Promotion of Crown-Gall Tumor Growth by Lysopine, Octopine, Nopaline, and Carnosine

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ABSTRACT

The growth of crown-gall tumors on primary bean leaves (*Phaseolus vulgaris* L. cv. "Pinto") was promoted by the addition of D-lysopine, D-octopine, L-carnosine, or nopaline. Assayed on tumors induced by *Agrobacterium tumefaciens* strain B6, the relative activity was octopine = carnosine > lysopine > nopaline; assayed on tumors induced by *A. tumefaciens* strain T-37, which induces tumors which form nopaline, the relative activity was nopaline = octopine = carnosine > lysopine. From one to three applications of carnosine or octopine gave equal additive increments in tumor growth, showing that a continual supply of these substances is required to maintain an increased rate of growth. At concentrations above 0.1 mM, pairs of these growth-promoting substances were less active than when applied singly. Inhibition of octopine-induced growth was obtained by applying 0.01 mM carnosine with 1 mM octopine and partial inhibition was obtained when carnosine was added 10 hr after octopine. Equimolar mixtures of lysopine, octopine, and carnosine, however, were at least as active in promoting tumor growth as any of the compounds added singly at equivalent concentrations. The activity of 0.1 to 0.5 mM lysopine, octopine, and carnosine was inhibited, respectively, by 1 mM L-lysine, L-arginine, and L-histidine and this inhibition was limited in each case to the basic amino acid corresponding to that of the growth factor. Arginine fully inhibited octopine-induced tumor growth when applied as much as 6 hr after octopine, indicating that this inhibition was not due to prevention of octopine uptake. Although four separate substances were found which promoted tumor growth, the molecular specificity required for activity of each compound was high. Evidence is presented which suggests that a tumor growth-promoting substance extracted from tumorous leaves is a carnosine-like derivative of L-histidine.

Several unique compounds have been isolated from crown-gall tumors which have not been detected elsewhere in the plant kingdom. Thus, octopine [N^2-(O-1-carboxyethyl)-L-arginine] and octopinic acid [N^2-(O-1-carboxyethyl)-L-ornithine] were isolated from these tumors by Ménagé and Morel (14, 15). A similar compound, lysopine [N^2-(O-1-carboxyethyl)-L-

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1 This investigation was supported by United States Public Health Service Research Grant CA-05387 from the National Cancer Institute.

*Abbreviations: TGF: tumor growth factor; S.U.: microscope ocular scale units.*
Table I. Relative Growth-promoting Activity of Octopine, Lysopine, Carnosine, and Nopaline on Strain B6-induced Tumors

<table>
<thead>
<tr>
<th>Additions to B6-inoculated Leaves</th>
<th>Conc of Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 μM</td>
</tr>
<tr>
<td>Increase in tumor diameter (S.U.)&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Lysopine</td>
<td>1.1</td>
</tr>
<tr>
<td>Octopine</td>
<td>1.0</td>
</tr>
<tr>
<td>Carnosine</td>
<td>1.2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Lysopine</td>
<td>0.8</td>
</tr>
<tr>
<td>Octopine</td>
<td>0.8</td>
</tr>
<tr>
<td>Carnosine</td>
<td>0.8</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
</tr>
<tr>
<td>Octopine</td>
<td>0.7</td>
</tr>
<tr>
<td>Nopaline</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Control tumor diameter: experiment 1 = 5.0; experiment 2 = 5.1; experiment 3 = 5.6. SE: experiment 1, range = 0.17-0.34, mean = 0.25; experiment 2, range = 0.18-0.22, mean = 0.20; experiment 3, range = 0.14-0.18, mean = 0.16.

Table II. Effect of Lysopine, Octopine, and Nopaline on the Growth of Strain T-37-induced Tumors

<table>
<thead>
<tr>
<th>Additions to T-37-inoculated Leaves</th>
<th>Conc of Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 μM</td>
</tr>
<tr>
<td>Increase in tumor diameter (S.U.)&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Lysopine</td>
<td>1.0</td>
</tr>
<tr>
<td>Octopine</td>
<td>0.8</td>
</tr>
<tr>
<td>Nopaline</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<sup>1</sup> Control tumor diameter = 6.0. SE: range = 0.17-0.27, mean = 0.22.

