

# Cytokinins in Seeds of Pumpkin

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## ABSTRACT

Extracts of seeds of pumpkin (*Cucurbita pepo* Linn.) contain three chromatographically distinguishable cytokinins which are held on Dowex 50-W and are extractable by ethanol and *n*-butanol. Two of the active factors are precipitable by silver nitrate at acidic pH. The chromatographic behavior and the spectral characteristics of one of these cytokinins are similar to those of zeatin. However, the  $R_F$  values of the other two active compounds do not match with those of any of the known natural cytokinins.

In recent years, considerable interest has been shown in the isolation and characterization of natural cytokinins. Several free cytokinins have been identified in higher plants. They are: zeatin or 6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine (14, 18), 9- $\beta$ -D-ribofuranosylzeatin (16), 9- $\beta$ -D-ribofuranosylzeatin-5'-phosphate (17), all first isolated from immature corn kernels, and dihydrozeatin, reported to occur in lupin seeds (11, 12). Two other cytokinins, namely a *cis* isomer of zeatin riboside and 6-( $\gamma$ , $\gamma$ -dimethylallyl)adenosine, have been found as constituents of soluble RNA of plant tissues (6, 7). From this laboratory, two preliminary reports were communicated dealing with the occurrence of cytokinins in developing seeds of watermelon and pumpkin (21, 22). This paper gives results of further work on cytokinins in seeds of pumpkin and our attempts to identify these factors.

## MATERIAL AND METHODS

Fruits of pumpkin (*Cucurbita pepo* Linn.) were collected from plants growing on the bank of river Jamuna, Delhi. Seeds were separated from the pulp, fixed in 95% ethanol (300 ml for 100 g of seeds) and stored at 4°. When required, the material was homogenized in a Waring Blendor and filtered, and the residue was twice extracted with 2 volumes of 95% ethanol. The ethanol extracts were pooled and evaporated *in vacuo* at about 45°. The crude extract thus obtained was partitioned thrice with 2 volumes of petroleum ether each time to remove pigments and fatty substances.

The aqueous extract, equivalent to 250 g, fresh weight, of seeds, was reduced to 62.5 ml by evaporation *in vacuo*, and its pH was adjusted to 5.5. It was then percolated through a 2.5 × 40 cm column of Dowex 50W-X8 (20-50 mesh, H<sup>+</sup> form); elution was done with 1 liter of 2 N ammonia. The eluate was evaporated *in vacuo* to remove ammonia and to reduce its volume further. The final drying was done in a VirTis lyophilizer, and the freeze-dried eluate was dissolved in a small quantity of distilled water.

Descending paper chromatography was mostly done on Whatman No. 3MM chromatography paper which had previously

been washed with 1 N formic acid by allowing the acid to run down the papers for 2 to 3 days. The fractions were applied as streaks on the side of the paper. The following solvent systems were used: (a) water-saturated *n*-butanol; (b) *n*-butanol-N ammonia, 10:7; (c) *n*-butanol-acetic acid-water, 12:3:5; (d) isopropanol-water, 4:1; (e) isopropanol-water, 4:1 (0.1 ml of concentrated ammonia per liter of the tank volume was kept at the bottom of the chamber); (f) boric acid, 0.03 M, pH 8.4; and (g) distilled water.

Electrophoresis was carried out on Whatman No. 1 chromatography paper. The following buffers were employed: (a) ammonium formate-formic acid, 0.05 M, pH 3.5, and (b) potassium dihydrogen orthophosphate-dipotassium phosphate, 0.05 M, pH 7.8.

The chromatograms as well as the strips of paper employed for electrophoresis were divided into 10 or more segments, each of which was eluted with water. The eluates were incorporated in the nutrient media to test their activity.

Tobacco pith callus (var. Wisconsin 38) was employed as the bioassay material. Fractions to be tested were added as supplements to the revised medium of Murashige and Skoog (26) containing 2 ppm IAA and 100 ppm inositol. Pieces of callus ranging in weight from 48 to 60 mg were used as the inoculum. The final fresh weights were determined after 3 to 4 weeks.

