Journal of Chromatography, 214 (1981) 101–109
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,961

COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–RADIOIMMUNOASSAY FOR CYTOKININS*

E. M. S. MacDONALD, D. E. AKIYOSHI and R. O. MORRIS*
Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331 (U.S.A.)
(Received May 4th, 1981)

SUMMARY

The cytokinins isopentenyladenosine and ribosylzeatin were conjugated to bovine serum albumin and the conjugates used to raise antisera in rabbits. The resulting antisera had high specificity towards the cytokinin haptens and low cross-reactivity towards other purines. They were used as the basis for a radioimmunoassay for cytokinins, which, when applied in conjunction with high-performance liquid chromatography, allowed rapid and sensitive (to the picogram range) estimation and identification of multiple cytokinins from natural plant and bacterial sources.

INTRODUCTION

The rapid isolation, identification and quantitation of members of one of the major classes of plant hormones, the cytokinins, is a necessary prerequisite for many physiological studies concerned with their mode of action and metabolism. Conventionally, analytical methods have relied upon solvent extraction, ion exchange, and low-resolution chromatography on Sephadex LH-20 followed by the sensitive and specific tobacco callus bioassay of Murashige and Skoog[1].

Because the callus bioassay is labor-intensive and of long duration (up to six weeks) other methods of quantitation have been sought, the most accurate of which has been the mass spectrometric isotope dilution procedure of Summons *et al.*[2] which has the advantage of sensitivity and high precision. The equipment required is, however, not always readily available to many investigators. Less demanding procedures would be of value.

Recently, Weiler[3] developed a radioimmunoassay (RIA) for cytokinins and described its application to unfractionated plant extracts. We have taken the procedure one step further and combined radioimmunoassay with high-performance liquid chromatography (HPLC). The procedure is rapid, very sensitive, of high specificity and allows the immediate identification and quantitation of the multiple cytokinins present in plant and bacterial extracts.

* This work constitutes Paper No. 5847 from the Oregon State University Agricultural Experiment Station.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Preparation of hapten conjugates

trans-Ribosylzeatin (*t*-io^{6}Ado) and isopentenyladenosine (i^{6}Ado) were separately conjugated to bovine serum albumin (BSA) using the method of Erlanger and Beiser[4]. To each cytokinin (0.13 mmole, ≈45 mg) in 10 ml water was added $NaIO_4$ (0.9 mmole, 200 mg) at 4°C and the mixture incubated for 1 h in the absence of light. BSA (2 μmoles, 120 mg) was added, the pH adjusted to 9.3 with 5% K_2CO_3 solution and the mixture again incubated at 4°C in the dark for 1 h. The excess periodate was destroyed by adding ethylene glycol (0.9 mmole, 50 μl) and the Schiff bases were reduced overnight at 4°C with an excess of $NaBH_4$ (3.6 mmole, 135 mg). After adjusting briefly to pH 5.5 with 1 *M* formic acid to destroy excess borohydride, the solution was taken to pH 8.5, the conjugate dialysed exhaustively against phosphate-buffered saline (PBS) (NaCl, 0.15 *M*; sodium phosphate, 0.05 *M*, pH 7.0; EDTA, 1 m*M*) then concentrated by ultrafiltration (Immersible CX-10 ultrafilter, Millipore, Bedford, MA, U.S.A.) to approximately 8 mg ml^{-1}. Difference spectra of the conjugates *versus* BSA showed typical cytokinin absorption maxima at 268 nm. Calculation indicated an i^{6}Ado:BSA molar ratio of 17 and a *t*-io^{6}Ado:BSA ratio of 15. Conjugates were stored at −20°C and were stable indefinitely.

Immunization schedule

Each conjugate in normal saline at 4 mg ml^{-1} was mixed with an equal volume of Freund's adjuvant and used to raise antiserum in New Zealand white rabbits. The first injection of 1 mg conjugate with Freund's complete adjuvant was distributed intramuscularly and subcutaneously. Two subsequent weekly injections of 1 mg were in Freund's incomplete adjuvant and were followed by a rest period of five weeks and a final injection of 0.5 mg. Animals were bled at the end of the ninth week and serum was stored at −20°C.

