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Cell-Division Factors from *Vinca rosea* L. Crown Gall Tumor Tissue
(ribosyl-trans-zeatin/"cytokinesins"/cytokinins/adenine derivative)

CARLOS O. MILLER

Department of Plant Sciences, Indiana University, Bloomington, Ind. 47401

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**ABSTRACT** A cell-division factor has been precipitated from extracts of cultured *Vinca rosea* L. crown gall tumor tissue by using the mercuric acetate procedure previously employed by Wood and colleagues to obtain their "cytokinein I." On the basis of its mass spectrum, ultraviolet light absorbancy spectra, solubilities, chromatographic migration values, and growth activity, the factor is ribosyl-trans-zeatin, that is, 6-(4-hydroxy-3-methyl-trans-2-butenylamino)-9-β-D-ribofuranosylpurine. Ribosylzeatin has now been isolated from tumor tissue by four experimental techniques; any possibility that it is an artifact seems to have been eliminated. Contrary to the report by Wood and colleagues, synthetic ribosylzeatin is precipitated from an aqueous solution by mercuric acetate, provided the complete precipitation procedure is utilized. These facts and others discussed strongly support our suggestion that ribosylzeatin was present in the preparation ("cytokinein I") examined by Wood and colleagues in several biological assays. The reasons advanced by Wood and others for rejecting this suggestion have been found either not to be pertinent to the question or to have insufficient experimental bases.

In an earlier communication, we reported the isolation of ribosyl-trans-zeatin [6-(4-hydroxy-3-methyl-trans-2-butenylamino)-9-β-D-ribofuranosylpurine] from crown gall tumor tissue (1). We concluded that this compound, which is very effective in promoting cell division in certain cytokinin bioassays, is responsible for a large portion—although not all—of the cytokinin activity detectable in extracts from the tumor tissue. Recently, Wood et al. (2) have questioned the validity of this conclusion and have rejected our suggestion that ribosylzeatin may be present in a preparation obtained in their laboratory from similar tumor extracts. We now provide additional support for our conclusion and evaluate the bases for both our suggestion and the rejection of it by Wood and colleagues.

**MATERIALS AND METHODS**

**Tumor Tissue.** The *Vinca rosea* L. A6 line of crown gall tumor tissue originally supplied to us by Dr. Henry Wood of The Rockefeller University was grown as previously described (1).

**Bioassay.** Tests for cell-division factors were made by using the soybean callus assay as outlined earlier (1).

**Purification of a Cell-Division Factor.** With a Waring blender, frozen tumor tissue was ground up in 100-g batches in sufficient cold 95% ethanol to make a calculated final concentration of 70% ethanol. Each batch was immediately filtered through a layer of washed cotton. A total of 1500 g of tissue was extracted in this manner. Solvent was removed from the pooled extracts with an air stream to give a final volume of 400 ml. The temperature was never higher than that of the room. The preparation was next centrifuged and filtered so as to remove solids and fatty materials. A precipitate was obtained from the filtrate by the modified Neuberg method as used by Wood (3). The pH of the filtrate was adjusted to 7.2 with a saturated solution of Na₂CO₃ and then readjusted to pH 6.0 with a 25% solution (25 g/100 ml of solution) of mercuric acetate. Next, more of the sodium carbonate solution was added until a pH of 7.2 was regained. The mixture was refrigerated overnight and the precipitate was collected by centrifugation in the morning. Two further precipitates were collected the following 2 days, the preparation being kept cold throughout the procedure. The combined precipitates were suspended in 50 ml of H₂O and the mercury removed by bubbling hydrogen sulfide through the suspension and then centrifuging. Excess hydrogen sulfide was removed by bubbling air through the supernatant. An aliquot representing 2 g of tumor tissue was removed for chromatography and subsequent bioassay. Five grams of KH₂PO₄ were added to the remainder of the supernatant. After the KH₂PO₄ was in solution (pH 4.2), 35.0 g of K₂HPO₄ were added and dissolved. These salts were added slowly and with constant stirring and cooling in an ice bath so as to prevent heating. The final pH was 8.3. The solution was then extracted with seven successive 50 ml volumes of ethyl acetate. These volumes were combined and the ethyl acetate evaporated. The residue was taken up into 5 ml of H₂O, which was then applied to the top of a 42 X 2 cm column of polyvinylpyrrolidone (4, 5); the column was developed with 0.1 M KH₂PO₄. Combined fractions (4 ml each) were bioassayed for cell division activity. Most of the activity was detected in fractions 71-80 and 80-90, although a little was detected in fractions 61-70 and 35-49. Fractions 61-90 were pooled and the water was evaporated at temperatures below that of the laboratory to give a dry residue which included a large amount of KH₃PO₄. The active material was removed by repeated washing of the residue with ethanol and then subjected to chromatography on previously washed Whatman no. 1 filter paper. The first solvent used was water-saturated sec-butanol. A band of ultraviolet-quenching material centered at Rₚ 0.83 had most of the activity. This material was eluted with ethanol and then further purified on washed filter paper by using water as the solvent. A band of material was now located at about Rₚ 0.74. This was eluted with redistilled ethanol and studied for physical properties and biological activity.