## RESULTS

The cytokinin level in pumpkin seeds reaches its maximum around 11 days after pollination and declines as the seeds mature further (Fig. 1). The crude extract of 11-day-old seeds induced about 12,000% increase in the fresh weight of the callus at a concentration equivalent to 10 g, fresh weight, of seeds per liter of medium (Fig. 2). After petroleum ether extraction, only the aqueous phase was active. Treatment of the aqueous extract with Dowex 50-W ion exchange resin resulted in its separation into an eluate and an effluent. The eluate enhanced the growth of the callus to a marked extent whereas the effluent was inactive (Fig. 2).

Paper chromatography of the Dowex 50-W eluate of seed extract (equivalent to 20 g, fresh weight, per liter of medium) employing Whatman No. 3MM paper in water-saturated *n*-butanol gave evidence for the existence of three cytokinins (Fig. 3a). Their  $R_F$  values were 0.17 to 0.23 (factor Ia<sup>1</sup>), 0.3 to 0.36 (factor II), and 0.78 to 0.85 (factor III). In other solvents, too, an essentially similar distribution of active factors was observed. In chromatograms developed in *n*-butanol-ammonia, the  $R_F$  values of the active factors were 0.1 to 0.12, 0.23 to 0.3, and 0.69 to 0.89 (Fig. 3b). In the *n*-butanol-acetic acid-water system, the cytokinins were located at  $R_F$  0.19 to 0.38, 0.48 to 0.54, and 0.66 to 0.93 (Fig. 3c). When isopropanol-water was used as the solvent,

<sup>1</sup> This compound is called Ia since it is different from factor I of watermelon seeds (22), which otherwise contain similar cytokinins as reported here.

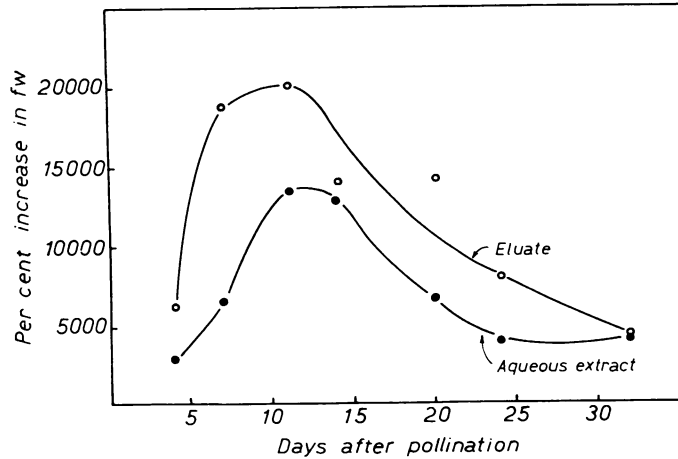


FIG. 1. Cytokinin activity of aqueous extract and the Dowex 50-W eluate obtained from seeds at different developmental stages tested at a level equivalent to 20 g of fresh seeds per liter of medium. Per cent increase refers to fresh weight of tobacco callus pieces.

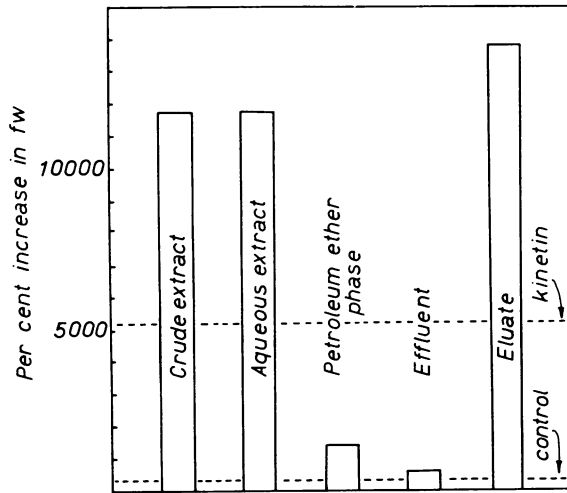


FIG. 2. Cytokinin activity of extracts of seeds, 11 days after pollination, determined at various stages of purification. Note that in some cases the extracts, tested at a level equivalent to 10 g of fresh seeds per liter of medium, are more active than kinetin at a 0.03 mg/liter concentration, which is optimal.

only two active zones were observed at  $R_F$  0.57 to 0.61 and 0.63 to 0.86 (Fig. 3d). However, when the same solvent was run in an atmosphere of ammonia, the eluate resolved into three active compounds which migrated to  $R_F$  0.44 to 0.54, 0.65 to 0.68, and 0.78 to 0.9 (Fig. 3e).

