reduction of ρ to no less than 0.166, or 60 percent of its initial value, and less than 5 percent of the lignin becomes soluble (Fig. 3b). Stage II, the dissolution stage, requires the cleavage of B groups, and is achieved by acid hydrolysis, with or without subsequent sulfonation.

Contrary to our previous work (*3*), our new model appears to obviate the need for considering the breaking of lignin-carbohydrate bonds as a necessary step in solubilizing lignin. Furthermore, like the Szabo and Goring model (*6*), it indicates how the increasing molecular weight of soluble lignin during delignification is a natural consequence of the statistical course of depolymerization and does not require invoking a hypothesis of "condensation" of small molecules.

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8. In view of its yield, Brauns' native lignin comprises mainly primary chains which are not cross-linked (see Fig. 3a). The molecular weight distribution of this lignin was estimated from data of C. L. Hess [*Tappi* **35**, 312 (1952)], as $M_w/M_n = 1.04$. Applying this distribution factor to some of the calculations described in this report made no significant difference in the results.
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12. 1-Ethoxy-1-(3′-methoxy-4′-hydroxyphenyl)-2-(2″-methoxyphenoxy)-3-hydroxypropane.
13. 1-Ethoxy-1-(3′,4′-dimethoxyphenyl)-2-(2″-methoxyphenoxy)-3-hydroxypropane.
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Lysopine and Octopine Promote Crown-Gall Tumor Growth in vivo

Abstract. *Growth of tumors induced on primary leaves of bean plants by* Agrobacterium tumefaciens *was increased by the addition of lysopine and octopine. A detectable response was observed when as little as 1 microgram of these compounds was added per leaf, and the mean volume of the tumors was increased two- to threefold when greater amounts were applied. The specificity of the response and the unique association of these compounds with the tumors suggest that endogenous lysopine and octopine contribute to the growth characteristics of these tumors.*

Crown-gall tumors produce octopine [*N*-α-(1-carboxyethyl)-L-arginine] (*1*) and lysopine [*N*-α-(1-carboxyethyl)-L-lysine] (*2*). Octopine has not been detected in normal plant tissues (*3*) and, while lysopine is a normal constituent of plants, its concentration in these tumors is about 24 times greater than in nontumorous tissues (*4*). No physiological function for these compounds related to the tumorous characteristics of these tissues, however, has been reported. Our results show that small amounts of octopine and lysopine can promote the growth of crown-gall tumors.

The growth of individual crown-gall tumors induced by *Agrobacterium tumefaciens* (strain B6) on primary pinto bean leaves is less than maximum when fewer than 60 to 80 tumors are formed on each leaf (*5*). A bioassay for tumor growth based on this observation was devised and used to demonstrate the presence of tumor growth promotion by extracts of tumorous leaves and the absence of such activity in extracts of control leaves (*6*). A modification of this bioassay was used to show the promotion of tumor growth by lysopine and octopine. The primary leaves of 7-day-old pinto bean plants were inoculated with cells of strain B6 sufficient to initiate about ten tumors per leaf. Substances tested for promotion of tumor growth were spread over each leaf (0.1 ml per leaf) on the 3rd day after inoculating the bacteria, and at 6 days after infection the diameter of tumors was measured at ×30 magnification with an ocular micrometer in a dissecting microscope. Each sample was tested on 14 to 16 leaves, and a maximum of three tumors was selected and measured on each leaf following a prescribed pattern designed to assure a random choice of tumors in each sample of leaves. Coded solutions were used in some experiments to assure that these procedures were not prejudicial to the results. Only the tumors with a nearly circular perimeter were measured, since this shape was found to be maintained during the 3-day period of growth and the measurements of such tumors were more readily made and interpreted volumetrically. The results obtained with this bioassay were similar to those obtained when the daily growth of several specific tumors was followed in the previous bioassay (*6*). The validity of the modified assay is further attested to by the degree of specificity evident in the limited number of compounds that gave this growth response and the proportionality found between the concentration of octopine or lysopine and the resulting amount of tumor growth. A more complete description of the bioassay will be published elsewhere.

Table 1 presents results from a single tumor growth experiment in which lysopine and octopine (*7*) showed growth-promoting activity. A spherical model was estimated to closely approximate the volume occupied by the tumors because they were selected to be circular in a plane parallel to the leaf surface, and such tumors are nearly circular in cross sections perpendicular to the leaf surface. The mean volume of tumors at day 6, estimated from the

measurements of diameter, was more than double that of the controls on leaves treated with lysopine and nearly three times that of the controls on leaves treated with octopine. These differences in growth occurred in the 3-day period following the single application of about 20 μg of these compounds to each leaf. A lower homolog of lysopine, octopinic acid [*N*-α-(1-carboxyethyl)-L-ornithine], and lysine, which is a precursor of lysopine, did not promote tumor growth. Some 80 additional compounds, including arginine, pyruvate, γ-guanidinobutyric acid, citrulline, ornithine, the common amino acids, nucleic acid bases, several vitamins, and carbohydrates, failed to promote tumor growth in this bioassay. Auxin, cytokinin, and gibberellin also failed to promote the growth of these tumors (*6*, *8*). The activity of lysopine and octopine thus appears highly specific.

