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COMPARISON OF REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH SEPHADEX LH-20 FOR CYTOKININ ANALYSIS OF TOMATO ROOT PRESSURE EXUDATE*

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SUMMARY

Rapid preparative purification and fragmentation of a cytokinin complex from tomato root pressure exudate is achieved using Bondapak C_{18}/Porasil B, a preparative reversed-phase high-pressure liquid chromatography column packing material. Bondapak C_{18}/Porasil B achieved the same resolution of the cytokinin complex from tomato root pressure exudate as Sephadex LH-20 in approximately one-thirtieth of the separation time.

The tomato root pressure exudate was found to contain at least eight cytokinin-like components. The total cytokinin activity of the root pressure exudate was estimated to be between 17.0 and 26.0 μg of kinetin equivalents per liter of exudate.

INTRODUCTION

High-pressure liquid chromatography (HPLC), within the last five years, has been intensively studied and used in such areas as medical physiology, pharmacology, organic synthesis, and biochemistry. Only recently has HPLC captured the attention of plant hormone physiologists[1–5]. Because of its speed, efficiency, and ease of quantification[6–8], HPLC offers many advantages for the purification and identification of plant hormones.

The major research efforts in most fields have been directed at improving the capabilities of HPLC for analytical analysis. All reported uses of HPLC for plant hormone analysis have been analytical in nature[1,2,5]. Few studies have been published in any field dealing with the preparative capabilities of HPLC, even though the major uses of classical column chromatography are largely preparative in nature. In the past, the cytokinins have been purified from plant material primarily by paper chromatography[9–17], ion-exchange chromatography[10,13,14,18], thin-layer chromatography[14,15,19–21], and conventional column chromatography using Sephadex LH-

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20[20,22–31]. The need for a rapid preparative purification of plant hormones is evidenced by the classical forms of chromatography being employed.

Preparative HPLC requires a totally porous packing. For ease of operation and column stability, permanently bonded stationary phases are desirable. In an earlier paper[1], the use of permanently bonded pellicular ion-exchange packings for plant hormone analysis was explored. It was found that they lacked the capacity to handle crude plant samples. At the time of this present study no permanently bonded, porous body support, ion-exchange packing materials were commercially available. The only porous packings available were adsorption packings and a reversed-phase liquid partition packing, Bondapak C_{18}/Porasil B (Waters Ass., Milford, Mass., U.S.A.). The reversed-phase liquid partition packing was chosen to reduce the possibilities of sample alteration, a common problem with adsorption chromatography.

Bondapak C_{18}/Porasil B is a permanently bonded, hydrolytically stable HPLC column packing material. The primary mode of separation is reversed-phase liquid partition achieved by chemically bonding a non-polar aliphatic functional group onto the external surface of the porous spherical silica bead[32]. The silica support has a surface area of 125–250 m^2/g of packing and has pore diameters of 100–200 Å. The silica support also functions as a solid adsorbent and as a molecular-size separation packing. Thus, three chromatographic modes of separation may occur when using Bondapak C_{18}/Porasil B.

Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) has been used for the purification of cytokinins from plant extracts. Sephadex LH-20 is a hydroxypropylated version of Sephadex G-25 and is commonly used for gel filtration with organic solvents. Sephadex LH-20 not only separates substances according to size, but also exhibits a number of gel–solute interactions which can be used for partition and adsorption chromatography. For the separation of cytokinins using Sephadex LH-20, plant hormone physiologists have used a mobile phase of either distilled water[20,23] or, more commonly, a mixture of water and a lower alcohol, usually 35% ethanol (v/v)[13,22,24,26–29,31]. In this study, 35% ethanol (v/v) was used as the mobile phase. Under these mobile phase conditions, partition and adsorption chromatography are considered to be the predominant modes of separation, with gel filtration playing little or no role[33].

The objectives of this investigation were to characterize the cytokinin content of the root pressure exudate of tomato and to compare preparative reversed-phase HPLC with Sephadex LH-20 for the ease and efficiency of purifying cytokinins for bioassay.

METHODS AND MATERIALS

Plant material

Tomato plants (*Lycopersicon esculentum* Mill. var. New Yorker) were grown in a greenhouse in 13-cm plastic pots containing a 2:1:1 soil–peat–sand mix for 10 to 12 weeks under a 16-h photoperiod. The natural photoperiod was supplemented by 9 h of illumination with high-intensity mercury-vapor lamps producing 240 microeinsteins m^{-2} sec^{-1} at plant height. The greenhouse temperatures were 18° at night and 24° throughout the day. The plants were fertilized once a week with 200 ml of a 200 ppm solution of 20:20:20 commercial fertilizer.