Table III. Effect of Repeated Applications of Octopine or Carnosine on Tumor Growth

<table>
<thead>
<tr>
<th>Treatment (0.5 mM)</th>
<th>Total No. of Applications</th>
<th>Mean Tumor Diameter (S.U.)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Increase in Tumor Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>Octopine, day 3</td>
<td>1</td>
<td>6.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Octopine, day 3, 4</td>
<td>2</td>
<td>6.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Octopine, day 3, 4, 5</td>
<td>3</td>
<td>6.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Carnosine, day 3</td>
<td>1</td>
<td>6.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Carnosine, day 3, 4</td>
<td>2</td>
<td>6.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Carnosine, day 3, 4, 5</td>
<td>3</td>
<td>6.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

<sup>1</sup> SE: range = 0.13-0.21, mean = 0.16.

received the same amount of growth factor (e.g., Table VI) suggests the reproducibility may be greater than this value indicates. Most of the results are presented as the difference in tumor diameter between experimental samples and the controls. An increase in tumor diameter from 5.5 to 5.9 S.U. represents a 24% increase in tumor volume and from 5.5 to 6.5 S.U., a 64% increase in volume.

Tumor Growth Factor Preparations. These were prepared by methanol extraction of tumorous leaves as described by El Khalifa and Lippincott (5) and further purified by removing ethyl acetate soluble materials.

Chemicals. D-Octopine, d-lysophine, and l-carnosine were obtained from Sigma Chemical Co., St. Louis, Missouri. Lysopine was obtained from Dr. R. Manasse of the Boyce Thompson Institute, Yonkers, New York and nopaline from Dr. G. Morel, Centre National de la Recherche Agronomique, Versailles, France. Other amino acid derivatives were obtained from Mann Biochemicals, New York and Cyclo Chemical Co., Los Angeles, California.

RESULTS

Because the purification characteristics of a tumor growth-promoting substance from tumorous bean leaves suggested that it might contain histidine, 14 different histidine derivatives were tested for effect on tumor growth. At 1 mM, only carnosine (β-alanyl-L-histidine) showed significant activity. Carnosine, as shown in Table I, was as effective in promoting the growth of bean leaf tumors as octopine, and both were more effective than lysopine. Previous studies showed that 3-fold higher concentrations of lysopine were necessary to obtain growth effects equal to that obtained with octopine (13). Because of the limited supply of natural nopaline, it was tested only once with strain B6 tumors. On the basis of this test, however, it appears to be about 100-fold less effective than octopine in promoting the growth of these tumors. Nopaline applied to tumors initiated by strain T-37, however, was fully as effective as octopine in promoting tumor growth (Table II). Both the strain B6 and T-37 results have been confirmed with a synthetic mixture of DL-nopaline.

Successive daily applications of TGF were previously found necessary to maintain the high rate of growth of the treated tumors (5). Table III shows that the growth response to octopine and carnosine behaved similarly. The increase in tumor volume based on a spherical model (11, 13) resulting from these additions is shown in Figure 1. Essentially a constant increment in tumor volume was obtained from each application of carnosine or octopine despite the fact that the three applications were at different times relative to the time of measurement and the second and third applications were made to tumors of increasing size. The curve for the carnosine addi-
tions is linear and extrapolates to the control tumor volume with no growth factor additions. The octopine results are not significantly different, though it is possible that the somewhat lower activity obtained after three additions was due to partial breakdown of octopine, as a single solution was used throughout the experiment.

