrates. With glycylglycine, the treated stems showed more than double the activity of the control stems; the treated leaves less than half the activity of the non-treated leaves; and the treated roots slightly less activity than the non-treated root tissue. The same trends were observed with cystinyl diglycine as the substrate. The

### TABLE I

**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.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Non-treated replicates</th>
<th>Treated replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stems</td>
<td>1.73</td>
<td>1.89</td>
</tr>
<tr>
<td>Leaves</td>
<td>1.62</td>
<td>1.93</td>
</tr>
<tr>
<td>Roots</td>
<td>1.02</td>
<td>1.05</td>
</tr>
</tbody>
</table>

*Expressed as the increase in milligrams of amino nitrogen per plant for five hours of reaction at 48° C.
activity of the treated stem tissue was double the activity of the control
tissue; the activity of the treated leaves was less than half that of the non-
treated leaves; and the activity of the treated roots was slightly less than
the activity of the non-treated roots. Neither cystinylglycylglycine nor
chloroacetyl-L-tyrosine substrates showed evidence of enzymatic hydrolysis
when stem, leaf, or root enzyme preparations of red kidney bean plants were
employed.

Glycylglycine and cystinylglycine are substrates which are utilized for the
detection of dipeptidase activity. Chloroacetyltyrosine is a specific
substrate for carboxypeptidase, and cystinylglycylglycine for amino-
polypeptidase activity (5). Evidently no enzymes are present in the immu-
ture bean plants which give carboxypeptidase or aminopolypeptidase activ-
ity. Dipeptidase activity was observed in both treated and non-treated
tissue. It is of interest that the proteolytic activity determined with hemo-
globin, glycylglycine and cystinylglycine substrates exhibited the same
trends and that the differences between the treated and non-treated tissue
were approximately of the same order of magnitude.

The lower proteolytic activity in the treated leaf tissue was not surpris-
ing since there is a slightly lower amino acid content in the treated leaves
(16). There was also evidence of an inhibition of the growth of the leaf
tissue in the treated plants as compared to the controls. The results of the
determinations in the stem tissue indicate that there is either an accumula-
tion of protein or free amino acids and other products of protein metabolism
in the treated plants. To clarify this problem, the nitrogen components of
the stem tissue were partially fractionated, and the nitrogen in each frac-
tion was determined. The results are summarized in table III. The pro-

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Substrate</th>
<th>Non-treated replicates</th>
<th>Treated replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem</td>
<td>Glycylglycine</td>
<td>0.50</td>
<td>0.45</td>
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<tr>
<td></td>
<td>L-Cystinylglycine</td>
<td>1.62</td>
<td>1.18</td>
</tr>
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<td></td>
<td>L-Cystinylglyclyclyglycine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Chloroacetyl-L-tyrosine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Leaf</td>
<td>Glycylglycine</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>L-Cystinylglycine</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>L-Cystinylglyclyclyglycine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Chloroacetyl-L-tyrosine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Root</td>
<td>Glycylglycine</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>L-Cystinylglycine</td>
<td>0.20</td>
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</tr>
<tr>
<td></td>
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<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Chloroacetyl-L-tyrosine</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE NITROGEN CONTENT OF THE STEMS OF RED KIDNEY BEAN PLANTS (DRY WEIGHT BASIS).*

<table>
<thead>
<tr>
<th></th>
<th>Non-treated replicates</th>
<th>Treated replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>3.02</td>
<td>4.61</td>
</tr>
<tr>
<td>Soluble nitrogen (80% ethanol)</td>
<td>1.44</td>
<td>1.82</td>
</tr>
<tr>
<td>Insoluble nitrogen (80% ethanol)</td>
<td>1.58</td>
<td>2.79</td>
</tr>
</tbody>
</table>

*Nitrogen determined by Kjeldahl method.

Protein nitrogen was taken to be that portion of the total nitrogen which was insoluble in 80% ethanol while the soluble fraction probably consisted of a large extent of amino acids and other nitrogenous products. The results indicate that the major portion of the increased nitrogen content of the treated stems is in the protein fraction although there is an indication that a small portion of the increase in nitrogen is due to free amino acids or other protein breakdown products.

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

These results confirm the postulate indicated by Sell et al. (14) that one factor associated with the effect of 2,4-D is the utilization of carbohydrate in the synthesis of protein. This increase in protein (N × 6.25) may be responsible for the proliferation of the stems in 2,4-D treated plants. Whether the protein in the treated stems is the same as in the non-treated stems is a problem requiring further investigation. Previous reports have indicated that the amino acids glycine and cystine give high nitrogen values with the Van Slyke method (6). The error in the amino nitrogen content reported in this paper because of cystine, and the major portion of the error due to glycine, is eliminated due to the method of determination of proteolytic activity. The error remaining due to glycine is too small to affect the results appreciably.

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LITERATURE CITED