Each plant was decapitated immediately below the cotyledonary node and root pressure exudate collected under reduced pressure of approximately 350 mm of Hg for 70 h in 10-h increments. The root pressure exudate was stored frozen until analysis, and will be hereafter referred to as exudate.

Extraction procedure

The collected exudate was pooled, filtered, and taken to dryness by flash evaporation at 35–40°. The residue was redissolved in deionized distilled water, filtered and adjusted to pH 9.0 with 1 *N* KOH, and partitioned seven times against equal volumes of water-saturated redistilled *n*-butanol as indicated in Fig. 1. The aqueous phase of the pH 9.0 partition was adjusted to pH 2.5 with 1 *N* HCl and partitioned seven times against water-saturated redistilled *n*-butanol. This partition scheme fragmented the exudate into three portions: (1) *n*-butanol pH 9.0 phase, (2) *n*-butanol pH 2.5 phase, and (3) aqueous pH 2.5 phase. The *n*-butanol pH 9.0 phase was divided and chromatographed by conventional liquid chromatography (LC) and HPLC. All the phases were subjected to HPLC and were first microfiltered through a 5-μm pore size Millipore PTFE filter on top of a 0.2-μm-pore size Fluoropore Millipore Filter into 10-ml erlenmeyer flasks. Two 1.0-ml rinses of 5% methanol in 0.05 *N* acetic acid were used to sequentially wash each test tube and filter assembly. The washes were added to the original filtrates.

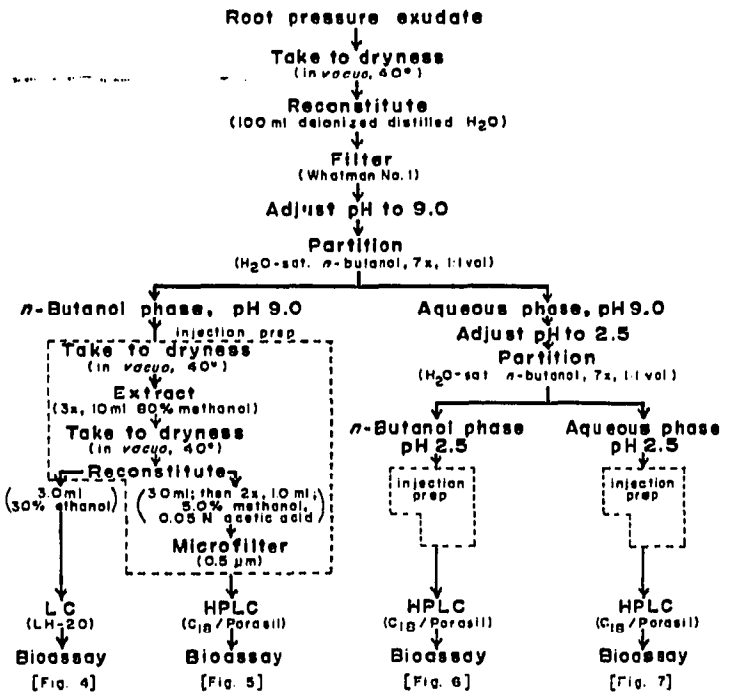

Fig. 1. Partition scheme for the fractionation and purification of the cytokinin content of tomato root pressure exudate. The injection preparation (in dashed box) was the same for all HPLC samples.

Chromatography

Liquid chromatography. A 100 × 1.27 cm I.D. glass column (Chem-Inert) was slurry packed with Sephadex LH-20. Three milliliters of sample were added to the column and eluted with 35% (v/v) ethanol in water at a flow-rate of 0.2–0.3 ml/min. The solvent flow was controlled by using a Millipore pressure reservoir with 3–5 p.s.i. nitrogen. The transmittance of the effluent at 254 nm was monitored with a UV detector (LKB Uvicord I).

