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Chromatographic Analysis of a Cytokinin from Tissue Cultures of Crown-Gall[1]

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ABSTRACT

Extracts from tissue cultures of crown-gall from *Parthenocissus tricuspidata* (Sieb. and Zucc.) Planch. exhibited cell division activity in the soybean cytokinin assay. The chromatographic migration of one component, responsible for most of the activity, is similar to that of zeatin ribonucleoside. In addition, acid hydrolysis of the active region taken from chromatograms and of an eluate from a cation-exchange resin column resulted in the production of an active free-base-like derivative. Since the derivative and the parent compound lose activity after $KMnO_4$ treatment, they are believed to possess an unsaturated constituent essential for biological activity. The major active factor present in crown-gall from *Parthenocissus tricuspidata* is therefore distinct from the nicotinamide derivative reported to be present in *Vinca rosea* L. tumor cells.

During the past decade numerous reports pertaining to the isolation, chemical characterization, and biological properties of zeatin (8, 11, 13–16, 19) and other *N*-6-substituted aminopurines (5, 7, 12, 17, 18) provide strong evidence that this category of compounds is the predominant type which promotes cell division in numerous higher plant species. In addition, several of these cytokinins have been successfully isolated from transfer RNA fractions of various sources (1–4, 6, 9, 10). Conversely, two nicotinamide derivatives extracted from normal and tumor cells of *Vinca rosea* L. have only been partially characterized chemically but are presumed to be representative of a new class of compounds which promote cell division (20, 21). An additional report indicates that a compound(s) of this type is present in habituated tissue of *Nicotiana tabacum* L. and crown-gall tissue of *Opuntia* sp. (22). Furthermore, the active nicotinamide-type compound is considered to be the major active cell division compound in tumor tissues and is synthesized in the absence of exogenous cytokinin as part of the changes which accompany the transformation of normal cells to those which exhibit an autonomous growth habit (22). Miura and Miller (17), however, have presented strong evidence that the cell division compounds present in a cytokinin-independent strain of soybean cotyledon callus tissue are all *N*-6-substituted aminopurines.

Obviously, additional information concerning the exact chemical nature of the cytokinin(s) in crown-gall as well as in normal tissues is important to the successful elucidation of the mechanism of cell division in plants. Therefore, results pertaining to the partial chemical characterization, and chromatographic properties of the active material from crown-gall of *Parthenocissus tricuspidata* are reported herein.

MATERIALS AND METHODS

Crown-Gall Tissue Cultures. Crown-gall tissue from *Parthenocissus tricuspidata* (Sieb. and Zucc.) Planch. was cultured on a modified White's medium consisting of the following (mg/l): $Ca(NO_3)_2 \cdot 4H_2O$, 288; Na_2SO_4, 200; KCl, 65; KNO_3, 80; $NaH_2PO_4 \cdot H_2O$, 19; $MnSO_4 \cdot H_2O$, 5.8; $ZnSO_4 \cdot 7H_2O$, 2.7; H_3BO_3, 1.5; KI, 0.75; $MgSO_4 \cdot 7H_2O$, 736; nicotinic acid, 0.5; pyridoxine-HCl, 0.2; thiamine-HCl, 0.2; $Na_2EDTA \cdot 2H_2O$, 13.4; $FeSO_4 \cdot 7H_2O$, 9.9; sucrose, 20,000; agar, 7,500.

After preparation of the medium, it was adjusted with NaOH to pH 5.2. Agar was dissolved in the medium by heating, and the hot liquid was then poured as 100-ml aliquots into 250-ml Erlenmeyer flasks which were subsequently stoppered with nonabsorbent cotton and autoclaved for 15 minutes at 121 C and 15 psi. The crown-gall tissue (approximately 40 mg/piece) was planted aseptically on the solidified medium and cultured in the dark at room temperature for approximately 28 days.

Preparation of Extracts. Crown-gall tissue was macerated in a Waring Blendor for 1 min with sufficient 95% ethanol to produce a final 70% ethanolic extract. The extract was filtered through cheesecloth and the filtrate, henceforth referred to as an ethanolic extract, was initially analyzed for cytokinin activity or further extracted.

1-Butanol extracts were prepared from the ethanolic extract by evaporating the latter to dryness *in vacuo* at 47 C. The residue was redissolved in twice-distilled water and the resulting aqueous solution (pH 5.5) was washed three times with equal volumes of 1-butanol. The organic phase, henceforth referred to as the 1-butanol extract, was used in all chromatographic procedures unless otherwise specified.

