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Ribosyl-*trans*-Zeatin, A Major Cytokinin Produced by Crown Gall Tumor Tissue

(crystalline cell division factor/plant hormone/adenine derivative/*Vinca rosea*)

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ABSTRACT A cell division factor in crystalline form has been isolated from ethanolic extracts of cultured *Vinca rosea* L. crown gall tumor tissue. The crystalline material shows the melting point, mass spectrum, ultraviolet-light absorbancy spectra, solubilities, chromatographic migration values, ion-exchange behavior, and growth activities expected of ribosyl-*trans*-zeatin, that is, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine. This compound, which appears to be a major cell-division factor in the extracts, has also been crystallized from liquid media in which the tumor tissue had been growing and apparently was the only active compound in such media.

Tumor cells resulting from the infection of plants by *Agrobacterium tumefaciens* (Smith and Townsend) Conn may be obtained in a bacteria-free condition and cultured with continued cell division on synthetic media (1, 2). In contrast to normal cells, cells which have been fully converted to the tumorous state grow without the addition of plant hormones to the nutrient medium. Partially converted cells may require a supply of auxin but no cytokinin; both hormones are essential for the culture of normal cells (3). The possibility that tumor cells may be synthesizing supplies of hormones adequate for growth was supported years ago by demonstrations that crown-gall tumor tissues yield extracts which markedly promote cell division (4, 5). Furthermore, in 1954 Braun and Naf (5) showed that at least one active compound must be something other than a hormone of the auxin type. In that year, kinetin (6-furfurylaminopurine) was discovered (6); since it promotes cells division in the same type of tobacco tissue as that used by Braun and Naf for bioassay, it appeared possible that the nonauxinic factor from the tumor tissue is a substituted adenine. Considerable chromatographic data and some chemical evidence obtained by Tegley, Witham, and Krasnuk (7) definitely support this possibility. On the other hand, Wood and Braun and associates (8) have argued that the activities of the tumor tissue extracts cannot be due to substituted adenines, and Wood (9) recently has concluded that at least one factor is a substituted purinone. Although the two groups of investigators used different species of tumor cells, we felt that the factor in question likely would be common to both species. This expectation was supported by unpublished chromatographic observations in this and F. H. Witham's (private communication) laboratories. The data indicated that the species used by Wood and Braun yields cell-division factors having the same migration values as those observed with the naturally occurring adenine compounds known as zeatin and its derivatives. Because of the lack of agreement on the question, a program to isolate an active compound from an A6 line of *Vinca rosea* L. tumor tissue, kindly supplied by Dr. Henry Wood, was started in this laboratory. The result is that ribosyl-*trans*-zeatin, a substituted adenosine already known as a cytokinin, has been isolated and found to be responsible for at least a major portion of the cell division activity of extracts made from tumor tissue.

MATERIALS AND METHODS

Bioassay. Cell-division factors were detected and measured by means of the Acme soybean callus-tissue assay (10). A comparison of bioassays in which the extracts were sterilized by autoclaving with bioassays in which the material was added through a Millipore filter indicated that autoclaving did not change the levels of activity. Therefore, all media were routinely sterilized by autoclaving. Fresh weights of soybean tissue pieces obtained after a growth period of about 28 days indicated the amount of factor present in the medium.

Crystallization from Tissue Extract. The *V. rosea* A6 line of crown gall tumor tissue supplied by Dr. Wood of The Rockefeller University was grown on a White's medium modified as reported by others (7), except that all vitamins were at levels five times as high (upon recommendation of F. H. Witham). The tissues were cultured at 27° for 6–7 weeks. Immediately after harvest, the tissues were frozen. Although early tests indicated that cold (4°) and hot (80°) extracts showed the same biological activities, extraction was routinely made at low temperatures. Sufficient cold 95% ethanol was added to the frozen tissue to make a final concentration of 70% (actually somewhat higher since not all of the weight of the tissue was due to water). The mixture was allowed to stand overnight in a cold room before filtering through cotton. The liquid was squeezed from the tissue; this gave a clear, yellow solution which was then subjected to isolation procedures or used for chromatograms or for estimates of biological activity. This filtrate was quantitatively compared to ribosyl-*trans*-zeatin for cell division activity in the soybean callus-tissue assay. Estimates of activity varied from one bath to another but ranged from 2 to 3.4 μmol equivalents (0.7–1.2 mg) of the ribonucleoside per kg tissue. When added to a liter of assay medium, the extract from 25 mg of tissue or even less gave a detectable response.