High-pressure liquid chromatography. Two 1.0 m × 6.5 mm I.D. stainless-steel columns were packed with a reversed-phase packing material (Waters Ass. Bondapak C_{18}/Porasil B) by the tap-fill method described by Kirkland[34]. After sample injection through a 5-ml external loop injection valve (Valco 7000 series), gradient elution was accomplished by using acetate buffer and methanol delivered by two constant-flow non-pulsed high-pressure pumps (Waters Ass., Model 6000) controlled by a solvent programmer (Waters Ass., Model 660). The solvent flow-rate was 9.9 ml/min at 1500 to 3000 p.s.i. The detection system was a UV monitor (Chromatronix Model 230) fitted with a 20-μl flow cell (Chromatronix MB-100).

Bioassay

The fractions from the two columns were taken to dryness before reconstituting the residue in phosphate buffer. For the fractions from the Bondapak column, the samples were dried until all odors of acetic acid were removed. Cytokinin-like activity of collected column fractions was detected and quantified using the cucumber cotyledon bioassay described by Fletcher and McCullagh[35].

Results of the bioassay are presented as histograms. The absorbance values measured the amount of chlorophyll produced by the cucumber cotyledons for each fraction. The amount of chlorophyll produced was proportional to the logarithm (base 10) of the concentration of cytokinin-like material eluted. The absorbance values were corrected by subtracting the chlorophyll absorbance of the control tissue. Negative values indicate that specific fractions had less chlorophyll than did control tissue. The right-hand ordinate expresses absorbance above control in terms of kinetin equivalents in micrograms per liter of exudate found in each fraction.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF CYTOKININ RETENTION ON SEPHADEX LH-20 AND BONDAPAK C_{18}/PORASIL B

k' = Capacity factor; N = number of theoretical plates; HETP = height equivalent to a theoretical plate; io^{6}Ado = zeatin riboside; io^{6}Ade = zeatin; i^{6}Ado = isopentenyl adenosine [also known as 6-(γ,γ,-dimethylallylamino)-purine riboside]; i^{6}Ade = isopentenyl adenine [also known as 6-(γ,γ-dimethylallylamino)-purine].

Cytokinin	*k'*		*N*		*HETP (mm)*	
	LH-20	*C_{18}/P*	*LH-20*	*C_{18}/P*	*LH-20*	*C_{18}/P*
io^{6}Ado	2.5	3.4	1,024	1,089	0.98	1.84
io^{6}Ade	3.3	4.1	1,104	784	0.91	1.81
i^{6}Ado	4.4	5.4	1,315	3,009	0.76	0.66
i^{6}Ade	5.9	6.5	1,638	3,136	0.61	0.64

RESULTS AND DISCUSSION

The *n*-butanol pH 9.0 phase of tomato exudate was purified for bioassay by LC and HPLC using Sephadex LH-20 and Bondapak C_{18}/Porasil B columns, respectively. The distribution of cytokinin-like activity in the various fractions of the *n*-butanol pH 9.0 phase and the retention characteristics of known cytokinin standards were the basis of comparison of the two chromatographic techniques.

The chromatographic behavior of zeatin, zeatin riboside, isopentenyl adenine, and isopentenyl adenosine on Sephadex LH-20 and Bondapak C_{18}/Porasil B is presented in Figs. 2 and 3, respectively. It can be seen that the order of elution was the same from the two columns, indicating similar modes of separation for both packing materials. Table I contains data which summarize and compare the fundamental column parameters involved in the cytokinin separations presented in Figs. 2 and 3. It is apparent that the resolution obtained by the two columns was essentially the same and that 100% separation of all components was obtained (Table II). The chromatographic technique using the Sephadex LH-20 packing material conformed to those reported in the literature[22–31,33].

The optimized mobile phase for the Bondapak C_{18}/Porasil B column consisted of a linear gradient starting with 0.2 *N* acetate buffer at pH 2.8 and terminated with 100% methanol. The gradient was delivered in 1 h at a flow-rate of 9.9 ml/min. The acetate buffer was used to stabilize pH and to overcome shifts in capacity factor (k') associated with cytokinin sample mass. It was found that the k' for zeatin was pH dependent, thus requiring a buffer. The functional groups within the pores of Bondapak C_{18}/Porasil B were not deactivated in the commercial preparation[32], and appeared to act as adsorption sites. The buffer seemed to mask these adsorption sites and stabilized k' for sample masses of cytokinin standards ranging from 100 ng to 100 μg per injection and for sample volumes ranging from 20 μl to 5 ml. Neither peak geometry nor resolution was significantly affected by using injection volumes within this range. Sample masses of up to 250 mg of partitioned exudate gave reproducible histograms of cytokinin-like activity and several of the major peaks reproducibly corresponded