In all the solvents, the cytokinins cochromatographed—namely zeatin, 6-( $\gamma,\gamma$ -dimethylallylamino)purine (9, 10), and triacanthine (also a naturally occurring compound, but reported to be active only after autoclaving; References 13, 29)—migrated between  $R_F$  0.8 and 0.9. As can be seen, the  $R_F$  of factor III was quite similar to that of zeatin and triacanthine but different from 6-( $\gamma,\gamma$ -dimethylallylamino)purine. In another solvent system, namely *n*-butanol-HCl, factor III migrated to  $R_F$  0.72 to 0.89; here the  $R_F$  of zeatin was 0.78 and of triacanthine 0.67. Thus, it seems evident that, chromatographically, factor III resembles zeatin most closely.

In almost all the solvents, a heavy ultraviolet-absorbing band

was observed in the same region as factor II. This band gave a positive Gerlach and Döring test (6), claimed to be specific for detecting adenine and its derivatives. Chromatography and spectral data of the eluate of this band revealed the ultraviolet-absorbing compound to be adenosine. Three other ultraviolet-absorbing bands were identified to be due to adenine, guanine, and guanosine.

**Properties of Active Factors of Pumpkin Seeds.** When the aqueous extract of seeds, at pH 7.0, was partitioned with *n*-butanol, the major activity was observed in the *n*-butanol phase. Paper chromatography in water-saturated *n*-butanol revealed