Preparation of ^{3}H-labeled isopentenyladenosine and ribosylzeatin dialcohols

The method of Weiler[3] was followed with some modification. *t*-io^{6}Ado was purified by preparative HPLC on octadecyl silica to remove traces of the *cis*-isomer. It was then oxidized to the dialdehyde with periodate for 45 min (*t*-io^{6}Ado, 1.5 μmoles, 0.5 mg; $NaIO_4$, 3 μmoles) and immediately separated from excess periodate by application to a small octadecyl silica column (C_{18} Sep-Pak, Waters Assoc., Milford, MA, U.S.A.). After washing with water, the dialdehyde was eluted with methanol, dried *in vacuo*, dissolved in methanol (20 μl), $NaCO_3$–$NaHCO_3$ buffer (pH 9.3, 1 *M*, 100 μl) was added and reduction was accomplished with NaB^3H_4 (50 mCi, sp. act. 8.9 Ci mmole^{-1}, Amersham, Arlington Heights, IL, U.S.A.) in 0.5 *M* NaOH (25 μl) for 30 min. Excess borohydride was decomposed with acetic acid and the ^{3}H-labeled dialcohol was applied to a small octadecyl silica column, washed with water to remove ^3H_2O and finally eluted with methanol. Analytical HPLC indicated only one radioactive species with a specific activity of ≈2.7 Ci mmole^{-1}. It was stored in methanol at −70°C. Labeled isopentenyladenosine dialcohol was prepared similarly.

Radioimmunoassay

Reactions were carried out in polypropylene 1.5 ml microcentrifuge tubes

(West Coast Scientific, Berkeley, CA, U.S.A.) to which additions were made strictly according to the following sequence: PBS containing gelatin (0.1 %, w/v) 250 μl; ovalbumin in PBS (1 %, w/v) 50 μl; [^{3}H]cytokinin dialcohol (1–2 pmole, ≈5000 cpm) 50 μl; and standard or unknown cytokinins (10 pg–10 ng) 50 μl.

After mixing, diluted antiserum (50 μl) was added using an amount which would have been sufficient to bind 50 % of the [^{3}H]cytokinin in the absence of added unlabeled competitor. Following an incubation at 20°C for 20 min, the antibody was precipitated by the addition of 90 % saturated ammonium sulfate (600 μl), collected by centrifugation, washed with 50 % saturated ammonium sulfate (400 μl) and dissolved in water (400 μl). Radioactivity was determined by scintillation counting in a standard cocktail (NEF 963; New England Nuclear, Boston, MA, U.S.A.) on a Packard Tricarb Model 3375 counter.

Standard curves and competition experiments

Cytokinins were dissolved in methanol at 1 mg ml^{-1} and serially diluted in methanol directly into the assay tubes to give the desired amount in each tube. After removal of methanol *in vacuo*, assay buffer was added as described above. Alternatively, the methanolic solutions were diluted into PBS containing 0.1 % gelatin and the assay was performed without preliminary drying.

Extraction of cytokinins from natural sources

Agrobacterium tumefaciens. *Agrobacterium tumefaciens* (strain C58, obtained from J. Schell, Rijksuniversiteit, Ghent, Belgium) was grown on 200 ml AB minimal medium[5] to mid-log phase (A_{660} 0.56). The culture was chilled rapidly, the cells removed by centrifugation (8000 *g* for 20 min), the supernatant passed through a short octadecyl silica column (C_{18} Sep-Pak) which was washed with water and then eluted with methanol (4 ml). The methanol was removed *in vacuo* and the cytokinins subjected to HPLC. This trace-enrichment procedure allows recovery of over 90 % of the cytokinins present[6].

Nicotiana crown gall

A cloned shoot teratoma line BT37 incited by *N. tabacum* var. Turkish (a generous gift from A. Braun, Rockefeller University, New York, U.S.A.) was grown on Murashige and Skoog[1] medium in the absence of hormones under continuous light. Cytokinins were extracted by homogenization in methanol and the extract was purified through DEAE-cellulose and octadecyl silica according to Akiyoshi and Morris[7]. The crude extract was subjected directly to HPLC.

Reversed-phase HPLC

Samples were applied to a 250 × 4.6 mm, 5-μm spherical particle octadecyl silica column (Ultrasphere; Altex, Berkeley, CA, U.S.A.) in a starting buffer of 0.1 *M* triethylammonium acetate, pH 3.35 containing 15 % (v/v) methanol. The column was eluted with a linearly increasing concentration of methanol to 80 % (v/v) in the same buffer over a period of 30 min at a flow-rate of 1.0 ml min^{-1}, followed by an increase to 100 % methanol over 3 min.

