Cytokinins in Vinca rosea L. Crown Gall Tumor Tissue as Influenced by Compounds Containing Reduced Nitrogen

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ABSTRACT
Several compounds containing reduced nitrogen markedly increased the yields of cell-division compounds extractable from an A6 Vinca rosea L. crown gall tumor tissue. Caein hydrolysate, several amino acids, and ammonium salts were effective. Both trans-zeatin and ribosyl-trans-zeatin were substantially increased in total amount per culture and in concentration. These two compounds have been identified by several criteria including mass spectra. The reduced nitrogen treatments also caused the appearance of a cytokinin not previously detected in this tissue; it has not yet been identified. The tumor tissue rapidly absorbed [8-14C]adenine from a liquid medium. Within 1 hour, the tissue converted some of the adenine to zeatin and ribosylzeatin, and greater degrees of conversion occurred in 2-, 4-, and 8-hour periods. The tissue grown on a medium containing ammonium chloride accumulated considerably greater quantities of the two cytokinins made from the labeled adenine during each incubation period.

Extracts of crown gall tumor tissue are known to contain factors which stimulate division of plant cells (3, 21). In following up on evidence provided by Tegley et al. (22), Miller (11–13) isolated ribosyl-trans-zeatin from crown gall tumor tissue of Vinca rosea L. and detected two other compounds which, on the basis of evidence available, could have been zeatin and a phosphate derivative of the nucleoside. It then became of interest to determine the pathways of synthesis of the cytokinins and to discover factors or conditions which control the rate of synthesis in tumor tissue. Such information might be helpful not only in understanding what happens when a normal cell is converted to the tumorous state but also in explaining cytokinin production in the normal plant. Our work has now led to the recognition that certain compounds containing reduced N2 can promote accumulation of cytokinins in the tumor tissue as they do in cultured root cells (18). The amounts of extractable ribosylzeatin and zeatin which are increased by such treatment, and a compound appears which we previously had not detected in the Vinca tumor tissue. Our studies have shown that radioactive adenine is incorporated into zeatin and ribosylzeatin during short incubation periods and that the accumulation of these compounds is promoted by prior culture on a medium containing ammonium chloride.

MATERIALS AND METHODS

Tissues and Bioassays. The A6 line of Vinca rosea L. tumor tissue was kindly supplied by Dr. Henry Wood of The Rockefeller University and was cultured as already reported (11) except as noted. The bioassays of cytokinin activity were performed with soybean callus tissue as previously outlined (11).

Chromatography. Paper chromatography was performed in an ascending fashion on Whatman No. 1 filter paper. Thin layer chromatography was done using 1-mm thick layers of silica gel (Merck PF-254). Solvent systems (v/v) employed were: (A) sec-butanol saturated with water; (B) water; (C) 1-butanol- benzene-water-methanol (2:1:1:1.2); (D) 1-butanol-concentrated ammonium hydroxide-water (86:5:14); (E) tert-butanol-water (5:2); (F) chloroform-thymol (9:1) (17); (G) 1-butanol-acetic acid-water (12:3:5); and (H) upper phase of ethylacetate-1-propanol-water (4:1:2).

Column chromatography on PVP was exactly as previously described (12), and on LH-20 was essentially as described by Armstrong et al. (1). With the latter, the column was 2.5 × 35 cm (50 g of gel) and elution was with 35% ethanol at a flow rate of 50 ml/hr. Samples were loaded onto the LH-20 column in 20-ml volumes and 9-ml fractions were collected.

Purification of Cell-division Factors. Method 2 of Miller (12) was modified slightly and used in a comparative purification of cell-division factors. Fifteen hundred grams of frozen tumor tissue were added to a mixture of cold ethyl acetate and a blend of 150 g of KH2PO4 and 1050 g of K2HPO4. The salts dissolved as the tissue thawed. After thawing was completed, the ethyl acetate layer was removed, and the remaining aqueous layer was further extracted with seven successive 500-ml volumes of cold ethyl acetate; the ethyl acetate layers were pooled. The aqueous layer was next extracted with five successive 500-ml volumes of sec-butanol which were combined. After removal of the solvents, the ethyl acetate and butanol fractions were each further purified on PVP columns, and the appropriate fractions were subjected to additional chromatography. Crystallizations were attempted in the usual manner (11) and any crystals were washed with water and ethanol.

Mass Spectra. A Varian CH-7x single focusing mass spectrometer was used at 70 ev to obtain low resolution mass spectra.