Chromatographic Technique and Solvents. Extracts from the crown-gall tissue were applied as a streak on Whatman No. 1 filter paper. The chromatograms were developed in an ascending fashion for all experiments, and the migration of activity in crown-gall extracts was always compared with that of known cytokinins.

The solvents used in the development of chromatograms were as follows: water-saturated *sec*-butanol; 0.03 M borate, pH 8.4; ethylacetate-1-propanol-H_2O (4:1:2, v/v, upper layer); and 20% ethanol.

Tissue Culture Techniques and Assay. The soybean (*Glycine max* [L.] Merrill var. Acme) cotyledon callus bioassay devel-

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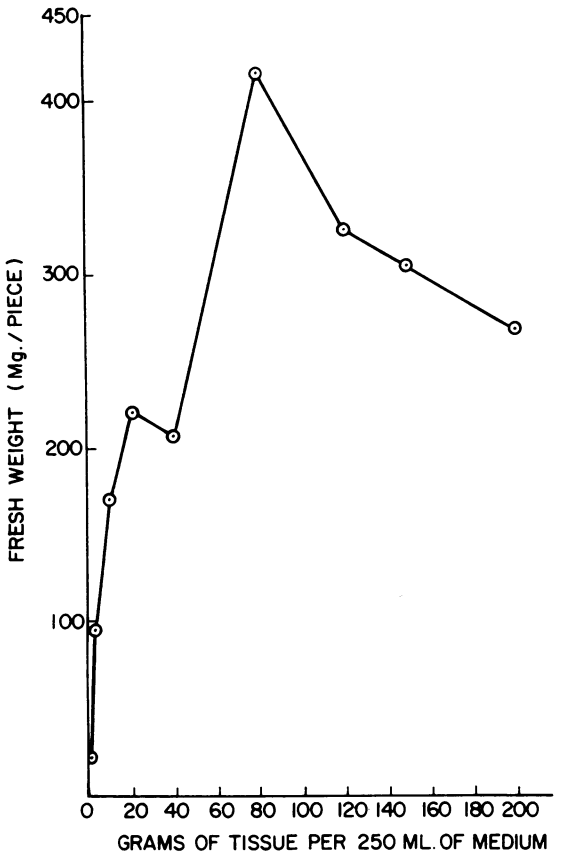

FIG. 1. Bioassay of the 1-butanol extract of crown-gall tissue at various concentrations.

oped by Miller (11, 14, 15) served as the main assay system for all experiments. Basal medium for the bioassays consisted of the following (mg/l): KH_2PO_4, 300; KNO_3, 1000; NH_3NO_3, 1000; $Cu(NO_3)_2 \cdot 3H_2O$, 0.35; $Ca(NO_3)_2 \cdot 4H_2O$, 500; $MgSO_4 \cdot 7H_2O$, 71.5; KCl, 65; $MnSO_4 \cdot H_2O$, 14; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1; KI, 0.75; $ZnSO_4 \cdot 7H_2O$, 3.80; H_3BO_3, 1.6; myoinositol, 100; nicotinic acid, 0.5; pyridoxine-HCl, 0.2; thiamine-HCl, 0.2; $Na_2EDTA \cdot 2H_2O$, 13.4; $FeSO_4 \cdot 7H_2O$, 9.9; sucrose, 30,000; Bacto-agar, 10,000; α-naphthalene acetic acid, 2.0; and, in some cases, kinetin at 0.5 mg (for controls).

The medium, which was usually prepared in multiple strength, was adjusted with NaOH to pH 5.8 and was divided into equal portions and supplemented with extracts or portions of chromatograms corresponding to specific R_F regions. The pH was then readjusted after the medium was brought to single strength with twice-distilled water. The agar was dissolved and 50-ml aliquots were poured into 125-ml Erlenmeyer flasks which were stoppered with nonabsorbent cotton plugs and autoclaved at 15 psi and 121 C for 15 min.

Three pieces of soybean callus tissue (approximately 2–5 mg/piece) were planted in each flask of hardened medium. Cultures were maintained at 30 C under constant illumination (about 40 ft-c) for 28 days. At the completion of each experiment, the tissues were removed from the agar and weighed.