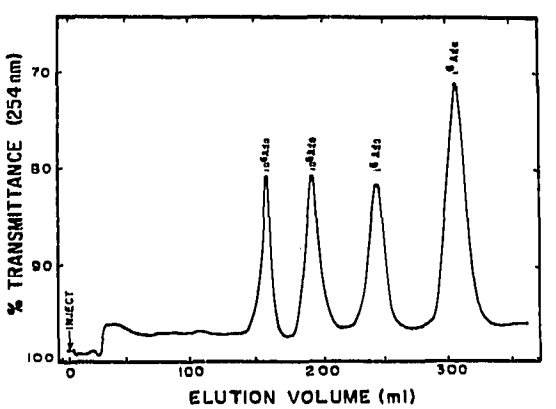

Fig. 2. A chromatogram of the retention characteristics of zeatin riboside (io^{6}Ado), zeatin (io^{6}Ade), isopentenyl adenosine (i^{6}Ado), and isopentenyl adenine (i^{6}Ade) on Sephadex LH-20. Mobile phase, 35% ethanol; column, 100 × 1.27 cm I.D.; flow-rate, 0.25 ml/min; injection volume, 2.0 ml.

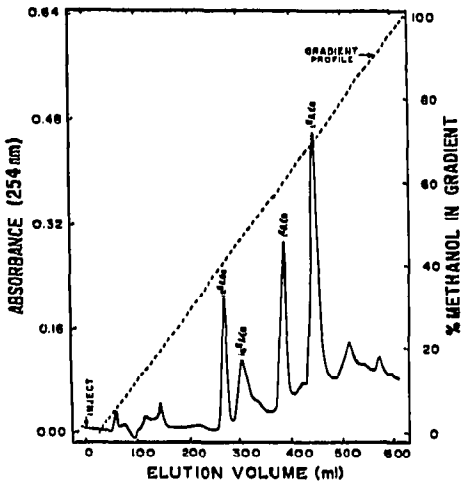

Fig. 3. A chromatogram of the retention characteristics of zeatin riboside (io^{6}Ado), zeatin (io^{6}Ade), isopentenyl adenosine (i^{6}Ado), and isopentenyl adenine (i^{6}Ade) on Bondapak C_{18}/Porasil B. Mobile phase, linear gradient of 0.2 *N* acetate buffer at pH 2.8 to 100% methanol; elution time, 1 h; two connected columns, 1.0 m × 6.5 mm I.D. each; flow-rate, 9.9 ml/min at 1500 to 3000 p.s.i.; injection volume, 5 ml.

to those of the cytokinin standards. This implied that sample sizes were within the limits of the linear capacity of the column.

Injections of large sample volume were possible because of the following: (1) the relatively large internal diameter of the column (6.5 mm); (2) the totally porous packing material; and (3) the composition of the mobile phase at the time of sample injection which allowed almost complete retention of the cytokinin complex. In preparative or large-scale LC, large sample volumes and dilute solutions increase the linear capacity of the column compared with that obtained when using small injection volumes of concentrated solutions[7].

Isopropanol, methanol, and ethanol were all found to be adequate for the nonpolar modifier of the mobile phase. Isopropanol was the least desirable of the three solvents because of its high viscosity. Although isopropanol has a desirable elutrophic value (0.84 E^0 Al_2O_3), it imposes definite flow restrictions whether using a constant-flow or constant-pressure chromatograph. A mobile-phase velocity of 0.5 cm/sec

TABLE II

RESOLUTION AND RELATIVE RETENTION ACHIEVED FOR CYTOKININ STANDARDS SEPARATED BY SEPHADEX LH-20 AND BONDAPAK C_{18}/PORASIL B

R_s = Resolution factor; α = relative retention; io^{6}Ado = zeatin riboside; io^{6}Ade = zeatin; i^{6}Ado = isopentenyl adenosine; i^{6}Ade = isopentenyl adenine.

Column packing	R_s			α		
	io^{6}Ade–io^{6}Ado	*io^{6}Ade–i^{6}Ado*	*i^{6}Ado–i^{6}Ade*	*io^{6}Ade–io^{6}Ado*	*io^{6}Ado–i^{6}Ado*	*i^{6}Ado–i^{6}Ade*
LH-20	1.7	2.0	2.3	1.3	1.3	1.3
C_{18}/Porasil B	1.2	2.8	2.1	1.2	1.3	1.2

TABLE III

MEAN OF RETENTION TIMES OF CYTOKININ STANDARDS SEPARATED ON BONDAPAK C_{18}/PORASIL B

Mobile phase, 0.05 *N* acetic acid to 0.05 *N* acetic acid in ethanol 95% (v/v); gradient, linear, 1 h; flow-rate, 9.9 ml/min.