Tracer Studies. The incorporation of [8-14C]adenine (17.9 μCi/μmole, Calbiochem) into compounds having cytokinin activity was studied in fairly short term experiments. Ten grams of tumor tissue grown under stated conditions were added aseptically to 10 ml of modified White's medium (11, 22) (adjusted to pH 6 before autoclaving) in each 50-ml Erlenmeyer flask. The flasks were shaken at 140 rpm on a gyratory shaker. At times indicated under “Results,” the tissue was separated from the liquid and washed with water. Extraction from the tissue was achieved by making the tissue 80% with respect to ethanol. The mixture was placed in the refrigerator for several hours, and the extract then was separated from the tissue by filtering through glass wool. The extracts were examined both by filter paper and column chromatography. Sections from paper chromatograms were placed in scintillation vials, and 5 ml of scintillation fluid (5 g PPO and 0.1 g POPOP/l toluene) were added. For fractions from the LH-20 column, aliquots were added to scintillation vials, and an equal volume of scintillant was added.
tion vials along with 10 ml of scintillation fluid (5 g of PPO, 0.1 g of POPOP, and 100 g of naphthalene/l dioxane). Counts were made in a Beckman LS-100 scintillation counter.

RESULTS

Increasing Extractable Cell-division Activity. In an effort to increase the production of cytokinins by the tumor tissue, various substances have been added to the basal medium on which the tissue is ordinarily grown. In early experiments, casein hydrolysate (acid) at 500 or 5000 mg/l increased the yield of cytokinin activity (per g of tissue) extractable into 80% ethanol. Since the hydrolysate also increased the growth of the tissue, the yield of activity per piece and per flask was very substantially increased. At the same time, glycine and L-aspartic acid also caused increases in total cytokinin activity even though they inhibited growth of the tumor tissue. In later tests run at concentrations of 0.38 and 3.8 mM, L-asparagine, L-glutamate, ammonium chloride, ammonium nitrate, proline, and L-arginine all were found to increase extractable cytokinin activity at the higher concentration and all decreased growth of the tumor tissue at this concentration. L-Lysine severely inhibited growth at the higher concentration and increased the cytokinin content at the lower concentration. L-Leucine, L-isoleucine, L-valine, L-cysteine, L-serine, L-threonine, and L-histidine failed to increase the amounts of cytokinin activity and all inhibited growth even more severely than those compounds listed above, with the exception of L-lysine. Urea was very severely inhibitory to growth but some increase of cytokinin content of the tissue was noted. α-Ketoglutaric acid, malate, potassium nitrate, and succinate affected neither growth nor the yield of cytokinin activity. The data obtained with the inorganic compounds are presented in Table I. The one common feature of the compounds active in increasing cytokinin activity is the presence of reduced nitrogen. Although the active compounds mentioned in the table inhibited growth by about 20 to 30%, the cytokinin activity per g was several times as high as in the controls. Since the simple ammonium salts gave the increased cytokinin contents, subsequent experiments have been done with ammonium chloride.

Chromatographic Analysis of Extracts. Extracts (80% ethanol) from tissues grown for 7 weeks on either the basal medium or on basal medium with 3.8 mM NH₄Cl were chromatographed with solvent system A. The results of the bioassays of the chromatograms are presented in Figure 1. The outstanding changes due to growth on the ammonium chloride are the marked increase in activity in the ribosylzeatin range (Rₖ 0.76-0.87) with a broadening at the upper end of the range, increased activity at an Rₖ around 0.16 where slight activity has been previously detected (11) with tissue grown on the basal medium, and the presence of previously undetected activity at an Rₖ of about 0.47.

In a further analysis of the active material in the upper parts of the chromatograms run in the same fashion, the sections from Rₖ 0.6 to the front were eluted with 95% ethanol, and the eluates were developed on paper with solvent B, a system known to separate zeatin from ribosylzeatin. The results of the bioassays of these chromatograms are presented in Figure 2. The material derived from tissue grown on the ammonium medium not only shows more activity at the ribosylzeatin region (Rₖ 0.6-0.8) than does the material from tissue grown on the basal medium but also shows considerable activity in the region to which zeatin (Rₖ 0.4-0.6) is known to migrate. An increased accumulation of both compounds under the influence of the reduced nitrogen therefore appeared to be possible.