The alcohol extract was run through a column containing 600 ml of the cation exchanger, Dowex 50W-X8, in the hydrogen ion form. Only traces of activity were ever detected in the effluent. After very thorough washing of the resin with 95% ethanol, materials were eluted with 2 liters of 6 N ammonium hydroxide in 50% ethanol; this procedure gave slightly less than complete recovery of activity. All operations with the

exchanger were performed in the cold room with previously cooled solutions. Elution with the ammonium hydroxide was made slowly and with vigorous mixing to prevent heating. That this precaution was effective was evident from the usual failure to detect free zeatin which can arise from its nucleoside quite easily if heating takes place. The eluent was reduced by use of an air stream to a volume of 70 ml, the temperature remaining below that of the laboratory. Most of the active material was then salted out into ethyl acetate by adding 6 g of K_2HPO_4 to each 10 ml of the aqueous solution and layering with the organic solvent. The aqueous layer was extracted seven times with equal volumes of ethyl acetate. After reduction of the combined ethyl acetate layers to 70 ml, the factor was extracted back into water by using six successive layerings of equal volume. The combined aqueous layers were evaporated to dryness and the material was then dissolved in ethanol and subjected to chromatography. Sequential chromatography was performed as follows with active material eluted from the indicated regions: (A) with water-saturated *sec*-butanol on 1 mm-thick layers of silica gel (11) (Merck PF-254) prepared in this laboratory (R_F 0.50–0.60), (B) with the same solvent on Whatman no. 1 paper (R_F 0.76–0.87), (C) with water on paper (R_F 0.70–0.80), (D) with the upper layer from a 4:1:2 mixture of ethyl acetate, *n*-propanol, and water (12) on a silica gel layer (R_F 0.36–0.44), (E) with the same solvent on paper (R_F 0.45–0.55), and then (F) with water on paper (R_F 0.72–0.80). The paper used in the last two steps had previously been exhaustively washed in alcohol and water. Active material was eluted with 95% ethanol. The final alcohol eluent was reduced to about 1 ml and permitted to evaporate very slowly. Crystals (melting range 177–179°) formed where the alcohol crept up the side of the 5-ml beaker. These were washed with a small volume of ethanol and then removed to another small beaker where there were dissolved in a small volume of ethanol. Crystals again formed when evaporation took place.

Crystallization from Culture Medium. Ultilizing information from F. H. Witham (private communication) that tumor cells leak hormones into an agar culture medium, we have succeeded in isolating a cell division factor by culturing *Vinca* cells in 13-liter carboys, each containing 10 l of medium without agar. Aeration was accomplished essentially according to the method of Tulecke and Nickell (13). After 3 weeks of culture, the liquid was filtered through a layer of cotton and the tissue discarded. Threshold activity was detected in the clear, colorless filtrate when used at a concentration as low as 0.2 ml/liter of assay medium. Since the threshold concentration of ribosyl-*trans*-zeatin in the assay is about 5×10^{-11} M (14), 1 liter of filtrate may contain up to the equivalent of 0.25 μmol (0.09 mg) of this nucleoside. The filtrate was run through a Dowex 50 cation-exchanger column and crystals were obtained by subjecting the eluted material to the same sequence of purification steps as described above for the tissue extracts. After the first crystallization the melting range was 178–180°.

Examination of Ribosylzeatin Isomers. The *cis* and *trans* isomers of ribosylzeatin were separately exchanged onto and removed from Dowex 50 resin by the procedures described above. After removal of the ammonium hydroxide, each isomer was examined for the presence of the other isomer by the method of Playtis and Leonard (11) in which a thin layer of silica gel (Merck product used here) is developed with a 9:1 mixture of chloroform and methanol. In each instance, only the starting isomer was detected (*cis* isomer at R_F 0.18 and *trans* isomer at R_F 0.12). The two isomers were also subjected to the ethyl acetate-water fractionation procedure. Examination by the chromatographic method showed each isomer to be unaffected. Each isomer was chromatographed with the systems used in isolation of the growth factor. The R_F values for the *cis* and *trans* compounds, respectively, were as follows for the indicated systems: (A) 0.56, 0.55; (B) 0.82, 0.81; (C) 0.75, 0.75; (D) 0.40, 0.38; (E) 0.51, 0.49; and (F) 0.75, 0.75.