Cytokinin	*Retention time (min)* *
Zeatin riboside	20.10 ± 0.19
Zeatin	25.67 ± 0.74
Isopentenyl adenosine	31.20 ± 0.22
Isopentenyl adenine	43.48 ± 0.34

* ± Standard deviation based on six observations.

(equivalent to a flow-rate of 9.9 ml/min) proved to be a good compromise between pump capability, resolution, peak height for detector sensitivity, and separation time.

Optimization of the mobile phase yielded reproducible retention times for the cytokinin standards. Table III contains the means and standard deviations of the retention times of the cytokinin standards run on Bondapak C_{18}/Porasil B. The means were based on six observations run over a two-week interval with crude plant samples applied between runs and with new batches of the mobile phase.

The Sephadex LH-20 fragmented the *n*-butanol pH 9.0 phase of the partitioned exudate into three major (statistically significant) and four minor fractions having cytokinin-like activity (Fig. 4, Table IV). Bondapak C_{18}/Porasil B resolved from the same phase five major (statistically significant) and two minor fractions having cyto-

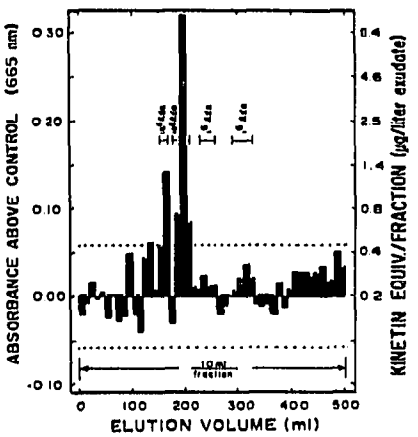

Fig. 4. Fractionation of cytokinin-like activity in the *n*-butanol pH 9.0 phase of partitioned tomato root pressure exudate on a Sephadex LH-20 column. Bars indicate cytokinin-like activity in the cucumber cotyledon bioassay. Column, 100 × 1.27 cm I.D.; mobile phase, 35% ethanol; sample volume, 3.0 ml containing 1 liter equivalent of root pressure exudate; flow-rate, 0.25 ml/min. Bars extending through the dashed line are significantly different from the control at the 95% levels using Dunnett's *t* test for multiple comparisons[42,43]. Bracketed lines indicate elution zones for the following cytokinin standards: zeatin riboside (io^{6}Ado), zeatin (io^{6}Ade), isopentenyl adenosine (i^{6}Ado), and isopentenyl adenine (i^{6}Ade).

TABLE IV

ELUTION ZONES AND KINETIN EQUIVALENTS OF CYTOKININ-LIKE COMPONENTS IN THE *n*-BUTANOL pH 9.0 PHASE OF PARTITIONED TOMATO ROOT PRESSURE EXUDATE FRACTIONATED ON SEPHADEX LH-20

Elution zone (ml)	*Kinetin equivalents (μg/liter exudate)*
90–100	0.4*
130–140	0.9
160–170	1.7
180–210	11.7
220–250	0.8*
300–340	0.9*
480–500	1.0*

* Not significantly different from the control at the 95% level using Dunnett's *t* test for multiple comparisons[42,43].

kinin-like activity (Fig. 5, Table V). These minor fractions must be seriously considered as zones of cytokinin-like activity either because of near statistical significance or because of elution in zones of known naturally occurring cytokinins. Tables IV and V contain the data for the elution zones of the biologically active fractions and their estimated kinetin equivalents for Sephadex LH-20 and Bondapak C_{18}/Porasil B. Sephadex LH-20 separated seven biologically active fractions containing a total of