Isolation of Zeatin and Ribosylzeatin. In order to definitely identify the cell-division compounds and to determine their amounts as influenced by the reduced nitrogen, comparative extracts of 1500 g each of tissues grown on the basal medium or on the medium containing the ammonium chloride were performed. By subjecting the ethyl acetate extracts to further purification as outlined under "Materials and Methods," 0.11 mg of ribosylzeatin was obtained from the tissue grown on basal medium and 1.14 mg was obtained from the ammonium-grown tissue. These yields were estimated by measuring absorbance and assuming a molar extinction coefficient of 19,000 at 268 nm. The presumed ribosylzeatin from the control tissue was not crystallized; however, it had the expected Rₖ values in all the isolation chromatograms and ran with synthetic ribosyl-transzeatin with solvent systems C, D, and G giving Rₖ values of 0.63, 0.57, and 0.73. The compound exhibited UV peaks in 95% ethanol, 0.1 N HCl and 0.1 N NaOH of 268, 264, and 268 nm,
Although a considerable amount of it is produced by the ammonium-grown tissue, we have not yet identified the compound which moves to RF 0.47 with solvent A. We have found that it goes mainly into the sec-butanol layers in our procedures for isolation.

**Labeling Experiments.** Since zeatin and ribosylzeatin are derivatives of adenine, it seemed reasonable that adenine may be a precursor of the cytokinins. This is especially so in view of the results of other investigators with other species (4, 15). Therefore, the incorporation of [8-14C]adenine by the tumor tissue into various compounds has been and continues to be examined. The radioactive adenine was added at a level of 2 x 10^6 cpms/culture in some experiments and 3.4 x 10^6 cpms/culture in others. After 1, 2, 4, or 8 hr of incubation on the shaker, the tissues were extracted. Cold zeatin, ribosylzeatin, adenine, and adenosine (0.4 amole each) were added to the extract from each flask, and the extract was developed on the LH-20 column. Zeatin and adenine were not clearly separated and were eluted in tubes 27 to 30. Likewise, ribosylzeatin and adenosine were not separated and were eluted in tubes 22 to 25. Definite peaks of radioactivity as well as peaks of UV absorbance were detected in the tubes mentioned for all periods of incubation (Fig. 3) with both the tissue grown on the basal medium and that grown on the medium containing ammonium chloride. The data of the figure are for a beginning level of 2 x 10^6 counts/flask but essentially the same patterns were seen in another experiment using the higher level of radioactivity. In order to learn for certain the nature of the compounds contributing to the radioactivity in the peaks, further purification was achieved. In the experiment of Figure 3, eluents representing the zeatin-adenine and ribosylzeatin-adenosine peaks were dried down, and aliquots of the materials were chromatographed on paper with solvent C for zeatin and solvent A for ribosylzeatin; these systems separated the cytokinins from adenine and adenosine which might be expected to be present. The exact positions of the four compounds were detected by the UV quenching primarily due to the added nonradioactive compounds. The spots were eluted with ethanol. Equal portions of each were used for determination of radioactivity. The counts for 2 and 8 hr for both tissues and for both levels of beginning radioactivity are given in Table II. Both the zeatin and the ribosylzeatin regions exhibited very considerably higher activities for material from ammonium-grown tissue than did those from the tissue grown on the basal medium. Chromatography of the remaining portions of the samples on solvent D (Fig. 4) showed that for each experiment in Table II, the large majority of counts ran with the UV-quenching (cytokinin) spots with much higher activities obtained from the ammonium-grown tissue. Although similar data are not given for incubation periods of 1 and 4 hr, such data were obtained and gave the same type of results but with quantitative differences. In this and various other experiments, zeatin fractions from the LH-20 column have been chromatographed with solvent systems A, B, C, D, E, G, and H with peaks of radioactivity coinciding with UV-absorbing spots (apparently zeatin) at RF values of 0.92, 0.51, 0.74, 0.62, 0.95, 0.79, and 0.62. In chromatography of the ribosylzeatin fraction with solvent systems A, B, C, D, E, G, peaks of radioactivity have coincided with UV-absorbing spots (apparently ribosylzeatin) at RF values of 0.80, 0.70, 0.65, 0.59, 0.94, and 0.72.