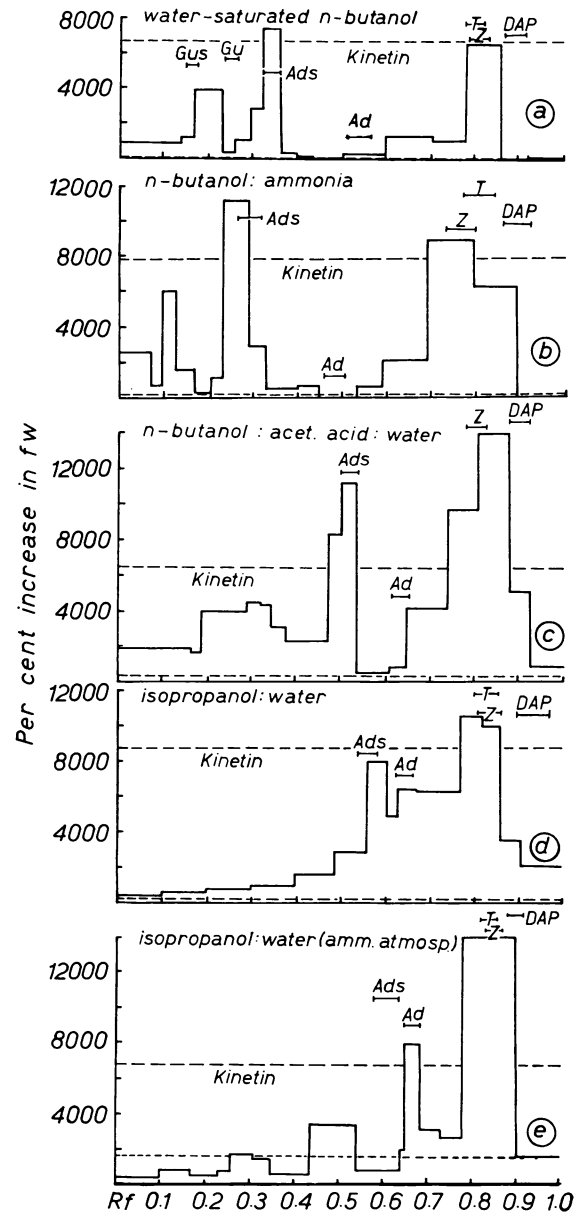


FIG. 3. Distribution of the activity of the Dowex 50-W eluate of seed extract after paper chromatography in different solvent systems. The short lines above the bars in the histogram represent the positions of adenine (Ad), adenosine (Ads), guanine (Gu), guanosine (Gus), zeatin (Z), triacanthine (T), and 6-( $\gamma,\gamma$ -dimethylallylamino)purine (DAP), cochromatographed. The upper broken line in each figure represents the extent of growth of callus on media containing kinetin and the lower, growth on the basal medium. Equivalent weight of tissue, 20 g/liter of medium.

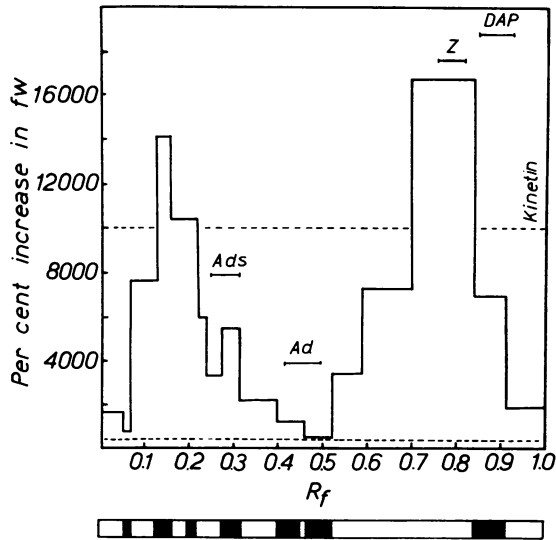


FIG. 4. Activity of the *n*-butanol phase of the aqueous extract (equivalent to 40 g, fresh weight, of seeds per liter of medium) after chromatography in water-saturated *n*-butanol.

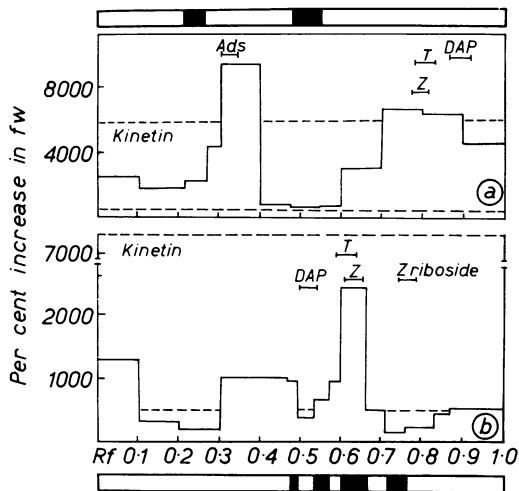


FIG. 5. a: Chromatography and bioassay of the Dowex 50-W eluate, after silver nitrate precipitation in water-saturated *n*-butanol (equivalent weight of tissue 20 g/liter of medium); b: rechromatography of factor III in distilled water. The solid areas in the horizontal bars show the location of ultraviolet absorbing bands in the two chromatograms.

the presence of all the three cytokinins in the butanol-extractable fraction of the seed extract (Fig. 4).

Most of the active material of pumpkin seed extract was precipitable by silver nitrate at pH 3.3 and could be redissolved in dilute hydrochloric acid (see Reference 22, for methodology). The silver nitrate-precipitated fraction of the Dowex 50-W eluate was chromatographed at a level equivalent to 20 g, fresh weight, of seeds per liter of medium using water-saturated *n*-butanol as the solvent (Fig. 5a). Both factors II and III were present in this fraction. It is not clear whether factor Ia was precipitated or not since no sharp peak of activity was observed at its expected position.

**Probable Identities of Active Factors.** To establish the identity of active factors of pumpkin, 3.9 kg of 11-day-old seeds were processed according to techniques described above, and the active material was chromatographed after precipitation by silver nitrate.