The bacterial culture filtrate sample was dissolved in 15 μl methanol, injected and fractions (200 μl) were collected over 34 min. Each fraction was divided into four aliquots which were dried individually *in vacuo* and stored at −20°C prior to analysis. The plant sample was treated similarly.

RESULTS

Radioimmunoassay for cytokinins

Conjugates of i^{6}Ado or *t*-io^{6}Ado with BSA were readily made via the appropriate dialdehydes using the method of Erlanger and Beiser[4]. Up to 17 moles of cytokinin could be attached to 1 mole of protein. Using the immunization schedule described, antisera of reasonable titers were obtained which, on Ouchterlony double diffusion, gave single precipitin lines. The sera were used directly in RIA without further purification.

Typical standard curves obtained in an assay for ribosylzeatin are illustrated in Fig. 1. The lower curve was obtained by serially diluting a stock methanolic solution of ribosylzeatin (1 $\mu g\ \mu l^{-1}$) into PBS containing 0.1% gelatin to obtain the desired level of cytokinin in each assay tube. The upper curve was obtained by diluting the same cytokinin stock solution serially in methanol, lyophilizing each sample directly in the assay tube, re-dissolving in PBS–gelatin and performing the assay. Significant losses of ribosylzeatin to the walls of the reaction vessels were observed in this latter procedure.

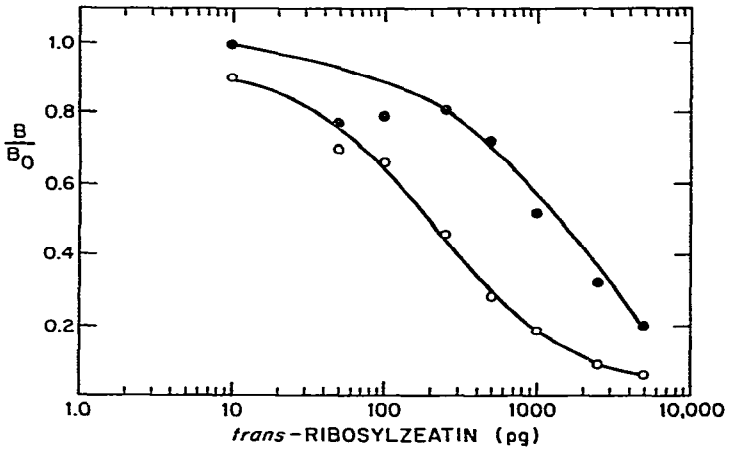

Fig. 1. Standard curve for RIA of ribosylzeatin. The assay was performed as described in the text. Dilutions of standard *t*-io^{6}Ado were made either in methanol and subsequently lyophilized (●) or directly in PBS (○).

In subsequent experiments (not shown) it was found that glass and polypropylene surfaces strongly absorbed both labeled and unlabeled cytokinins. Absorption was consistently worse with polypropylene tubes originating with some manufacturers. Care was therefore taken to select those to which it was minimal. For routine assays, tubes and pipet tips were pre-saturated with a non-cross-reacting purine such as adenosine. The limit of detection of ribosylzeatin (when diluted in PBS–gelatin) was about 10–20 pg. From dried samples, which more closely resemble those originating from HPLC, it was of the order of 100–200 pg, a ten-fold decrease in sensitivity.

Antiserum specificity

Both antisera exhibited a high degree of specificity when different cytokinins were allowed to compete with the radiolabeled dialcohols, cross-reactivity with a number of purines is illustrated in Table I. Antiserum to i^{6}Ado–BSA did not significantly cross-react with high levels of adenosine, *t*-io^{6}Ado or *trans*-zeatin but there was considerable cross-reactivity towards isopentenyladenine. Interestingly (Fig. 2B), this anti-serum bound significant levels of 2-methylthio-isopentenyladenosine (ms^{2}i^{6}Ado) and the 5′-phosphate of isopentenyladenosine (i^{6}AMP).