Further evidence supporting the identification of the cytokinins was obtained by a procedure involving crystallization and sequential chromatography with specific activities being determined after various steps. The supposed zeatin and ribosylzeatin fractions from the LH-20 column were chromatographed as before with solvents C or A, and the proper regions were eluted. Cold zeatin (previously cleaned by running on silica gel with solvent F) was added to the putative radioactive zeatin and cold ribosylzeatin (likewise cleaned) to the radioactive material thought to contain ribosylzeatin. At this point, specific radioac-
Fig. 3. Comparative profiles of radioactivities eluted from LH-20 columns. Incubations were with $2 \times 10^6$ cpm [8-14C]adenine. Ribosylzeatin and zeatin were eluted in fractions 22 to 25 and 27 to 30, respectively. N6-(Δ2-isopentenyl)adenosine and N6-(Δ2-isopentenyl)adenine were eluted in fractions 32 to 37 and 40 to 44. Biological activity from ammonium-grown tissue extracts was eluted in tubes 6 to 35 with high activity in pooled fractions 11 to 15 and 16 to 20 but higher in 21 to 25 and 26 to 30. The indicators for basal- and ammonium-grown tissues (B and A) are followed by the hours of incubation in the presence of [14C]adenine. The numbers at tops of broken columns are counts/min·ml of extract.
tivities of the mixtures (cpm/μmole) were determined by making counts and measuring the absorbances at the absorption maxima. They were 1165 for zeatin and 1420 for ribosylzeatin. The materials were now crystallized; specific radioactivities did not change. The zeatin was next run sequentially with solvents B, D, G, A, H, and E. The specific radioactivities after the last four systems were 948, 978, 896, and 1019, respectively. The ribosylzeatin was developed sequentially with solvents B, D, G, C, and E. The specific radioactivities after the last three systems were 1532, 1491, and 1444.

Potassium permanganate oxidation of adenine type of cytokinins may be diagnostic both for the presence of the double bond in the side chain and of adenine or adenosine (9, 14). The materials from an 8-hr incubation of ammonium-grown tissue which had been purified through the LH-20 and solvent C or A steps were treated with 0.01% KMnO4 and chromatographed with solvent G. These materials contained nonradioactive zeatin or ribosylzeatin added after the incubations. The new patterns of both UV-quenching and radioactive spots coincided very well. For zeatin, both absorbance and radioactivity peaks were observed at the position of adenine (Rf 0.59) and at an unidentified locus (Rf 0.40). For ribosylzeatin, the same was noted at the position of adenosine (Rf 0.50) and also at an unidentified locus (Rf 0.41). The unidentified spots have been observed in earlier tests (9, 14).

Media from the 1- and 2-hr incubation experiments were examined in order to see if a difference in the rate of 14C-adenine uptake was a causative factor in the differential accumulation of the cytokinins. In these experiments, the amount of radioactivity left in the media was significantly less with the tissues grown on the basal medium than with those grown on the ammonium medium. When the medium from the 1-hr incubation of ammonium-grown tissue was run on the LH-20 column, the resulting profile showed only very minor amounts of radioactivity not associated with the adenine elution position. Thus, the higher counts in the media with ammonium-grown tissue are probably not due to a disposal of labeled metabolites into the medium subsequent to uptake.

**DISCUSSION**

The influence of the ammonium chloride on amounts of extractable cytokinin activity has been demonstrated by the bioassays of crude extracts (Table I), by the comparative chromatograms of Figure 1, and by purifications of zeatin and ribosylzeatin. Since the growth of the tumor tissue is inhibited by the ammonium salt (a fact noted much earlier by Braun [2]), interpretation of the data might seem somewhat complicated. An examination of experiment A in Table I shows that the total activity is higher for the ammonium-grown tissue not only per g of tumor tissue but also per piece and per flask. If all of the tissue from a flask containing basal medium is extracted and bioassayed at the equivalent of 4 g tumor tissue/assay medium, 727 ml of bioassay medium may be prepared and will support growth of the soybean bioassay tissue to a final weight of 42 mg/piece. If the same is done with a flask of ammonium-grown tissue except that the bioassay is performed at a level of extract equivalent to only 0.4 g tumor tissue/assay medium, 5400 ml of the bioassay medium may be prepared and growth of the soybean tissue will be supported to a final weight of 54 mg/piece. Obviously, there is much more activity/flask when the ammonium chloride is included. This is not a matter of diluting out inhibitors since at 0.4 g of basal tissue/flask growth is even less.

Comparing the data given in this paper with earlier results from this laboratory for similarly grown tissue (11), we noted two discrepancies. The height of the peak (basal tissue, Rf 0.7-0.9) in Figure 1 is only one-twentieth that reported earlier, and the amount of ribosylzeatin actually purified from tissue grown on the basal medium is only half that reported previously. Additional growth of the tumor tissue may have produced compounds which are not extracted by the LH-20 solvent system used to purify the cytokinin.

We do not yet know the nature of the compound from the ammonium-grown tissue which runs at Rf 0.47 in solvent system A. We have purified small amounts of it and the UV spectra resemble those of ribosylzeatin. The compound moves to the region in which Tegley et al. (22) have detected cytokinin activity in extracts of Parthenocissus tricuspidata tumor tissue. Its chromatographic behavior resembles that of glycosyl derivatives of zeatin (16), but we cannot yet say that it is such a derivative.