**Mass Spectral Studies.** After removal of the ethanol the purified material was examined in a computerized Varian
Table 1. Chromatographic distribution of cell-division factors precipitated by the mercuric acetate/sodium carbonate procedure

<table>
<thead>
<tr>
<th>RF region*</th>
<th>Average fresh weight/piece of soybean callus (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.1</td>
<td>13 ± 0.9</td>
</tr>
<tr>
<td>0.1-0.2</td>
<td>14 ± 1.3</td>
</tr>
<tr>
<td>0.2-0.3</td>
<td>12 ± 0.6</td>
</tr>
<tr>
<td>0.3-0.4</td>
<td>14 ± 1.1</td>
</tr>
<tr>
<td>0.4-0.5</td>
<td>15 ± 1.3</td>
</tr>
<tr>
<td>0.5-0.6</td>
<td>20 ± 3.1</td>
</tr>
<tr>
<td>0.6-0.7</td>
<td>15 ± 1.3</td>
</tr>
<tr>
<td>0.7-0.8</td>
<td>44 ± 2.5</td>
</tr>
<tr>
<td>0.8-0.9</td>
<td>89 ± 6.7</td>
</tr>
<tr>
<td>0.9-1.0</td>
<td>45 ± 3.5</td>
</tr>
</tbody>
</table>

* On a paper chromatogram developed with water-saturated sec-butanol.
† Value for tissues cultured on basal medium without cell-division factors was 12 ± 1.4. Values are given ± SEM.

MAT CH-7 mass spectrometer at 70 electron volts and various temperatures.

Precipitation of Synthetic Ribosyltranszeatin. Two slightly different procedures were used to see if the mercuric acetate method will precipitate ribosyltranszeatin. In the first, about 1 mg of synthetic ribosyl-\(\text{trans}\)-zeatin was dissolved in 50 ml H\(_2\)O and 5 ml of the 25% mercuric acetate solution was added. The solution was put in the refrigerator overnight and then examined for precipitate; there was none. After an ultraviolet light absorbance spectrum was obtained for the solution, its pH was adjusted to 7.2 by adding sodium carbonate solution; a precipitate formed. This mixture was centrifuged after it had been kept in a refrigerator for 24 hr. The supernatant was then examined for the presence of ribosyltranszeatin as indicated by ultraviolet light absorbance. In the second procedure, about 1 mg of synthetic ribosyltranszeatin was dissolved in 0.1 M KH\(_2\)PO\(_4\). This solution was adjusted to pH 7.2 by adding sufficient sodium carbonate solution, and then readjusted to pH 6.0 with the 25% solution of mercuric acetate. After an adjustment back to pH 7.2 with sodium carbonate solution, the mixture was refrigerated for 24 hr and an additional precipitate was removed by centrifugation. The supernatant was examined with a spectrophotometer for the presence of the ribosyltranszeatin.

RESULTS

The small aliquot from the solution remaining after removal of the mercuric sulfide was chromatographed on paper with water-saturated sec-butanol serving as the solvent. The paper was cut into ten equal strips parallel to the origin and to the front. The results of the bioassay, run at a concentration of the preparation equivalent to 10 g of tumor tissue per liter, are presented in Table 1. The peak of activity was located between \(R_F 0.8\) and \(R_F 0.9\). Synthetic ribosyl-\(\text{trans}\)-zeatin also migrated to this region in a second chromatogram run at the same time.