When initial combinations of octopine plus lysopine failed to promote tumor growth as much as either compound applied singly, more systematic experiments were designed. As shown in Table IV, combinations of octopine plus lysopine, or of lysopine plus carnosine, each at a concentration of 0.1 mM or lower were somewhat more effective than when applied singly. Less growth promotion, however, was observed with a 1 mM concentration of one growth factor plus a second at 0.1 mM. All possible combinations of carnosine, octopine, and lysopine at these concentrations gave less growth than when these compounds were applied separately at 1 mM.

Titration of the inhibitory effect of carnosine applied with octopine is shown in Figure 2. Significant inhibition of the growth-promoting effect of 1 mM octopine was obtained with 10 μM carnosine and even greater inhibition was observed as the concentration of carnosine was increased. Carnosine applied singly as elsewhere, however, promoted tumor growth.

Figure 3 shows that application of carnosine as much as 10 hr after octopine still gave partial inhibition of octopine-induced growth. By 24 hr, carnosine was no longer inhibitory, although this result might be the sum of an inhibition effect plus direct growth promotion by carnosine. The data are consistent with results (ref. 5; Table III) which indicate that most of the growth increase occurs during the first day after application of these substances.

The results in Table V show another unique aspect of the effect of these growth factors when added in combination. Whereas each promoted tumor growth when applied individually and pairs of these substances inhibited tumor growth, the combined application of all three promoted tumor growth as much or more than any individual compound.

Although neither lysine, arginine, nor histidine, among other common amino acids, was found to promote tumor growth in this bioassay (11, 13), it was of interest to see if the amino acids corresponding to those which occur in octopine, lysopine, and carnosine would affect the growth-promoting properties of the latter. Table VI shows that arginine applied with octopine inhibited the growth-promoting effect of the latter substance, while histidine was without effect and lysine was only partially inhibitory. In other experiments, lysine had no effect on the growth-promoting activity of octopine. The activity of lysopine, however, could be completely inhibited by lysine, whereas arginine and histidine were without effect. Similarly, only histidine of these three basic amino acids inhibited the growth obtained with carnosine. However, neither pyruvate, L-lactate, D-lactate, L-alanine, D-alanine, nor β-al-

**Table IV. Antagonistic Effects of Combinations of Octopine, Lysopine, and Carnosine on Tumor Growth**

<table>
<thead>
<tr>
<th>Conc of Compound Varied</th>
<th>Octopine Varied</th>
<th>Lysopine Varied</th>
<th>Carnosine Varied</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lyso (0.1 mM)</td>
<td>Octo (0.1 mM)</td>
<td>Carn (0.1 mM)</td>
</tr>
<tr>
<td></td>
<td>— +</td>
<td>— +</td>
<td>— +</td>
</tr>
<tr>
<td>Mean tumor diameter (S.U.)</td>
<td>5.72 ± 0.05</td>
<td>5.94 ± 0.03</td>
<td>5.6 ± 0.05</td>
</tr>
<tr>
<td>50 μM</td>
<td>6.0 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>6.2 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>0.3 mM</td>
<td>6.2 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>1 mM</td>
<td>6.4 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>6.0 ± 0.2</td>
</tr>
</tbody>
</table>

1 SE (left to right): range = 0.17–0.27, mean = 0.20; range = 0.18–0.28, mean = 0.23; range = 0.17–0.21, mean = 0.18.

*30 μM octopine.*

Fig. 2. Inhibition of octopine-induced tumor growth by various concentrations of carnosine. Except the control, all tumorous leaf samples were treated with 1 mM octopine or this concentration of octopine plus the indicated concentrations of carnosine. SE: range = 0.16–0.25, mean = 0.20.