Titration curves (Fig. 1) suggest that octopine is at least two to three times more active than lysopine in promoting tumor growth. The endogenous levels of these two compounds in the tumorous leaves, however, may account for these differences rather than any inherent difference in their specific activity. Application of 1 μg of either compound (approximately 0.1 ml of $5 \times 10^{-5}M$ solutions) to an infected leaf can result in detectable changes in tumor growth. The in vivo amount of lysopine in crown-gall tumors of stems is reported to be about 50 μg per gram, wet weight (*4*). The weight of these bean leaves minus the petiole at the time of lysopine application varies from 0.2 to 0.3 g. Since only a small portion of the leaf is actually tumor tissue (1 percent at most), the endogenous level of lysopine in these infected leaves is probably less than 1 μg per leaf. Data on the amount of octopine in crown-gall tumors in vivo is not available, but tumor tissue cultures grown on a medium containing 0.3 percent arginine yielded 100 μg of

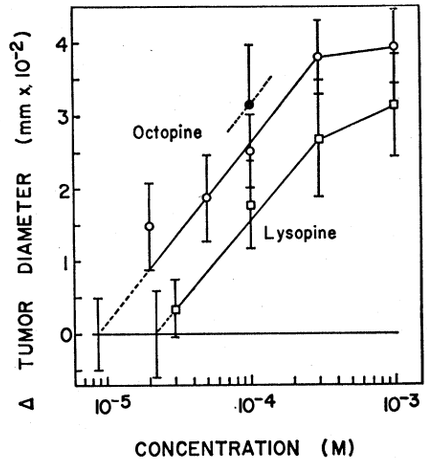

Fig. 1. Relation between the concentration of lysopine or octopine applied to tumor-bearing leaves and the promotion of tumor growth. Two experiments are shown; in one only octopine was tested (open circles); in the second, both lysopine (open squares) and octopine (closed circle) were tested. The diameters of the tumors on control leaves were 0.17 ± 0.005 mm (open circles) and 0.19 ± 0.006 mm (squares and closed circle).

octopine per gram of tissue (*1*). The in vivo level of octopine, thus, is probably no greater than that of lysopine and, since normal tissues apparently contain no octopine, the amount of this compound in the infected leaves is probably less than that of lysopine.

The approximate linear relation between mean tumor diameter and the logarithm of the concentration of lysopine or octopine applied is similar to the results obtained in this assay with partly purified extracts from tumorous leaves. The substance responsible for the tumor growth–promoting activity of these extracts is unknown, but the extraction and purification steps successfully applied in the isolation of this growth factor indicate that it is neither lysopine nor octopine.

In this bioassay for tumor growth–promoting activity, test substances are applied to the leaves after tumor initiation is complete (*9*) and no significant change in the number of tumors per leaf was associated with these promotions of tumor growth by lysopine or octopine. Similar results were obtained with the partly purified growth factor isolated from tumorous leaves (*6*), and additional evidence indicates that it is not active in the tumor initiation process (*10*). The absence of reports on the tumor initiation properties of lysopine and octopine suggests that such tests have been negative, and consequently, that these compounds are not tumorigenic. Further studies are in order, however, to fully eliminate the possibility that these substances may participate in the initiation process.

These results add to those of previous investigators which show that the amount of several normal growth factors is greatly increased in crown-gall tumors (*11*) and that their concentration presumably contributes to the growth characteristics of the tumors. Similar kinds of response have been found in studies of many animal tumors (*12*). Knowledge of those growth factors which are unique to tumors, their biosynthesis, and mode of action should lead to applications aimed at controlling tumor growth via the specific pathways which contribute to their abnormal growth properties.

Table 1. Size of tumors initiated by *Agrobacterium tumefaciens* on primary bean leaves treated with various compounds (0.1 ml of $10^{-3}M$ solutions) at day 3 and measured at day 6 after inoculation. The standard errors for the six measurements of diameter varied from $\pm$ 0.006 to $\pm$ 0.014 mm.

Treatments	Amount per leaf (μg)	Number of leaves	Number of tumors measured	Mean tumor diameter (mm)	Estimated mean tumor volume ($mm^3 \times 10^3$)
None		14	42	0.18	3.0
None		16	48	.19	3.1
Lysine	15	16	47	.19	3.1
Octopinic acid	20	16	47	.19	3.1
Lysopine	22	16	47	.24	7.2
Octopine	23	16	48	.26	9.2

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