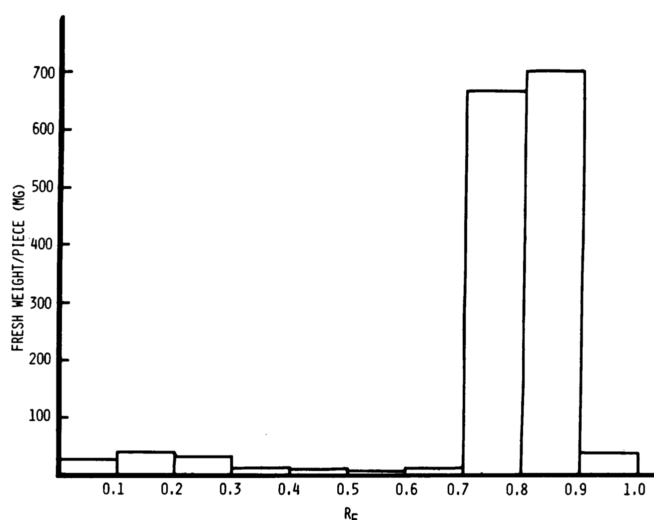

FIG. 1. Soybean callus-tissue bioassay of a chromatogram of crude ethanolic extract of tumor tissue. Chromatogram developed with water-saturated *sec*-butanol. An equivalent of 10 g of tissue per liter of assay medium was used.

RESULTS

Number of Active Compounds. Chromatography of the alcohol extract from the tumor tissue and subsequent bioassay yielded distribution patterns on the chromatograms very much like those previously seen with extracts of maize tissue known to contain zeatin derivatives (15). For example, on a paper chromatogram developed with water-saturated *sec*-butanol, activity was detected at R_F values around 0.2 and 0.8, the fast moving material being in much greater quantity (Fig. 1). The faster moving material, when rechromatographed on paper with water, was detected at R_F 0.7–0.8 but sometimes gave a bit of activity at R_F 0.5. Upon incubation with alkaline phosphatase, the slow moving material was converted to a substance with the same R_F as the fast moving factor. The pattern thus is exactly what one expects with a group of zeatin derivatives, with the most abundant material behaving like ribosylzeatin. Similar chromatography of the filtered medium from the carboys showed activity only at the position of the apparent nucleoside. In all other isolation and purification steps, including apparent exchange onto Dowex 50 resin as a cation, the fast moving material acted like ribosylzeatin.

Crystals from the Tissue Extract. An extract from 1200 g of tissue having an estimated total activity equivalent to 4.1 μmol (1.44 mg) of ribosyl-*trans*-zeatin was purified by the

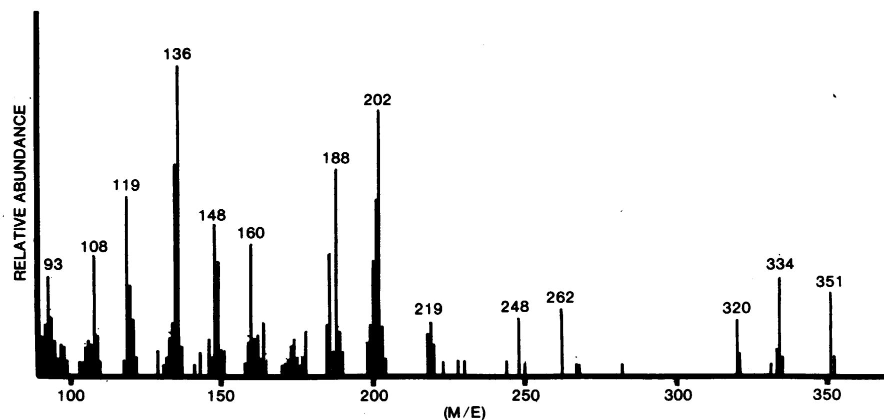

FIG. 2. Low-resolution mass spectrum of crystals isolated from *V. rosea* tumor tissue. The probe temperature was 200°. Much of the peak at 149 is known to be due to a constant contaminant in the Varian MAT CH-7 mass spectrometer.