Fig. 3. Time course of the sensitivity of octopine-induced tumor growth to inhibition by carnosine. Except the control, all tumorous leaf samples received 1 mM octopine at time 0 or this treatment plus 0.1 mM carnosine applied at the indicated times. Tumor diameter measurements were at 72 hr. SE: range = 0.12–0.20, mean = 0.16.
Table V. Growth Promotion by a Mixture of Lysopine, Octopine, and Carnosine Relative to the Growth Obtained with These Compounds Applied Singly or in Pairs

<table>
<thead>
<tr>
<th>Additions to Infected Leaves at Day 3</th>
<th>Change in Tumor Diameter (S.U.) at Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>D-Lysopine</td>
<td>+0.3</td>
</tr>
<tr>
<td>D-Octopine</td>
<td>+0.4</td>
</tr>
<tr>
<td>L-Carnosine</td>
<td>+0.5</td>
</tr>
<tr>
<td>Lysopine + octopine</td>
<td>+0.1</td>
</tr>
<tr>
<td>Lysopine + carnosine</td>
<td>−0.1</td>
</tr>
<tr>
<td>Octopine + carnosine</td>
<td>−0.1</td>
</tr>
<tr>
<td>Lysopine + octopine + carnosine</td>
<td>+1.1</td>
</tr>
</tbody>
</table>

1 All substances at 0.5 mM.
2 Diameter of control tumors (S.U.): experiment 1 = 6.2; experiment 2 = 5.8; experiment 3 = 5.7. se: experiment 1, range = 0.14-0.29, mean = 0.17; experiment 2, range = 0.12-0.18, mean = 0.15; experiment 3, range = 0.16-0.20, mean = 0.18.

Table VI. Influence of Basic Amino Acids on the Promotion of Tumor Growth by Octopine and Lysopine

<table>
<thead>
<tr>
<th>Additions to Infected Leaves at Day 3</th>
<th>Mean Tumor Diameter (S.U.) at Day 6</th>
<th>Change in Tumor Diameter</th>
<th>Increase in Diameter %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octopine</td>
<td>6.5</td>
<td>+0.8</td>
<td>100</td>
</tr>
<tr>
<td>Octopine + L-arginine</td>
<td>5.5</td>
<td>−0.2</td>
<td>0</td>
</tr>
<tr>
<td>Octopine + L-lysine</td>
<td>6.2</td>
<td>+0.5</td>
<td>62</td>
</tr>
<tr>
<td>Octopine + L-histidine</td>
<td>6.5</td>
<td>+0.8</td>
<td>100</td>
</tr>
<tr>
<td>Lysopine</td>
<td>6.5</td>
<td>+0.8</td>
<td>100</td>
</tr>
<tr>
<td>Lysopine + L-lysine</td>
<td>5.7</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Lysopine + L-arginine</td>
<td>6.5</td>
<td>+0.8</td>
<td>100</td>
</tr>
<tr>
<td>Lysopine + L-histidine</td>
<td>6.5</td>
<td>+0.8</td>
<td>100</td>
</tr>
</tbody>
</table>

1 Concentrations: amino acids, 1 mM; octopine, 0.2 mM; lysopine, 0.5 mM.
2 se: range = 0.11-0.18, mean = 0.15.

Table VII. Reversal of the Antagonistic Interaction between Octopine and Lysopine by Lysine

<table>
<thead>
<tr>
<th>Additions to Infected Leaves at Day 3</th>
<th>Mean Tumor Diameter (S.U.) at Day 6</th>
<th>Increase in Tumor Diameter (S.U.)</th>
<th>Increase in Diameter %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Octopine</td>
<td>5.6</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>Octopine + lysine</td>
<td>5.7</td>
<td>0.7</td>
<td>117</td>
</tr>
<tr>
<td>Lysopine</td>
<td>6.0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Lysopine + lysine</td>
<td>5.3</td>
<td>0.3</td>
<td>30</td>
</tr>
<tr>
<td>Octopine + lysopine + lysine</td>
<td>5.3</td>
<td>0.3</td>
<td>30; 50</td>
</tr>
</tbody>
</table>

1 Concentrations: octopine, 0.1 mM; lysopine, 0.3 mM; lysine, 1 mM.
2 se: range = 0.14-0.20; mean = 0.18.
3 Values relative to lysopine standard; octopine standard.