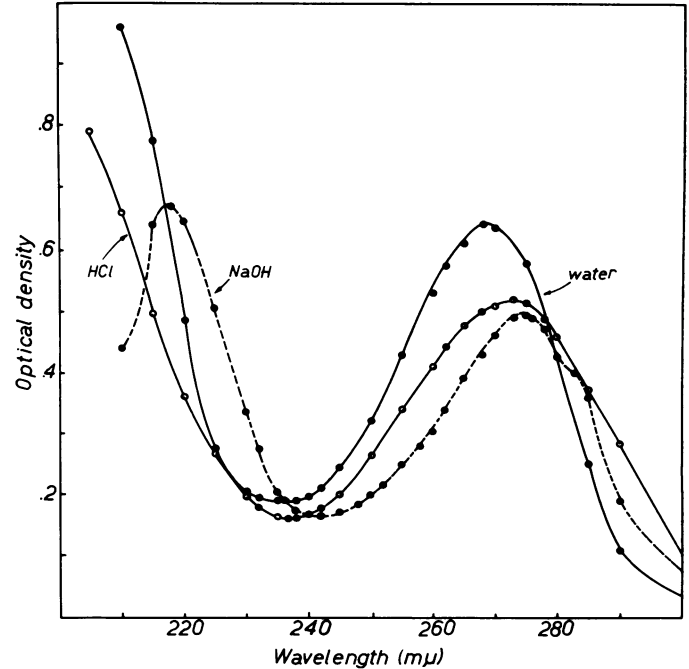


FIG. 6. Ultraviolet absorption spectrum of factor III in water, acid, and alkali. The addition of acid and alkali caused slight dilution of the original sample.

The zones corresponding to factor III were eluted with water, and the concentrated eluate was rechromatographed in the isopropanol-water (in ammonia atmosphere) system. Finally, when factor III was chromatographed with distilled water as the solvent, cytokinin activity, which was observed at  $R_F$  0.61 to 0.66, coincided with an ultraviolet-absorbing band (Fig. 5b). The  $R_F$  of zeatin was 0.63. Zeatin riboside, which otherwise moves to the same place as zeatin in most of the solvents, had a higher  $R_F$  (0.76) here. The spectral characteristics of the eluate of this band in water were as follows:  $\gamma_{max}$  268.5  $m\mu$ ;  $\gamma_{min}$  236  $m\mu$ . The addition of HCl resulted in a shift of the wave length of maximum absorption to 273  $m\mu$ . When the solution was made alkaline, maximum absorption was observed at 217 and 275  $m\mu$  and minimum at 242  $m\mu$ . Also, there was a shoulder between 280 and 285  $m\mu$  (Fig. 6). These spectral data are very similar to those of zeatin (15, 19). The amount of factor III on the basis of its ultraviolet absorption was calculated to be about 40  $\mu g$  in 1 kg of seeds.

As mentioned earlier, there was a close similarity in the mobility of adenosine and factor II on paper chromatograms. In electrophoretic runs at two pH values—3.5 and 7.8—again the active factor moved to the same place as adenosine. However, the fact that factor II is different from adenosine is clear from the following results: (a) factor II is precipitable by silver ions at acidic pH<sup>2</sup>; (b) in certain solvents such as isopropanol-water (in ammonia atmosphere), factor II moves more slowly than does adenosine; and (c) adenosine itself shows only very weak cytokinin activity. In order to obtain an authentic ultraviolet absorption spectrum of factor II, therefore, it became essential to separate this cytokinin from adenosine. In one experiment, factor II was fractionated through Dowex 1 ( $Cl^-$  form) according to the method of Cohn (3). Five-milliliter fractions were collected, and their optical density was measured at 260  $m\mu$ . To test cytokinin activity, 10 to 20 fractions were mixed, adjusted to pH 7.0, and extracted with double volumes of *n*-butanol. The butanol phases were evaporated, and the residues were incorporated into the

<sup>2</sup> In our experiments, for some reason not clear to us, small quantities of adenosine were also precipitated.