Our results with the incorporation of radioactive adenine are in agreement with those obtained with the fungus *Rhizopogon roseolus* (15) and with a strain of tobacco tissue (4). The results also give further support to our findings that the tumor tissue makes zeatin and ribosylzeatin and that growth on the ammonium medium increases the capacity of the tissue to accumulate such compounds. Evidence for the formation of radioactive cytokinins includes locations in the LH-20 elution profiles, positions on chromatograms developed with many solvent systems, co-crystallization with the cold compounds, constancy of specific radioactivities through a sequence of chromatographic purifications, and the finding that treatment with permanganate decreased the radioactivities of spots, even those in the zeatin position. It is not possible to identify the specific cytokinins which are involved in the various experiments, since the ammonium-grown tissue is inhibited by the ammonium salt.

### Table II. Influence of Ammonium Chloride and Time of Incubation on Amounts of Radioactive Materials Formed from Adenine which Chromatograph as Zeatin or Ribosylzeatin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue</th>
<th>Source</th>
<th>Radioactivity of Spots (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>B</td>
<td>A</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>Zeatin</td>
<td>B</td>
<td>A</td>
<td>1,000</td>
</tr>
<tr>
<td>Ribosylzeatin</td>
<td>B</td>
<td>A</td>
<td>5,100</td>
</tr>
<tr>
<td>8 hr</td>
<td>Zeatin</td>
<td>B</td>
<td>1,100</td>
</tr>
<tr>
<td>Ribosylzeatin</td>
<td>B</td>
<td>A</td>
<td>8,400</td>
</tr>
</tbody>
</table>

Two levels of radioactive adenine were used (2 x 10⁶ and 3.4 x 10⁶ cpm). Tissues were grown on basal (B) or on ammonium chloride (A) media and then incubated with adenine for 2 or 8 hr. Materials were eluted from LH-20 column and then chromatographed with solvent systems which separate the cytokinins from adenine and adenosine. Counts given were calculated by multiplying actual counts by amount of cold cytokinin added at beginning of purification procedure and dividing by final amount of cytokinin as indicated by UV absorbance.

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Fig. 4. Comparative chromatography of extracts from basal- and ammonium-grown tissues incubated with $2 \times 10^6$ cpm [¹⁸-C]adenine for 8 hr. Zeatin- and ribosylzeatin-containing portions were eluted from chromatographs developed with systems C and A, respectively (see Table II), and the results of further chromatography with system D for aliquots of both factors are presented here. The positions of the UV quenching due to added cytokinins are indicated on the chromatograms. Basal-grown tissue (B); ammonium-grown (A); zeatin (Z); ribosylzeatin (RZ).

nate. As can be detected in Figure 3, the adenine was converted to several forms in addition to those of zeatin and ribosylzeatin. Those materials extractable into ethanol and the adenine left in the incubating medium accounted for only a rather small fraction of the beginning counts; much of the adenine had been incorporated into the ethanol-insoluble fraction. These various compounds and fractions will be studied in our continued efforts to delineate the steps of cytokinin synthesis in the Vinca tumor tissue. Eventually, we hope to compare such steps with steps in normal tissues which can or cannot form cytokinins.

We do not know how the reduced nitrogen compounds work. Both the stimulation of purine biosynthesis and the inhibition of purine breakdown (including perhaps that of cytokinins) should be considered. The formation of purines is known to be under the control of some amino acids and ammonia in animals and bacteria (6), and evidence has been presented which suggests that the same is true in plants (24). Although we have found that ammonium chloride promotes the accumulation of the zeatin base from exogenously supplied adenine, it may be that additional purines are required for such promotion. For example, if the adenine must be cycled through nucleic acids in order to form the cytokinin, other purines would be needed and an increase in their amounts could lead to greater cytokinin yields. Evidence has also been presented (23) that enzymes involved in the breakdown of purines in some bacteria and fungi are repressed by excess ammonia in the culture medium. Perhaps the same is true in Vinca tumor tissue. Regardless of this, the observations of Sargent and King (18) and those made by us...
make us wonder if some effects of application of reduced nitrogen salts to plants occur because of increased cytokinin concentrations.

**Noted Added in Proof.** In our discussion, we should have pointed out that P. Beutelmann (1973. Planta 112: 181-190) had reported that the presence of amino acids in the culture medium increased the amount of radioactive adenine converted to cytokinins by a moss hybrid tissue.

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**LITERATURE CITED**