Fig. 4. Effect of arginine addition at various times on the increase in tumor diameter obtained with octopine. Except the control, all tumorous leaf samples were treated with 0.3 mM octopine at time 0 or this treatment plus 1 mM arginine applied at the times indicated. Tumor diameter measurements were at 72 hr. se: range = 0.12-0.21, mean = 0.15.
nine plus pyruvate, lysine plus pyruvate, or histidine plus β-alanine failed to promote tumor growth. Octopinic acid, a one carbon lower homolog of lysopine, was inactive (13) as were the glycyrl-, alanyl- and γ-aminobutyryl- derivatives of lysine. The alanyl-derivative of arginine was also inactive. Because of the greater number of variants available the requirements for carnosine activity could be better defined. Thus, the L configuration of histidine was necessary for activity and methyl substitutions at either the 1 or 3 positions of the histidine moiety of carnosine (i.e., anserine and ophidine) rendered the molecule inactive. Substituting γ-aminobutyric acid for β-alanine, as in homocarnosine, led to loss of activity and several other compounds with various substitutions at the N2 position of L-histidine were also inactive. Both the L-histidine and β-alanine moieties of carnosine, therefore, appear to be specific requirements for growth promotion by this compound.

### DISCUSSION

Derivatives of the three principal basic amino acids found in organisms have now been shown to promote the growth of crown-gall tumors in vivo. In the case of lysopine and octopine, activity could be based on conversion to pyruvate plus the corresponding basic amino acid with the formation of NADH (7, 8, 17). The β-alanyl-portion of carnosine, however, is added and removed by separate mechanisms, both differing from those involved in the synthesis and degradation of octopine and lysopine (4). The growth-promoting activity of carnosine thus suggests a different explanation may be necessary to account for the action of these substances. The high concentrations of arginine, lysine, and histidine in histones and the suggested role of histones in regulating gene activity (1) presents at least a unifying target which may be relevant to the way in which these compounds affect tumor growth.

The presence of nopaline in these tumors appears to be an alternate of the octopine-lysopine type of tumor as suggested by the results of Petit et al. (16). They found that of tumors induced by 43 different strains of A. tumefaciens, 20 formed octopine plus lysopine, 19 formed nopaline, two formed all three, and in two cases none of these substances was detected in the tumors. In addition, when certain of these bacteria were grown in nopaline or octopine containing media, those which induced nopaline-forming tumors degraded nopaline but not octopine, whereas those which induced tumors containing octopine plus lysopine could degrade octopine but not nopaline. It is of interest, therefore, that nopaline-producing tumors induced by strain T-37 responded to lysopine and octopine as well as to nopaline. Tumors initiated by strain B6 which form lysopine and octopine, however, showed relatively little growth in response to nopaline, indicating that a limitation exists in the ability of octopine-producing tumors to respond to this substance. Because only those tumors which produce nopaline (T-37 induced tumors) show a strong growth response to this compound, whereas nopaline-producing tumors and octopine-producing tumors both respond to octopine, the action of nopaline is most probably on tumor tissue rather than on the surrounding host tissues which would be a constant background of both tumor types.

The molecular weight of the TGF extracted from tumorous bean leaves as estimated by molecular sieve chromatography would place it in the same molecular weight range as carnosine (11). When separated by ion exchange chromatography on sulfoethyl Sephadex, however, TGF elutes before histidine, whereas carnosine elutes after histidine. The high growth-promoting activity of carnosine, the degree of molecular specificity essential for its activity, plus the demonstration that the activity of both TGF and carnosine is inhibited by histidine indicate that TGF must be a closely related substance. Also, acid hydrolysis of purified TGF preparations yields a ninhydrin positive, imidazole positive compound with an Rs on thin layer chromatography identical with that of histidine. TGF, therefore, can be tentatively identified as a carnosine-like compound.