TABLE I

ANTISERUM SPECIFICITY: CROSS-REACTION WITH OTHER PURINES

Expressed as the ratio of labeled cytokinin dialcohol bound in the presence (B) or in the absence (B_0) of the competitor; $B/B_0 = 1$ indicates no competition. Abbreviations: Ado = adenosine; *c*-io^{6}Ade and *t*-io^{6}Ade = *cis*- and *trans*-zeatin; f^6 Ade = kinetin; i^{6}Ade = isopentenyladenine; i^{6}Ado = isopentenyladenosine.

Competitor added (ng)	*Cross-reaction (B/B_0)*								
	Anti-ribosylzeatin				*Anti-isopentenyladenosine*				
	Ado	*c-io^{6}Ade*	*f^{6}Ade*	*i^{6}Ado*	*Ado*	*t-io^{6}Ado*	*t-io^{6}Ade*	*f^{6}Ade*	*i^{6}Ade*
0.1	1.09	0.93	1.04	0.97	1.07	1.04	1.01	1.05	0.69
1.0	1.08	1.03	1.04	0.98	1.05	1.02	0.92	0.95	0.53
10	1.01	1.07	1.03	0.82	1.01	0.89	0.94	0.75	0.31

Antiserum prepared against *t*-io^{6}Ado–BSA (Fig. 2A, Table I) gave equally high specificity. Adenosine and kinetin did not react even when present at 10 ng per assay. i^{6}Ado was a weak competitor, showing cross-reactivity only at 10 ng, while zeatin and dihydrozeatin (Fig. 2) cross-reacted effectively. Surprisingly, there was no observable competition with *cis*-zeatin (Table I).

The extent of cross-reaction was estimated roughly on a molar basis by determining the concentration of competitor necessary to give a B/B_0 value of 0.5. The data are tabulated in Table II. Zeatin was approximately sixty percent as effective as ribosylzeatin in competing with the tritiated dialcohol for the antibody. Dihydrozeatin was only 30% as effective. No other purine gave significant competition.

For antisera to isopentenyladenosine, isopentenyladenine (i^{6}Ade) and i^{6}Ado appeared to compete with equal effectiveness. The phosphate, i^{6}AMP, and ms^{2}i^{6}Ado were moderate competitors, kinetin was less effective and no other substance tested gave any significant response.

Combination of HPLC and RIA

The above data show clearly that if an assay utilizing ribosylzeatin antiserum were to be applied to a natural extract containing multiple zeatin-like species, no meaningful result could be obtained. Nevertheless, the known[8] resolution of zeatin, its riboside, and glucosides on HPLC suggests that a combination of HPLC and RIA would provide a rapid, sensitive and highly specific method for determining many cytokinins. Two natural extracts, known to contain multiple cytokinins were therefore examined by HPLC–RIA.

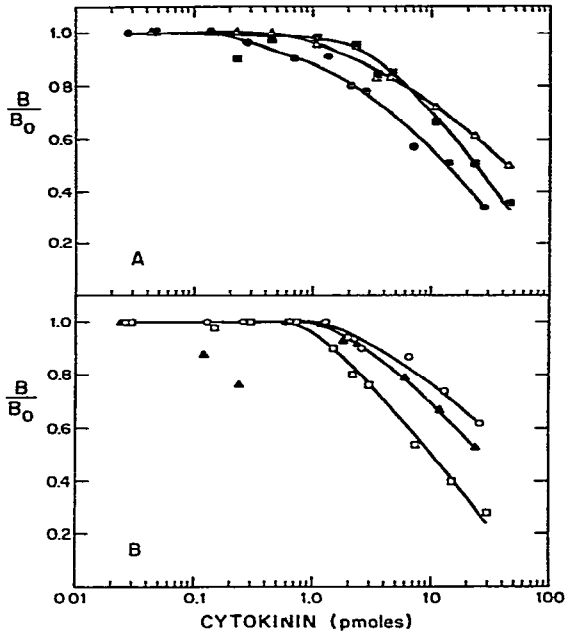

Fig. 2. Competition of various cytokinins for anti-ribosylzeatin or anti-isopentenyladenosine antiserum. (A) Anti-ribosylzeatin antiserum. ^{3}H-Labeled ribosylzeatin dialcohol (1.8 pmole) competed against *t*-io^{6}Ado (●), *t*-io^{6}Ade (■), or io^{6}h^{2}Ade (△). (B) Anti-isopentenyladenine antiserum. ^{3}H-Labeled isopentenyladenosine dialcohol (1.8 pmole) competed against i^{6}Ado (□), i^{6}AMP (▲) or ms^{2}i^{6}Ado (○).