procedures already given. Purified material obtained by this series of separations gave ultraviolet spectra identical to those of the synthetic ribosylzeatin. Assuming the factor to be ribosylzeatin and using a molar extinction coefficient of 1.9×10^4 at 268 nm, the yield after the last chromatographic step was estimated to be 1.41 μmol (0.494 mg). Further assuming the factor to be the *trans* isomer, this accounts for 34% of the beginning activity. Efficiency of recovery was also estimated by comparing growth responses on media containing several concentrations of the beginning extract and of the purified compound. Factor purified from 2.5 g of tissue, in one example, gave the same growth response as that given by the ethanolic extract from 0.77 g of tissue; the estimate of recovery of activity is therefore 31%. The two estimates therefore are in agreement. The crystals were colorless, had a melting point of 180–181° (recrystallization did not change the melting point), and gave the absorption spectra of ribosylzeatin (16) with maxima at 268 nm in 95% ethanol, at 268 and 217 nm in 0.1 N NaOH, and at 264 nm in 0.1 N HCl. The crystals showed activity in the soybean assay at concentrations as low as 5×10^{-11} M. The low resolution mass spectrum shown in Fig. 2 is in excellent agreement with that of ribosylzeatin (12, 14). Values from the high-resolution mass-spectral data which correspond to the peaks in the low resolution spectrum are given in Table 1, as are the closely corresponding values calculated for ribosylzeatin and its possible fragments. The migration values in the chromatographic systems used for isolation were those already listed for the *trans* isomer of ribosylzeatin, but these migration values were very nearly the same as for the *cis* isomer. However, when the crystals were examined with the system of Playtis and Leonard (11), only the *trans* isomer was detected, and this was true even when heavy applications of the material were made to the chromatograms. The crystalline material therefore is ribosyl-*trans*-zeatin or 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine.

TABLE 1. *Comparison of some masses measured for the isolated cell-division factor with masses calculated for ribosylzeatin*

Fragment composition	Calculated mass	Measured mass*
$C_4H_4N_4$	108.0436	108.0433
$C_5H_3N_4$	119.0358	119.0356
$C_5H_5N_5$	135.0545	135.0552
$C_5H_6N_5$	136.0623	136.0621
$C_6H_6N_5$	148.0623	148.0625
$C_7H_6N_5$	160.0623	160.0638
$C_6H_6ON_5$	164.0572	164.0567
$C_9H_8N_5$	186.0780	186.0772
$C_9H_{10}N_5$	188.0936	188.0931
$C_{10}H_{10}N_5$	200.0936	200.0930
$C_{10}H_{11}N_5$	201.1014	201.1007
$C_{10}H_{12}N_5$	202.1093	202.1101
$C_{10}H_{13}ON_5$	219.1120	219.1102
$C_{11}H_{14}O_2N_5$	248.1147	248.1140
$C_{12}H_{16}O_2N_5$	262.1304	262.1305
$C_{14}H_{18}O_4N_5$	320.1359	320.1330
$C_{15}H_{20}O_4N_5$	334.1515	334.1512
$C_{15}H_{21}O_5N_5$	351.1543	351.1535

* Measurements kindly supplied by Dr. A. L. Burlingame of the Space Sciences Laboratory, University of California, Berkeley. Measurements obtained with an A.E.I. MS-902 high resolution mass spectrometer.

Examination (11) of the liquids remaining after the two crystallizations showed the presence of the *trans* isomer of ribosylzeatin but not that of the *cis* isomer.

Crystals from the Culture Medium. The growth factor from seven carboys (total of 70 liters) was also purified to the crystalline state. After the last chromatographic step, the yield was estimated to be 4.48 μmol (1.7 mg), which accounts for about 27% of the initial activity and was in agreement with biological estimates. The crystals possessed precisely the characteristics mentioned above for the crystals from the tissue. The crystals from the two sources are therefore identical; the tissue apparently releases ribosyl-*trans*-zeatin into the culture medium. The liquids from which the crystals were obtained still contained some of the *trans* isomer but did not contain the *cis* form.

DISCUSSION

The data obtained with the crystalline material from two sources indicate quite precisely that the crystals are indeed those of ribosyl-*trans*-zeatin. The very close matching of the migration values for the main active substance in the crude extracts or the culture medium with the migration values of the purified material and of the synthetic ribonucleoside support the interpretation that the nucleoside is the major

active compound initially present. Further support is afforded by the high recovery (around 30%) of total activity in the form of the ribonucleoside even though considerable loss would be expected with each purification step. Absolutely no chromatographic evidence was obtained for the presence of compounds at any stage of purification other than those detected in the initial extracts. Furthermore, estimates of total activity made at each purification step provided no evidence for generation of new active compounds. To the contrary, loss of total activity occurred at each step. Nevertheless, one must examine the possibility that this particular compound was formed in some manner during isolation.

Since transfer ribonucleic acid may contain units capable of cytokinin action when separated from the macromolecules (17), one might suggest that the cell division factor was enzymatically generated from such a nucleic acid during the extraction or purification. This is unlikely for several reasons. The extract, made at low temperatures and consisting of more than 70% ethanol, had a pH of 6.2 when diluted to 50% ethanol; it therefore probably contained little if any of the nucleic acid and the enzymes which might break down the nucleic acid. Furthermore, in some assays we used 80% ethanol and in others made quick extractions with hot ethanol without influencing total activity. Additionally, the amount of ribosylzeatin recovered is rather high to have arisen during or after extraction from transfer ribonucleic acid (compare with yields from ribonucleic acid reported in ref. 12). Finally, the compound would not have been separated from the *cis* isomer by the isolation procedures nor formed from it; yet, transfer ribonucleic acid has to date been found to contain either only the *cis* form or a mixture of the two isomers (17, 11, 18).