Cation Exchange Column Chromatography. A crude 70% ethanolic extract equivalent to 4 kg fresh weight of crown-gall tissue was subjected to a Dowex AG 50W-X8, H^+ (100–200 mesh) cation-exchange resin column chromatographic procedure which is essentially that used by Miller in the isolation of zeatin (11, 15, 16).

Table I. *Chromatography of Several Cytokinins and the Active Component(s) from Crown-Gall*

Compound[1]	R_F Range in Different Solvents[2]			
	Water-*sec*-butanol	Borate	Ethyl-acetate-propanol-water	Ethanol (20%)
6-(γ,γ-Dimethylallyl-amino) purine	0.96–1.00	0.39–0.49	0.94–1.00	0.46–0.66
Dihydrozeatin	0.93–1.00	0.53–0.60	0.73–0.81	0.63–0.71
Zeatin	0.87–0.93	0.49–0.58	0.64–0.77	0.61–0.69
Zeatin ribonucleoside	0.80–0.88	0.86–0.91	0.45–0.59	0.67–0.75
Crown-gall tissue extract	0.80–0.90	0.80–0.90	0.50–0.60	0.70–0.80

[1] Each standard and 1-butanol extract of crown-gall was chromatographed separately but under the same laboratory conditions.

[2] The standards were detected at the R_F range given as ultraviolet absorbing bands. The ranges for the crown-gall tissue extract represent the migration of peak growth-promoting activity as determined in the soybean callus bioassay.

EXPERIMENTS AND RESULTS

During preliminary experiments, the crown-gall tissue was found to release into the culture medium a factor(s) which stimulated soybean callus proliferation. For purposes of this study, however, direct extraction of the culture medium was not practical because relatively higher and consistent yields of activity were obtained in 70% ethanolic extracts of the tissue. Similarly, reconstitution of the ethanol soluble materials in water followed by partitioning of activity into 1-butanol provided sufficiently clean material for further analysis.

To obtain information as to the levels of activity in the crown-gall tissue, 1-butanol extract was evaporated to dryness and the resulting solids were redissolved in twice-distilled water. Aliquots, corresponding to various gram equivalents of tissue extracted, were incorporated into soybean basal medium. The results of the bioassay indicated the presence of cytokinin(s) in the extract (Fig. 1). It is interesting to note that the level of activity detected in 1 g of crown-gall tissue, as compared to that elicited by zeatin, would represent exceedingly small amounts of a conventional cytokinin. In addition, chromatographic analysis of several known *N*-6-substituted aminopurines and 1-butanol extracts of crown-gall (Table I) indicates that the migration pattern of the active material in crown-gall is similar to that of zeatin ribonucleoside.

To determine whether or not the active material from crown-gall was due to the presence of one or more cytokinins, paper chromatographic techniques were employed which were, for the most part, those of Miura and Miller (17).

The first solvent used was WSB[2] and 25-g fresh weight of tissue of both the 1-butanol extract as well as the aqueous phase were chromatographed and bioassayed. As illustrated in both Figures 2 and 3 a major peak of activity (R_F 0.8–0.9) is present. This corresponds to the region of known migration for *N*-6-substituted purine free-base and ribonucleoside cytokinins (17 and Table I). Also, as shown in the bioassay of chromatograms of the aqueous phase (Fig. 3), a minor peak exists which corresponds to R_F region 0.4 to 0.5.

When the active region of WSB chromatograms (R_F 0.70–

[2] Abbreviations: EPW: ethylacetate-1-propanol-water; WSB: water-*sec*-butanol.

0.95) is eluted in 95% ethanol and rechromatographed in the borate solvent which is specific for separating the conventional free-base and nucleoside cytokinins, a single peak of activity is present (R_F 0.8–0.9) as illustrated in Figure 4. This migration corresponds to that of known active nucleosides (17 and Table I). Further substantiation of this idea resides in the results obtained when the active component(s) at the R_F region 0.70 to 0.95 from WSB chromatograms was eluted and subjected to acid hydrolysis.

For hydrolysis studies a 50 g eq of 1-butanol crown-gall extract was chromatographed in WSB and the active 0.70 to 0.95 R_F region was eluted in 95% ethanol. The ethanol extract was divided in half and each aliquot was evaporated to dryness. One aliquot was dissolved in 0.1 N HCl and treated for 1 hr at 100 C. The other portion, redissolved in water, remained as the control. After heating, the aliquots were cooled, adjusted to pH 7.0, evaporated to dryness, reconstituted in 95% ethanol and chromatographed in the borate solvent. The results shown in Figure 5 clearly indicate that the active material undergoes chemical modification so that the activity after presumed hydrolysis shifts to a lower R_F region associated with standard free-base migration.