After final purification on paper, the eluted material gave the absorption spectra of ribosyltranszeatin (6) with maxima at 208 nm in 95% ethanol, at 268 and 217 nm in 0.1 N NaOH, and at 264 nm in 0.1 N HCl. The low resolution mass spectrum obtained at 173° had mass to charge, m/e, peaks at 351 (molecular weight of ribosyltranszeatin), 334, 331, 320, 262, 248, 219 (molecular weight of zeatin), 202, 201, 200, 199, 188, 186, 160, 148, 136, 135 (molecular weight of adenine), 119, and 108; there were no other prominent peaks in this range. No peaks above 351 (and the smaller 352) were seen even though the probe temperature was increased to 220°. This pattern of peaks is commonly obtained with ribosyltranszeatin (1, 7, 8).

The \(R_F\) values already mentioned were also obtained with synthetic ribosyltranszeatin. When chromatographed on a thin layer of silica gel with the 9:1 mixture of chloroform and methanol (9), the compound from tumor tissue ran with synthetic ribosyl-\(\text{trans}\)-zeatin \((R_F 0.14)\), which distinguished it from the \(\text{cis}\) isomer, which ran at \(R_F 0.20\). The compound therefore is ribosyl-\(\text{trans}\)-zeatin. The yield from 1.5 kg of tissue was 0.14 mg (assuming a molar extinction coefficient of 208 nm of 19,000). This represents 13% of the beginning active material, which was estimated to be the biological equivalent of 1.05 mg of ribosyl-\(\text{trans}\)-zeatin.

Just as reported by Wood et al. (2), mercuric acetate did not precipitate synthetic ribosyltranszeatin from solution but did cause a shift of the absorption peak from 268 to 276 nm. However, when both mercuric acetate and sodium carbonate were added to either the water or potassium phosphate solution, the ribosyltranszeatin was removed from solution along with a copious precipitate. After removal of the precipitates, the absorbancies at 268 nm for both solutions dropped from 1.1 to 0.1 and absorbancies at 276 nm dropped to less than 0.1. Adjustments of the pH of either solution to a value of 6.0 failed to increase these absorbancies, and sodium carbonate did not substantially alter the absorption of a ribosyltranszeatin solution. It is clear that ribosyltranszeatin is removed from solution by the mercuric acetate/sodium carbonate procedure. Incidentally, free zeatin also is removed from solution by this procedure.

DISCUSSION

The basis for the questioning by Wood et al. (2) of our conclusion that the tumor tissue produced ribosyltranszeatin apparently was that the compound may be artifact formed by some isolation step rather than being a compound formed by the tissue. In particular, they criticized the use of the cation exchange resin in our preparative method. We were aware of Wood’s earlier misgivings (10) concerning such use and, although we did not share them (1), we took precautions to avoid action by the resin. Since our first report (1), three additional methods have been devised to isolate the main cell-division factor from the tumor tissue. The cation exchanger is used with none of these three methods; yet all have yielded ribosyl-\(\text{trans}\)-zeatin. Two of these procedures have been described in detail (5) and are summarized here. The first one was essentially the same as that given herein in Materials and Methods except that the mercuric acetate/sodium carbonate and hydrogen sulfide steps were not included. The second method utilized frozen tissue which was thawed in a mixture of potassium phosphates (to give pH 8.2) under a layer of ethyl acetate; the rest of the sequence of steps was as for the first. The yields of ribosyl-\(\text{trans}\)-zeatin obtained with these two procedures and with the mercuric acetate method were sufficient to account for an estimated 26%, 28%, and 13% of the beginning activity, respectively. The procedure using mercuric acetate, therefore, is less efficient in recovering the active factor than are the other procedures. All three methods of purification avoided pH values higher than 8.5. The lowest pH encountered with two of them was 4.5, but in the procedure using precipitation with mercuric acetate a pH of 2.1 was measured after the treatment with hydrogen sulfide. Temperatures at all steps...
for the three methods were those of the room or lower. None of the three appears to involve steps or conditions likely to cause the formation of ribosylzeatin. The results obtained with these methods agree completely with those we reported earlier (1). It is reasonable, therefore, to conclude that the tumor tissue produces ribosylzeatin and that this compound accounts for a large portion of the activity detected in extracts from tumor tissue. This, of course, does not preclude the existence of other active factors. In fact, we have reported the presence of two other factors active as cytokinins (1).