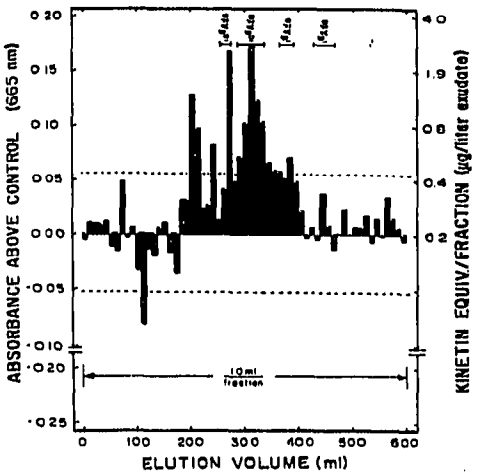

Fig. 5. Fractionation of cytokinin-like activity in the *n*-butanol pH 9.0 phase of partitioned tomato root pressure exudate on a Bondapak C_{18}/Porasil B column. Bars indicate cytokinin-like activity in the cucumber cotyledon bioassay. Columns, as in Fig. 3; mobile phase, linear gradient, 0% methanol, 0.2 *N* acetate buffer at pH 2.8 to 100% methanol; elution time, 1 h; flow-rate, 9.9 ml/min; sample volume, 5 ml containing 1 liter equivalent of root pressure exudate. Bars extending through the dashed line are significantly different from the control at the 95% levels using Dunnett's *t* test for multiple comparisons[42,43]. Bracketed lines indicate elution zones for the following cytokinin standards: zeatin riboside (io^{6}Ado), zeatin (io^{6}Ade), isopentenyl adenosine (i^{6}Ado), and isopentenyladenine (i^{6}Ade).

TABLE V

ELUTION ZONES AND KINETIN EQUIVALENTS OF CYTOKININ-LIKE COMPONENTS IN THE *n*-BUTANOL pH 9.0 PHASE OF PARTITIONED TOMATO ROOT PRESSURE EXUDATE FRACTIONATED ON BONDAPAK C_{18}/PORASIL B

Elution zone (ml)	*Kinetin equivalents (μg/liter exudate)*
70–80	0.3*
190–230	2.1
240–260	0.9
270–290	1.8
300–370	4.4
380–400	1.0
430–460	0.3*

* Not significantly different from the control at the 95% level using Dunnett's *t* test for multiple comparisons[42,43].

17.4 μg of kinetin equivalents per liter of exudate as compared with 10.8 μg for seven biologically active fractions isolated by Bondapak C_{18}/Porasil B. The discrepancy in the total kinetin equivalents isolated by the two methods occurs in the elution zone corresponding to that of the zeatin standard. From the Sephadex LH-20 column, 11.7 μg kinetin equivalents per liter were detected for the elution zone of zeatin as compared with 4.4 μg kinetin equivalents per liter from the Bondapak C_{18}/Porasil B. A possible explanation for this discrepancy is that an inhibitory substance(s) had a retention zone similar to zeatin, partially masking the biological activity associated with this fraction from Bondapak C_{18}/Porasil B (Table IV). Alternatively, Bondapak C_{18}/Porasil B may be fragmenting this fraction into components such as *cis*- and *trans*-zeatin, causing a larger elution zone, thus reducing the amount of activity associated with each fraction which would cause errors in quantification in the bioassay. The kinetin equivalents associated with the elution zone for zeatin riboside from Sephadex LH-20 were 1.7 μg per liter of exudate as compared with 1.8 μg per liter for Bondapak C_{18}/Porasil B. Both chromatographic methods isolated biological activity corresponding to isopentenyl adenine and isopentenyl adenosine. Except for the isopentenyladenosine zone from the Bondapak C_{18}/Porasil B column, these fractions were not statistically significant. Because the cucumber cotyledon bioassay is less sensitive to these naturally occurring components than to zeatin or zeatin riboside[36], they should be considered as potentially present and in higher concentrations than indicated by the estimated kinetin equivalents in Tables IV and V. Both chromatographic techniques isolated three zones of biological activity other than those of the naturally occurring cytokinin standards used in this study. These compounds were more polar than zeatin riboside, possibly with a polyhydroxylated isopentyl side-chain located on the N-6 position of the adenine ring[14] (*i.e.*, 6-(2,3,4,-trihydroxy-3-methylbutylamino)purine) and/or a glucose or ribose sugar substitution on the N-9 or N-7 position[29,37,38]. The total activity of these cytokinin-like components eluted from the Sephadex LH-20 column was 2.3 μg kinetin equivalents per liter of exudate and those eluted from the Bondapak C_{18}/Porasil B column were estimated to be 3.3 μg kinetin equivalents per liter. For both columns, two of the three cytokinin-like components were statistically significant at the 95% level.