Because the active material from crown-gall appeared to be chromatographically similar to a purine nucleoside cytokinin, its susceptibility to mild $KMnO_4$ oxidation was investigated (17). A 50-g eq of crown-gall 1-butanol extract was chromatographed in WSB, the 0.70 to 0.95 R_F region was eluted with 95% ethanol and divided in half. One aliquot was evaporated to dryness, redissolved in 2 ml of twice-distilled H_2O and treated with 2 ml of 0.01% aqueous $KMnO_4$, pH 7.0, for 15 min after which an excess of 95% ethanol was added. The other aliquot remained as a control and received only the ethanol. Both aliquots were evaporated to dryness, reconstituted in 95% ethanol and chromatographed in the borate solvent. As illustrated in Figure 6, the reduction of activity due to mild $KMnO_4$ treatment is substantial. This indicates that the nature of the cytokinin from crown-gall includes a point of unsaturation susceptible to the mild oxidative treatment.

A third solvent used was EPW because of its selective resolution of the highly similar forms of the *N*-6-substituted aminopurine cytokinins. When 25-g eq of a 1-butanol extract of crown-gall tissue were chromatographed in this solvent, only

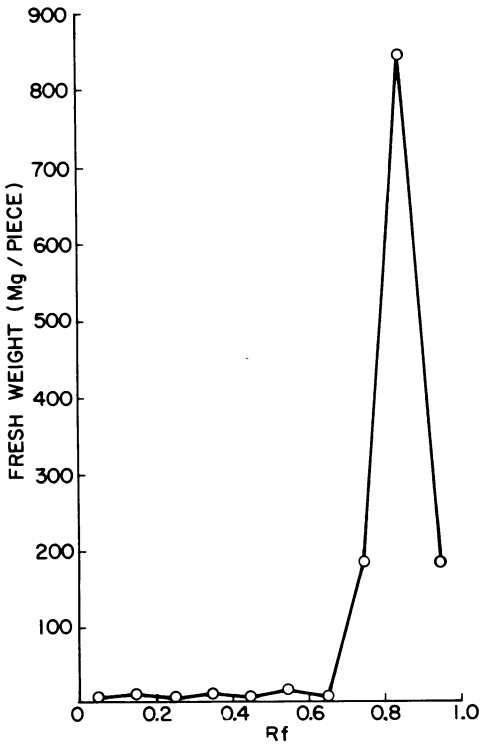

FIG. 2. Bioassay of chromatograms of 1-butanol crown-gall extract equivalent to 25 g fresh weight of tissue. The solvent used was water-saturated *sec*-butanol.

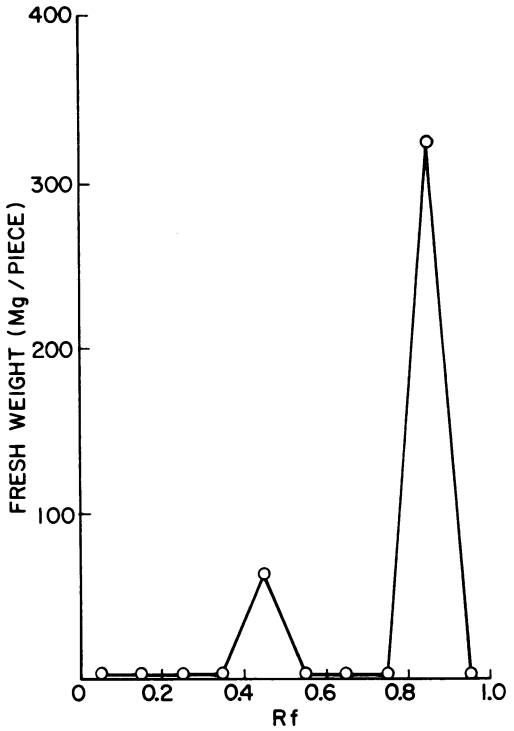

FIG. 3. Bioassay of chromatograms of approximately 250-g eq of the water phase remaining after 1-butanol extraction of crown-gall ethanolic extracts. The solvent used was water-saturated *sec*-butanol.