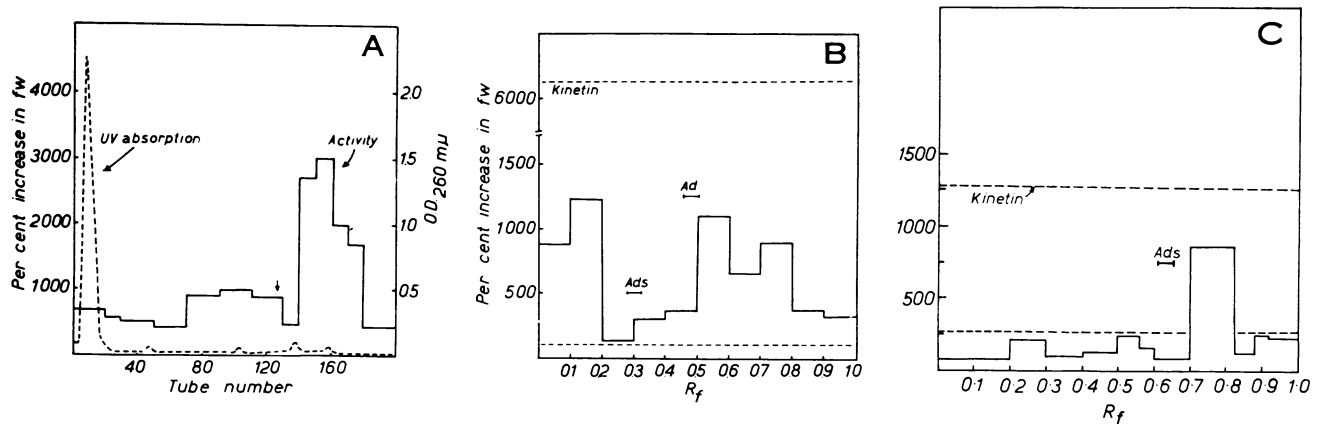


FIG. 7. a: Fractionation of factor II (equivalent to 36 g, fresh weight, of seeds) through Dowex 1 (Cl<sup>-</sup> form) resin. The resin column was eluted with a buffer the composition of which was 0.02 N ammonia-0.025 M NH<sub>4</sub>Cl. At the point indicated by the arrow, the molarity of NH<sub>4</sub>Cl in the buffer was raised to 0.1. Fractions were tested at a level equivalent to 20 g, fresh weight, of seeds per liter of medium. The increase in fresh weight of tobacco callus in the basal medium was 255%. b: Paper chromatography of fractions 140 to 180 of the experiment shown in part a in water-saturated *n*-butanol. The fractions were earlier combined and extracted with *n*-butanol, and the butanol phase was employed for chromatography. c: Rechromatography of factor II in 0.03 M boric acid, pH 8.0.

nutrient media. The results of this experiment are shown in Figure 7a. The first 50 ml of the eluate, which included the unadsorbed substances, contained adenosine, and thus most of the ultraviolet absorbing material: there was hardly any stimulation of growth in media supplemented with these fractions. The major activity appeared just after elution with the second buffer was begun (tubes 140–180). However, when the contents of these tubes were combined and rechromatographed in water-saturated *n*-butanol, instead of one, three peaks of activity were observed at R<sub>F</sub> values 0 to 0.2, 0.5 to 0.6, and 0.7 to 0.8 (Fig. 7b). Also, there was a considerable loss of activity. Therefore, although treatment with Dowex 1 separated the active factor from adenosine, it presumably resulted in chemical alteration of the original compound. Better separation could be achieved by successively chromatographing factor II (obtained from 3.9 kg of fractionated seeds) first with boric acid (0.03 M, pH 8.4) followed by isopropanol–water in an atmosphere of ammonia. With boric acid as the chromatographic solvent, activity was observed at R<sub>F</sub> 0.7 to 0.82 whereas the R<sub>F</sub> of adenosine was 0.63 (Fig. 7c). When the compound present at the former zone was eluted and rechromatographed in the isopropanol–water system (in ammonia atmosphere) there was no longer any ultraviolet-absorbing material in the active region, providing unequivocal evidence for the fact that factor II is different from adenosine. However, when the purified factor was subjected to further chromatography, the activity could not be correlated with any ultraviolet-absorbing band on the chromatogram.