Carnosine is a common constituent of vertebrate skeletal muscle where it exists in concentrations of the order of 0.1% of the wet weight (4). A single report, though lacking supporting data, has stated that carnosine is present in certain algae (9). While many proposals have been made concerning the physiological function of carnosine in muscle, its presence is still enigmatic. It is probably relevant to the activity of these

<table>
<thead>
<tr>
<th>Source of Extracts</th>
<th>Additions with Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Strain B6 inoculated leaves</td>
<td>0.9</td>
</tr>
<tr>
<td>Strain 15955 inoculated leaves</td>
<td>0.4</td>
</tr>
</tbody>
</table>

1 Control tumor diameter, 5.5 S.U. se: range = 0.11-0.18, mean = 0.15.

<table>
<thead>
<tr>
<th>Active Compounds</th>
<th>Compounds with no Activity at 1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Lysopine [N2-(D-1-Carboxyethyl)-L-lysine]</td>
<td>L-Lysine</td>
</tr>
<tr>
<td></td>
<td>D-Alanine</td>
</tr>
<tr>
<td></td>
<td>L-Alanine</td>
</tr>
<tr>
<td></td>
<td>Hydroxylsine</td>
</tr>
<tr>
<td></td>
<td>Octopinic acid [N2-(D-1-carboxyethyl)-L-ornithine]</td>
</tr>
<tr>
<td></td>
<td>Glycyl-L-lysine</td>
</tr>
<tr>
<td></td>
<td>L-Alanyl-L-lysine</td>
</tr>
<tr>
<td></td>
<td>L-α-Aminobutyryl-L-lysine</td>
</tr>
<tr>
<td>D-Octopine [N2-(D-1-Carboxyethyl)-L-arginine]</td>
<td>L-Arginine</td>
</tr>
<tr>
<td>Nopaline [N2-(1,2-Dicarboxypropyl)-L-arginine]</td>
<td>D-Alanine</td>
</tr>
<tr>
<td></td>
<td>L-Alanine</td>
</tr>
<tr>
<td></td>
<td>Homoarginine</td>
</tr>
<tr>
<td></td>
<td>L-Alanyl-L-arginine</td>
</tr>
<tr>
<td>L-Carnosine (β-Alanyl-L-histidine)</td>
<td>L-Histidine</td>
</tr>
<tr>
<td></td>
<td>β-Alanine</td>
</tr>
<tr>
<td></td>
<td>β-Alanyl-D-histidine</td>
</tr>
<tr>
<td></td>
<td>Anserine (β-Alanyl-L-methyl-L-histidine)</td>
</tr>
<tr>
<td></td>
<td>Ophidine (β-Alanyl-3-methyl-L-histidine)</td>
</tr>
<tr>
<td></td>
<td>Homocarnosine (γ-aminobutyryl-L-histidine)</td>
</tr>
<tr>
<td></td>
<td>L-Alanyl-D-histidine</td>
</tr>
<tr>
<td></td>
<td>N-Acetyl-L-histidine</td>
</tr>
<tr>
<td></td>
<td>N-Formyl-L-histidine</td>
</tr>
<tr>
<td></td>
<td>Glycyl-L-histidine</td>
</tr>
<tr>
<td></td>
<td>DL-Histidyl-DL-histidine</td>
</tr>
</tbody>
</table>
substances that octopine is also a muscle component where it occurs in the animal kingdom (17).

Our previous studies of different strains of Agrobacterium showed that TGF was induced by most tumorigenic strains, the three exceptions being strains of very low infectivity (12). Both nopaline and octopine type strains of A. tumefaciens induced TGF formation in infected leaves. Thus, the occurrence of TGF in these tumors is not limited to either the octopine type tumor as is lysopine (16) or to nopaline-forming tumors, but is produced by both.