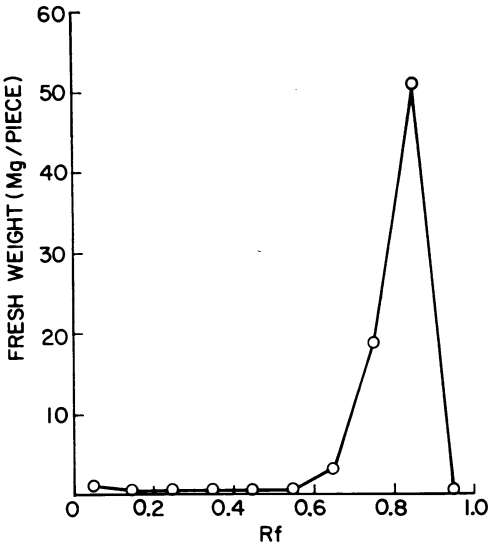

FIG. 4. Bioassay of chromatograms developed in 0.03 M borate, pH 8.4. 1-Butanol crown-gall extract equivalent to 25 g fresh weight of tissue was chromatographed in water-saturated *sec*-butanol, and the active material in the 0.70 to 0.95 R_F region was eluted and rechromatographed in borate.

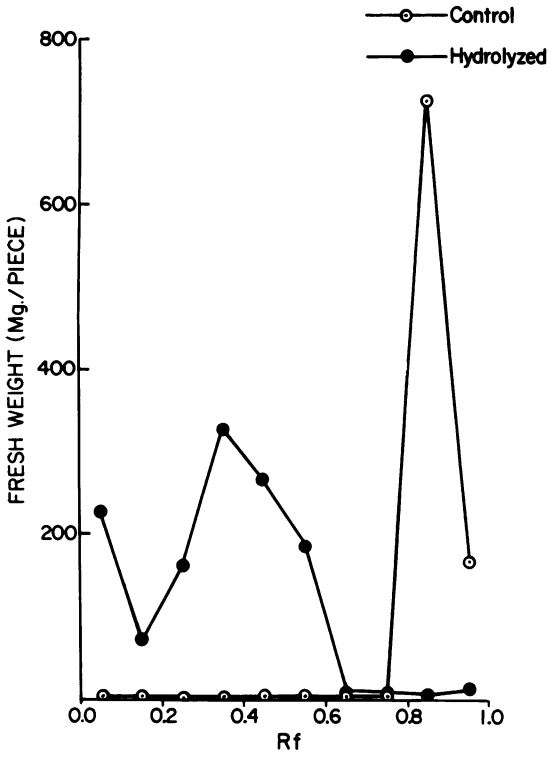

FIG. 5. Bioassay of chromatograms representing activity subjected to acid hydrolysis. An aliquot of the active material eluted from the 0.70 to 0.95 R_F region of chromatograms developed in water-saturated *sec*-butanol was treated with 0.1 N HCl at 100 C for 1 hr and rechromatographed in 0.03 M borate, pH 8.4.

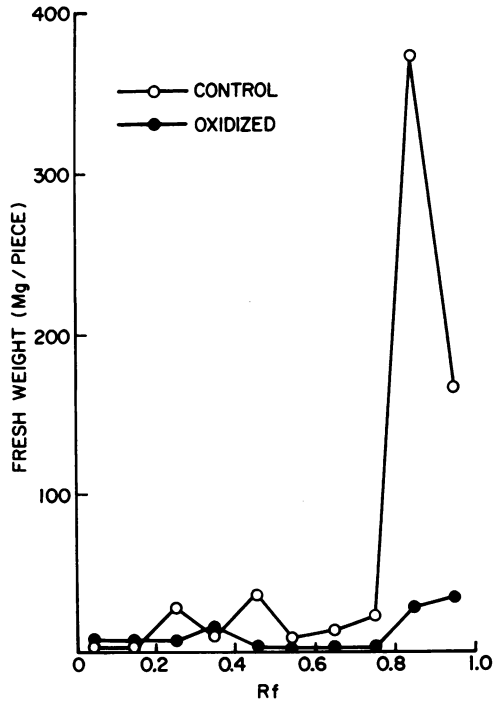

FIG. 6. Bioassay of chromatograms of the active material eluted from 0.70 to 0.95 R_F region of chromatograms developed in water-saturated *sec*-butanol, subjected to $KMnO_4$ treatment and rechromatographed in 0.03 M borate, pH 8.4.

one peak of activity was consistently observed between R_F 0.5 to 0.6 (Fig. 7). This migration pattern is similar to that repeatedly obtained for zeatin ribonucleoside standards (R_F range 0.45–0.59) which is consistent with published data (17).