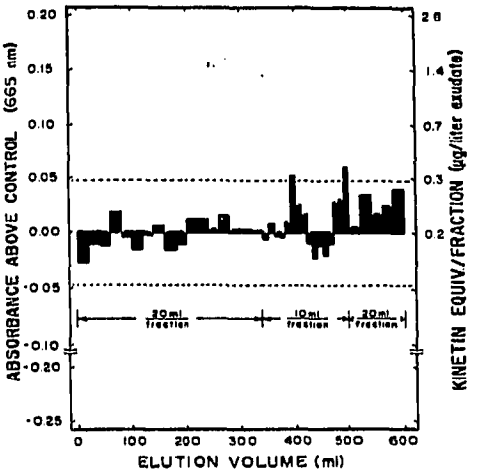

Fig. 6. Fractionation of cytokinin-like activity in the *n*-butanol pH 2.5 phase of partitioned tomato root pressure exudate of a Bondapak C_{18}/Porasil B column. Sample volume, 5 ml containing 1 liter equivalent of root pressure exudate. Separation conditions and statistics, see Fig. 5.

The separation time required to elute the seven zones of cytokinin-like activity from the Sephadex LH-20 column ranged from 30–35 h. In comparison, the time required to elute the seven zones of cytokinin-like activity from the Bondapak C_{18}/Porasil B column was 1 h.

After establishing the validity of Bondapak C_{18}/Porasil B for cytokinin analysis by comparing it with Sephadex LH-20, complete characterization of the cytokinin content of the exudate of tomato was undertaken using Bondapak C_{18}/Porasil B.

The data in Fig. 6 indicate the elution zones of biologically active components

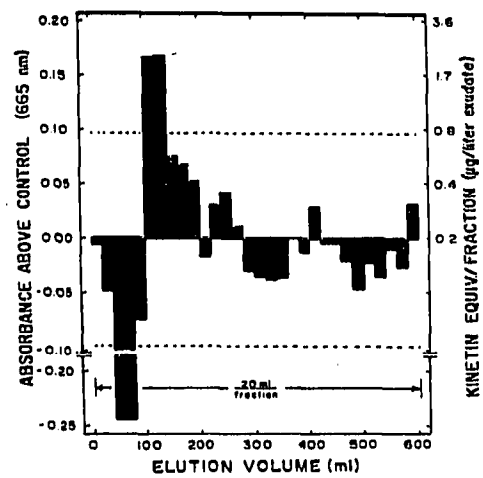

Fig. 7. Fractionation of cytokinin-like activity in the aqueous pH 2.5 phase of partitioned tomato root pressure exudate on a Bondapak C_{18}/Porasil B column. Sample volume, 5 ml containing 2 liter equivalents of root pressure exudate. Separation conditions and statistics, see Fig. 5.

of the *n*-butanol pH 2.5 phase of tomato exudate (Fig. 1). This phase contained a total of 2.0 μg kinetin equivalents per liter of exudate divided almost equally between two zones. Each zone was statistically significant at the 95% level. Cytokinins having functional groups with low pK_a values partition into this phase. Letham has reported N-6 substituted purines with a carboxylated side-chain[14].

The cytokinin-like components isolated in the aqueous pH 2.5 phase are presented in Fig. 7. One major zone of activity and one major zone of inhibition were found. The inhibitory zone occurred in the solvent front and was probably due to inorganic salts which were carried through the partition sequence. A statistically significant zone of cytokinin-like behavior was found to occur on the trailing edge of the solvent front and was estimated to contain 5.6 μg of kinetin equivalents per liter of exudate. It appeared that salts in the solvent front were masking approximately one third of the potential activity of this active zone. The aqueous pH 2.5 phase could contain as much as 7.0 μg of kinetin equivalents per liter of exudate. This zone of cytokinin-like activity probably corresponds to a cytokinin ribotide[39,40].

The exudate that was partitioned into three fractions contained at least eight cytokinin-like components and possibly even ten or more components, totaling 17.0 –26.0 μg of kinetin equivalents per liter. These values agree with reported values for other plants[10,25,28,31,39,41].