Some objection can be made (19) to the use of the cation exchange resin. We therefore have made several attempts to isolate the active compound without using the resin, but the tissue extract contains substances which move closely with known ribosylzeatin in all of the solvent and chromatographic systems mentioned in this report. From various chromatograms at positions to which the nucleoside moves, we have obtained bands of material showing absorption maxima at slightly above 270 nm and minima near 250 nm. This material was biologically active, but much less so than ribosyl-*trans*-zeatin (concentration of the material was computed on the assumption of an extinction coefficient at 270 nm equal to that of the zeatin ribonucleoside). In one attempt at isolation, when this material in cold solution was very quickly put through a small cooled Dowex 50 column, absorbance at 270 nm was reduced from 3.0 to 2.8. The effluent showed qualitatively the same spectral characteristics observed before treatment with resin and the material moved to the same loci on chromatograms. However, *all* of the biological activity was removed by the exchanger. This activity was recovered by eluting with the ammonia-alcohol mixture and the eluate showed the spectrum and biological effectiveness exhibited by ribosylzeatin. The *Vinca* tissue therefore contains material which has solubility properties very close to those of ribosylzeatin and isolation by means of solvent fractionation may not succeed in separating the two. Migration values of crude active material on paper chromatograms were not altered by exchange onto and off the resin when checked with water-saturated *sec*-butanol (R_F 0.8–0.9); a 3:2 mixture of *tert*-butanol and water (R_F 0.8–0.9); a 4:1:1 mixture of *n*-butanol, water, and glacial acetic acid (ref. 9) (R_F 0.6—0.7); the upper layer of an 86:14:5 mixture of *n*-butanol, water, and concentrated ammonium hydroxide (ref. 12) (R_F 0.5–0.6); and the upper layer of a 4:1:2 mixture of ethyl acetate, *n*-propanol, and water (ref. 12) (R_F 0.4–0.6). Thus, the formation of the nucleoside on the resin seems unlikely, although not impossible. That the adenine nucleus is formed during the procedure seems very unlikely. Therefore, a reasonable conclusion is that the adenine nucleus is present before the material is applied to the resin and is in such form as to be exchanged onto the column and to give the biological activity observed. On the basis of this reasoning, the probability that the active material in the tumor tissue is an adenine derivative mainly in the form of the ribosylzeatin seems quite high.

This conclusion is at variance with the contention of Wood *et al.* (8) that the active material cannot be an adenine compound. The purification of the material tested by these investigators relied largely upon counter-current fractionation with one solvent system and some additional chromatography on paper or thin layer preparations (9). In view of our observations that the tissue contains compounds which migrate closely with synthetic ribosylzeatin, there seems a real possibility that complete purification was not achieved. The claim for purity of the preparation is based on the observation of a single ultraviolet-absorbing band or spot on chromatograms and on a symmetrical counter-current distribution curve. The single band or spot might occur with a mixture of compounds moving at the same rate such as we have observed in so many systems. The symmetrical curve might be found when a small amount of one material is mixed with another and both have equal solubilities and very similar absorption spectra. In concluding that the active material could not be of the adenine type, Wood *et al.* (8) emphasized that the preparation did not cause leafy gametophyte formation from *Funaria* protonemata. However, for this particular test, a rather low concentration of the material from tumor tissue was used and, if the active compound constituted only a fairly small portion of the material, the actual concentration would have been smaller still. No data were given, and apparently no information was available in the literature at the time of manuscript preparation, as to the sensitivity of this particular test to ribosylzeatin. It may be that the active material simply was tested at too low a concentration to evoke a good response. Thus, the test was inconclusive and no real proof was offered to rule out the presence of an adenine type of cell-division factor. The assignment of even a general chemical structure to the active compound in their preparation and the application of a new class name (cytokinesins) (20) to such a structure seem unwarranted at this time.

The relationship, if any, between the production of ribosylzeatin by bacteria-free tumor tissue and the production of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine by the tumor-initiating *A. tumefaciens* (21, 22) is obscure. However, a study of the system which synthesizes zeatin and of the particular steps activated may indeed tell us more about the conversion from the normal to the tumorous state.

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