To further determine if the crown-gall factor was similar to an *N*-6-substituted purine nucleoside, its behavior on a

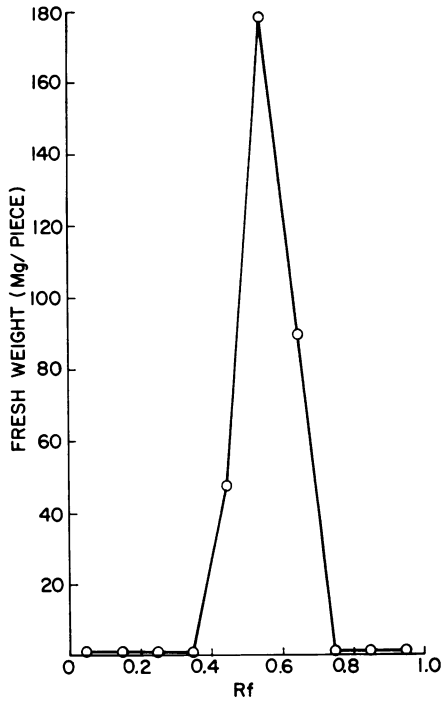

FIG. 7. Bioassay of chromatograms of 1-butanol crown-gall extract equivalent to 25 g fresh weight of tissue. The solvent used was ethylacetate-1-propanol-H_2O (4:1:2, v/v, upper layer).

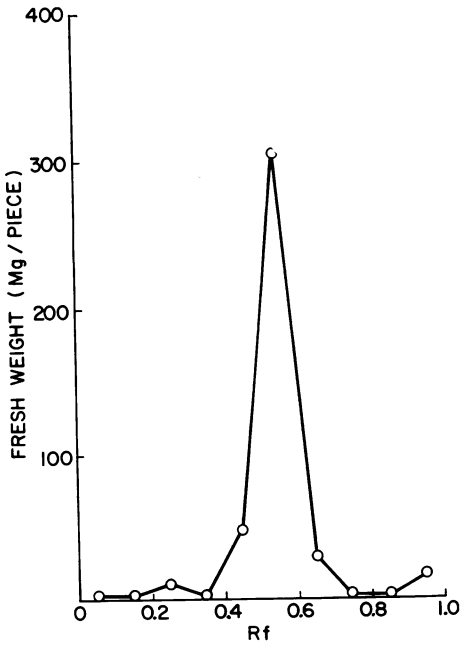

FIG. 8. Bioassay of chromatograms of the active material present in the NH_4OH eluate when crown-gall extract was subjected to cation-exchange column chromatography. The solvent used was 0.03 M borate, pH 8.4.

cation-exchange resin column was studied. A crown-gall ethanolic extract equivalent to 4 kg fresh weight of tissue was subjected to the cation-exchange resin chromatography procedure described by Miller (11). When a 25-g tissue eq of the final 6 N NH_4OH eluate from the column was chromatographed in the borate solvent, only one peak of activity was detected between R_F 0.5 to 0.6 (Fig. 8). This result is consistent with that obtained earlier by the acid hydrolysis of active regions eluted from paper chromatograms and strongly suggests that the active factor undergoes conversion on the resin column from the nucleoside to the free-base derivative and is in this feature similar to known *N*-6-substituted aminopurines.

Although not illustrated here, it should be noted that the cell division activity of the free-base-like derivative detected after acid hydrolysis or cation-exchange resin treatment was substantially reduced after mild $KMnO_4$ oxidation.

DISCUSSION AND CONCLUSION

A cell division factor present in extracts of crown-gall tissue from *Parthenocissus tricuspidata* (Sieb. and Zucc.) Planch. has been shown to exhibit chromatographic properties which are similar to those of an *N*-6-substituted aminopurine nucleoside.

The fact that the nucleoside-like factor and the free-base derivative, obtained after acid hydrolysis or resin treatment, exhibited a substantial loss of activity after mild oxidation is consistent with the idea that an unsaturated substituent necessary for activity is indeed present. Furthermore, all of the data suggest that the major active component present in the tumor tissue is zeatin-ribonucleoside or a closely related compound. This suggestion, however, is not absolute and studies relating to the isolation and further chemical and mass spectral analysis of the active component are essential.

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