REFERENCES

1 M. G. Carnes, M. L. Brenner and C. R. Andersen, in S. Tamura (Editor), *Plant Growth Substances 1973*, Tokyo, in press.
2 R. M. Pool and L. E. Powell, *HortScience*, 7 (1972) 330.
3 N. Mandava, *Amer. Lab.*, May (1973) 27.
4 L. E. Powell, *HortScience*, 7 (1972) 236.
5 R. M. Pool and L. E. Powell, in S. Tamura (Editor), *Plant Growth Substances 1973*, Tokyo, in press.
6 J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971.
7 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 1974.
8 P. R. Brown, *High-Pressure Liquid Chromatography —Biochemical and Biomedical Applications*, Academic Press, New York, London, 1973.
9 R. Sandstedt, *Physiol. Plant.*, 24 (1971) 408.
10 K. G. M. Skene and A. J. Antcliff, *J. Exp. Bot.*, 23 (1972) 283.
11 C. O. Miller, F. Skoog, F. S. Okumura, M. H. von Saltza and F. M. Strong, *J. Amer. Chem. Soc.*, 78 (1956) 1375.
12 W. J. Burrows, F. Skoog and N. J. Leonard, *Biochemistry*, 10 (1971) 2189.
13 R. Horgan, E. W. Hewett, J. G. Purse and P. F. Wareing, *Tetrahedron Lett.*, (1973) 2827.
14 D. S. Letham, *Phytochemistry*, 12 (1973) 2445.
15 D. S. Letham, C. W. Parker and M. E. Gordon, *Physiol. Plant.*, 27 (1972) 285.
16 D. S. Letham, J. S. Shannon and I. R. C. McDonald, *Tetrahedron*, 23 (1967) 479.
17 S. M. Bezemer-Sybrandy and H. Veldstra, *Physiol. Plant.*, 25 (1970) 1.
18 C. O. Miller, *Science*, 157 (1967) 1055.
19 D. C. Elliott and A. W. Murray, *Biochem. J.*, 130 (1972) 1157.
20 N. J. Leonard, in V. C. Runeckles and E. Sondheimer (Editors), *Recent Advan. Phytochem.*, 7 (1974) 21.
21 A. J. Playtis and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 45 (1971) 1.
22 J. W. Einset and F. Skoog, *Proc. Nat. Acad. Sci. U.S.*, 70 (1973) 658.
23 W. J. Burrows, F. Skoog and N. J. Leonard, *Biochemistry*, 10 (1971) 2189.

24 R. Horgan, E. W. Hewett, J. G. Purse, J. M. Horgan and P. F. Wareing, *Plant Sci. Lett.*, 1 (1973) 321.
25 R. K. Atkin, G. E. Barton and D. K. Robinson, *J. Exp. Bot.*, 24 (1973) 475.
26 F. Skoog, R. Y. Schmitz, R. M. Bock and S. M. Hecht, *Phytochemistry*, 12 (1973) 25.
27 J. van Staden and P. F. Wareing, *Physiol. Plant.*, 27 (1972) 331.
28 E. W. Hewett and P. F. Wareing, *Physiol. Plant.*, 29 (1973) 386.
29 E. W. Hewett and P. F. Wareing, *Planta*, 112 (1973) 225.
30 E. W. Hewett and P. F. Wareing, *Planta*, 114 (1973) 119.
31 E. W. Hewett and P. F. Wareing, *Physiol. Plant.*, 28 (1973) 393.
32 J. Little, Waters Associates, Millford, Mass., personal communication.
33 Anonymous, *Sephadex LH-20, Chromatography in Organic Solvents*, Pharmacia Fine Chemicals, Svegea AB, Uppsala, Sweden, 1970.
34 J. J. Kirkland, *J. Chromatogr. Sci.*, 7 (1969) 361.
35 R. A. Fletcher and D. McCullagh, *Planta*, 101 (1971) 88.
36 C. R. Andersen, M. G. Carnes and M. L. Brenner, unpublished results.
37 R. Yoshida and T. Oritani, *Plant Cell Physiol.*, 13 (1972) 337.
38 E. J. Fox, J. Cornette, G. Deleuze, W. Dyson, C. Giersak, P. Niu, J. Zapata and J. McChesney, *Plant Physiol.*, 52 (1973) 627.
39 D. J. Carr and D. M. Reid, in F. Wightman and G. Setterfield (Editors), *Biochemistry and Physiology of Plant Growth Substances*, Runge Press, Ottawa, 1968, p. 1169.
40 D. M. Reid and W. J. Burrows, *Experientia*, 24 (1968) 189.
41 H. Kende, *Proc. Nat. Acad. Sci. U.S.*, 53 (1965) 1302.
42 R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics*, McGraw-Hill, New York, 1960, p. 481.
43 C. W. Dunnett, *Biometrics*, 20 (1964) 482.