The greater tumor growth obtained by daily applications of these substances has some interesting implications relative to their mode of action. Because repeated applications are necessary to maintain tumor growth, these substances are apparently either metabolized or transported from the leaf so that their supply must be constantly renewed to maintain a high rate of growth. An essentially constant increment in tumor volume was obtained per application of growth factor with from one to three separate applications. Single applications of these growth substances of increasing concentration do not show a 1:1 relation between concentration and tumor volume (11, 13), however, but a linear-logarithmic type relation. The resulting system, therefore, appears to be saturable and to remain essentially fixed in amount over the 3 day period of these experiments, despite the increasing size of the tumors. This latter relation raises the interesting possibility that the growth-promoting activity of these substances is manifest not through direct action on the tumor cells but on the surrounding host cells by enhancing their ability to contribute to the growth of the tumor cells. In the case of nopaline, however, this possibility is apparently ruled out.

The inhibition of tumor growth by pairs of these growth-promoting substances was unexpected, particularly in view of their apparently different sites of action. Titration of this effect suggests an amplification mechanism must exist to account for the inhibition observed with low concentrations of one growth factor applied with 10-100-fold higher and non-growth-saturating concentrations of a second active compound. This type of interaction is inconsistent with a single site of action which could accommodate all these compounds. The reversal of the inhibition between pairs of these substances by adding a third growth factor is equally mystifying and suggests there is some critical metabolic system in the cell which is sensitive to each of these compounds so as to enhance or inhibit growth. The nature of this system is such that a large imbalance between one of these substances and the other two, or similar increases in the amount of all three, will increase tumor growth. Thus, depending upon the number and relative amount of these compounds in the leaf, tumor growth may be stimulated, inhibited or unchanged.

Since only the basic amino acid which respectively corresponds to that found to octopine, lysopine, and nopaline was inhibitory to each, at some point in the bioassay between application of these substances and increased tumor growth, it appears that three separate sites exist which can each distinguish one of these compounds or its corresponding basic amino acid. Addition of the amino acid to the leaves several hours after they were treated with these growth factors was also inhibitory, and this ability decreased with increasing time between addition of growth factor and amino acid. The rate at which the ability to obtain this inhibition decreased in the case of octopine plus arginine was slower than that of the inhibition of octopine-induced growth by carnosine. As the latter results appear to stem from secondary interactions after the compounds have gained entry to responding cells and most of the uptake of these substances probably occurs during the first 10 to 15 min after application while the leaves are still wet and the substances in solution, it appears therefore that the inhibiting effects of these amino acids are not due to prevention of uptake of the growth factor. Because high concentrations of these amino acids were necessary for significant inhibition, a reasonable explanation of these results is that the amino acid and the corresponding growth factor may be competing for the same internal site which is essential for activity. As substances which might be expected to compete with the other portion of these growth factors such as pyruvate, D- or L-alanine, D- or L-lactate, or β-alanine were not inhibitory, the specificity associated with the action of these substances would seem to reside in the basic amino acid portion of these compounds. The absence of growth-promoting activity of many very similar derivatives of arginine, lysine, or histidine, however, indicates that the nature of the substitution on the α-amino group of these compounds is critical to their activity. A possible explanation to account for this discrepancy is that compounds such as alanine or lactate are metabolized or transported from the leaf before they can exert a significant inhibitory effect.

The 2- to 3-fold variation observed in the amount of growth promotion obtained with identical concentrations of lysopine, carnosine, or octopine in different experiments may be explained by the inhibitors obtained with amino acids and with pairs of these growth factors. Both the endogenous levels of lysine, histidine, and arginine in the leaf and the relative as well as absolute amounts of these three growth factors produced by the tumors used for the bioassay could alter the degree of the growth response to exogenous applications. Other factors as well, however, probably contribute to this variability.

The number of growth-promoting compounds now associated with crown-gall tumors (2, 11, 13) and the variety of qualifying interactions we have shown between several of these, indicate that tumor growth is supported in a complex way via the presence or absence, and relative abundance, of each of these substances as well as of normal cellular constituents which can modify their activity. Both the bacterium, the host and the physical environment of the host must certainly contribute to each of these variables to provide a spectrum of tumors with different growth characteristics, possibly accounting for the various degrees of teratoma and normal organ formation arising from many Agrobacterium infections.

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LITERATURE CITED