Our suggestion that the preparation obtained by Wood et al. (13) might actually contain a small amount of ribosylzeatin (1) referred only to that material which was closely examined with several biological assays in the work cited when we made the suggestion. This material has been termed “cytokinin I” (11). The same investigators have reported purifying another material which they have named “cytokinin II.” According to their early reports, the second material was obtained in smaller amounts than was the first (12). This may be why they emphasized “cytokinin I” and did not report testing “cytokinin II” in several biological assays (13). Our suggestion that “cytokinin II” contains some ribosylzeatin was based on several points which are given in italics below and which are now discussed in light of additional evidence:

1. Evidence for production by tumor tissue of ribosylzeatin in substantial quantities had been obtained. The purification of ribosylzeatin from the tumor extracts by three additional techniques is solid support for the conclusion that it exists in the tissues. All available chromatographic data obtained with crude extracts still are consistent with the idea that it is the main active compound.

2. The published chromatographic migration values for “cytokinin I” were very close to those established for ribosylzeatin. Although some differences for migration of ribosylzeatin and “cytokinin I” in additional systems have now been reported by Wood et al. (2), the values really are quite close. Furthermore, the differences were seen with ultraviolet light and no biological analysis of chromatograms of “cytokinin I” alone in such systems was reported. The amount of ribosylzeatin in “cytokinin I,” if present, may be too small to be detected with the ultraviolet light, and only a fine, precise analysis could determine with certainty whether or not biological activity moves exactly with the ultraviolet-detectable material in these additional systems.

3. Because of the very similar migration rates of ribosylzeatin and “cytokinin I,” a separation of the two with the countercurrent distribution system or the subsequent chromatographic steps employed seemed unlikely. That the countercurrent distribution would not have separated the two has been acknowledged by Wood et al. (2).

4. The reliance on the Funaria protonema test for detecting the presence or absence of the adenine type of cytokinin was not justified since the test had an uncertain experimental basis. The Funaria protonema test has now been shown in two laboratories (14, 15) to be rather insensitive to ribosylzeatin; thus, the assertion by Wood et al. (13) that the active factor in the “cytokinin I” could not be an adenine type of cytokinin is not tenable.

In summary, our evidence for suggesting that “cytokinin I” may contain some ribosylzeatin has been strengthened.

In rejecting our suggestion, Wood and colleagues (2) made the following points (given in italics) which are now discussed as to their pertinence to the question being considered or as to their validity:

1. Ribosylzeatin was not precipitated from solution by mercuric acetate, whereas “cytokinin I” was obtained by a procedure using the mercury salt. It is true that mercuric acetate does not precipitate ribosylzeatin from a water solution. However, this is not the complete procedure used by Wood (3); sodium carbonate is also added as the pH is adjusted. When the complete procedure is used, ribosylzeatin indeed is precipitated from solution. Furthermore, the new isolation method reported herein used the mercuric acetate/sodium carbonate procedure and yielded ribosylzeatin as the main active compound. Thus, the mercuric acetate technique obviously does not separate “cytokinin I” from ribosylzeatin.

2. Hydrolysis of the “cytokinin I” preparation did not yield the products obtained when ribosylzeatin was similarly hydrolyzed. The sensitivity of the hydrolysis–chromatography analysis was not stated (2); therefore, the reader cannot know how much ribosylzeatin would have had to be present in order to give products which would have been detected.

3. Mass spectrometry of the products obtained by hydrolysis of “cytokinin I” did not show m/e peaks expected of adenine cytokinins. The mass spectrometry of products dealt, of course, only with detectable spots. This analysis should be of value in determining the nature of the major compound seen in “cytokinin I” but does not bear on the possibility that a small amount of ribosylzeatin is present.

4. Attempts at volatilization of “cytokinin I” in a mass spectrometer were unsuccessful and did not give evidence for the presence of ribosylzeatin, which can be volatilized. Wood et al. (2) state that the volatilization test would detect ribosylzeatin content as low as 1%. They further state that more than this amount would be required to give the biological activity observed. The latter statement is extremely important and should be supported by data. Curves showing fresh weight responses of tobacco tissue to various concentrations of cytokinins are known to be quite complex. Therefore, establishing the amount of biological activity of “cytokinin I” in terms of ribosylzeatin equivalents requires a comparison of fresh weights of tobacco tissue obtained with several concentrations of “cytokinin I” to those obtained simultaneously with several concentrations of ribosylzeatin. Without such biological data, one cannot know the significance of any of the physical data for the question at hand.