## DISCUSSION

From the data presented in this paper it is evident that pumpkin cytokinins are relatively polar, as they are soluble in ethanol. Furthermore, they appear to be basic in nature, as they are absorbed on Dowex 50-W, a cation exchange resin, and can be eluted by ammonia. However, the cytokinin activity in pumpkin seed extract must be largely due to compounds which are either weakly polar or which possess nonpolar groups in addition to the polar ones since, upon partitioning an aqueous extract (obtained by concentrating the ethanol extract) with *n*-butanol, the bulk of activity migrates to the *n*-butanol phase. Two of the cytokinins (factors II and III) are precipitable by silver ions at acidic pH, and this strongly suggests that they are purine derivatives. Since the extract which was not fractionated through Dowex 50-W contained active factors with R<sub>F</sub> values similar to those in the

chromatographed Dowex 50 eluate, it is concluded that the cytokinins occur naturally *per se* and are not artifacts.

The chromatographic properties as well as ultraviolet spectral characteristics of factor III are similar to those of zeatin. Dihydrozeatin, the cytokinin isolated from lupin seeds, also resembles zeatin in these respects (11, 12). However, the *Lupinus* factor is soluble in ether (24) whereas factor III isolated from pumpkin seeds as well as zeatin<sup>3</sup> is not. Thus we believe that factor III is probably zeatin.

As compared to factor III, the movement of the second factor is slow in all the solvents. This cytokinin is very similar to adenosine in its mobility on paper chromatograms. In electrophoresis, too, it was found to move to the same spot as adenosine. However, the two can be separated by repeated chromatography. It is interesting to note that factor II has R<sub>F</sub> values very different from those of the naturally occurring cytokinins cochromatographed: zeatin, triacanthine, and 6-( $\gamma$ , $\gamma$ -dimethylallylamino)purine. It is clear that this cytokinin cannot be zeatin ribonucleoside or zeatin ribonucleotide since the former compound moves close to zeatin in most solvents and the latter does not migrate significantly in a solvent like water-saturated *n*-butanol (26). The possibility of factor II being dihydrozeatin can also be ruled out because dihydrozeatin has R<sub>F</sub> values similar to those of zeatin (11, 12). The other naturally occurring cytokinins—6-( $\gamma$ , $\gamma$ -dimethylallyl) adenosine, *cis* isomer of zeatin riboside, and 6-(3-methyl-2-butenylamino)-2-methylthio-9- $\beta$ -ribofuranosyl purine—have been thus far isolated only as constituents of transfer RNA of yeast, bacteria, and higher plants (1, 2, 4, 6–8, 20, 28, 30). However, since factor II is precipitable by silver ions at acidic pH, it is unlikely that this cytokinin could be similar even to any of them. Thus, we believe that factor II is a new cytokinin in plants. Although it would be interesting to know the chemical nature of this compound, unfortunately at present we can say little about its identity. Factor II appears to be a purine derivative as it is precipitable by silver ions at acidic pH. This property also indicates that the 9-position on this compound is free. It is likely that a side chain similar to that in zeatin may be present at the 6-position which must be more polar than that in zeatin itself. However, these suggestions must remain tentative until further work is done and sufficient amount of factor II can be isolated to obtain an ultraviolet absorption spectrum.

<sup>3</sup> See Reference 25; the factor discussed in this paper was later identified as zeatin.

Factor Ia, which represents only a small part of the total cytokinin activity of pumpkin seeds extract, also has  $R_F$  values different from those of the cytokinins isolated and reported in the literature thus far. However, at the moment it is not possible to offer any comment regarding the nature of this cytokinin since it is not precipitable by silver ions at acidic pH. It may be observed here that in extracts of the lot of seeds which we used for our earlier experiments (21), we did not observe the presence of this active factor. This could be either due to the fact that in the experiments reported earlier the paper chromatograms were divided into rather large segments and factors II and Ia got merged or due to differences in the batch of the material.

Lastly, a brief mention may be made of the level of cytokinins in relation to seed growth. We propose to publish the details elsewhere, but we can say here that the level of cytokinins is low in both very young and old seeds.

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