5. Two major materials showing cell-division activity were obtained in their laboratory, whereas we detected predominantly the one compound. It is true that we investigated in detail only the one major compound. This compound was detected in initial extracts and accounted for more activity than did the other two compounds that we found. Wood and colleagues apparently base their evaluation of the relative importance of their two “cytokinins” on the weights of the final products and give no information concerning the relative cell-division activities of the two either in the final preparations or in the initial extracts. In their recent publication, Wood et al. (2) reported about equal yields of the two “cytokinin” preparations. Earlier (12), yields reported were much lower and the recovered amount of “cytokinin I” was higher than that of “cytokinin II.” Because such variations might arise from inconsistent efficiencies of recovery, it is best to determine the relative
importance of the active compounds in the crude extracts. This could be done by bioassying a chromatogram developed with a solvent system that clearly separates the active compounds. However, the finding of more than one main compound even in crude extracts would have no obvious bearing on the question of whether or not “cytokinins I” contains some ribosylzeatin.

6. Although ribosylzeatin may not have been separated from “cytokinins I,” it clearly was separated from “cytokinins II” in both the countercurrent distribution and in chromatographic systems employed. It is pertinent that the pattern of migration values for “cytokinins II” is very similar to that which we have observed for free zeatin (ref. 16 and unpublished data). However, our suggestion was that ribosylzeatin may be present in “cytokinins I” and we were not concerned with “cytokinins II.” The point therefore has no bearing on the question at hand.

7. We did not repeat the isolation procedure which they had employed to obtain the “cytokinin” preparations. We intentionally developed isolation procedures different from those used by Wood and colleagues. If the isolation procedures do not change the nature of the active material—and it is apparent that those we have used do not—then the main active substance (which we have shown to be ribosylzeatin) should be the same as determined with any other valid procedure.

8. We failed to mention the evidence showing that the adenine cytokinins and the “cytokinins” are functioning in very different ways to elicit biological responses in plant tissues; for example, we did not mention the effects on cAMP (adenosine 3′,5′-monophosphate). Even if the main compounds in the “cytokinin” preparations have some activity in cAMP metabolism, there is no proof that such activity controls cell division in the various cytokinin assays. Whether or not “cytokinins I” influences cAMP metabolism is of no significance in considering whether or not it contains some ribosylzeatin.

In conclusion, the evidence for the occurrence of ribosylzeatin in the crown gall tumor cells seems firm. The possibility that “cytokinins I” contains some ribosylzeatin which confers the cell-division activity is still open. Furthermore, the possibility that “cytokinins II” contains the free base, zeatin, should be considered even though zeatin has not been definitely identified in the tumor tissue extracts. The best proof that the compounds in the “cytokinin” preparations detected by physical means are actually the biologically active ones would be to determine their structures, synthesize them, and test them for cell-division activity. If success is not attained in this, the “cytokinins” could be examined further. For example, do their passage through a cation exchanger remove the biological activity? Can polyvinylpyrrolidone separate any components as determined by cell-division assays? Even if the synthetic compounds are obtained and prove to be active in cell division, however, one would still have to consider them of secondary importance in the tumor problem. Wood and Braun (17) already have stated that normal tissue produces “cytokinins” if the adenine type of cytokinins is supplied. Therefore, the likely conclusion would be that the tumor tissue produces “cytokinins” because it has made the adenine type of compounds such as ribosylzeatin. A striking difference between normal and tumor tissue in culture would be that the tumor tissue synthesizes enough zeatin compounds to grow, whereas the normal tissue does not.

I wish to thank Dr. Henry Wood of The Rockefeller University for a start of the Vinca rosea tumor tissue, Mr. John Schneider of Indiana University for the mass spectral studies, Ms. Robin Matthews for excellent technical assistance, and Dr. F. Skoog of the University of Wisconsin and Dr. N. J. Leonard of the University of Illinois for the sample of ribosyl-sis-zeatin synthesized in the latter’s laboratory. The work was supported by Grant GB 35232X from the National Science Foundation.