TABLE II

RELATIVE MOLAR CROSS-REACTIVITY OF CYTOKININS

The concentration of cytokinins at $B/B_0 = 0.5$ expressed as a percentage of the io^{6}Ado or i^{6}Ado concentration required to produce the same effect. ND = not determined. Abbreviations: *c*-io^{6}Ade and *t*-io^{6}Ade = *cis*- and *trans*-zeatin; io^{6}h^{2}Ade = dihydrozeatin; f^{6}Ade = kinetin; Ado = adenosine. For other abbreviations, see text.

Cytokinin	*Percent cross-reaction*	
	Anti-ribosylzeatin	*Anti-isopentenyladenosine*
t-io^{6}Ade	60	<5
t-io^{6}Ado	100	<5
c-io^{6}Ade	<1	ND
io^{6}h^{2}Ade	33	ND
f^{6}Ade	<1	<5
i^{6}Ado	<5	100
i^{6}Ade	ND	100
i^{6}AMP	ND	37
ms^{2}i^{6}Ado	ND	21
Ado	<1	<1

The first was a culture filtrate of the plant pathogenic bacterium *Agrobacterium tumefaciens*, the causative agent of plant crown gall tumors. It has been shown previously[9] that the bacterium produces both *trans*-zeatin and i^{6}Ado at concentrations

of approximately 200 ng l^{-1} when grown on defined medium. *A. tumefaciens* (strain C58) was therefore grown on a defined medium to mid-log phase and the culture filtrate was subjected to trace enrichment[6] in order to extract and concentrate the cytokinins. The enriched extract was then subjected to HPLC on microparticulate octadecyl silica and individual fractions from the column were lyophilized and examined by RIA. As seen in Fig. 3, a gradient of methanol in a weakly acidic buffer is capable of resolving almost all known cytokinins in a single 30-min analysis. The most polar cytokinins, zeatin and ribosylzeatin, for example, are eluted prior to the less polar such as ms^2i^6Ado. The resolution is sufficiently high to allow separation of the geometric isomers of both zeatin and ribosylzeatin.

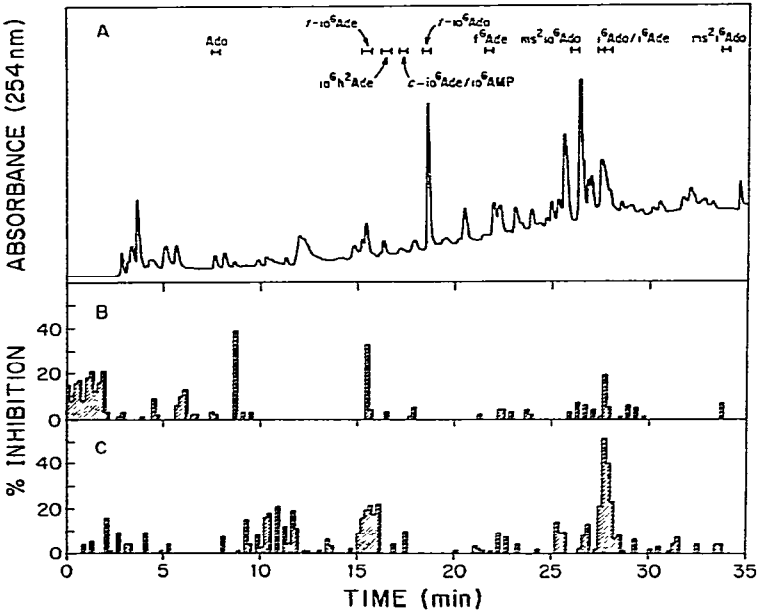

Fig. 3. Cytokinin-like substances in *Agrobacterium tumefaciens* culture filtrate. Chromatographic conditions as described in the text. The retention times of standard cytokinins are indicated by horizontal bars: (A) UV absorbance. (B) Assay with anti-ribosylzeatin. (C) Assay with anti-isopentenyladenosine.

RIA of individual fractions with both antisera confirmed the presence of *trans*-zeatin (retention time, t_R = 15.4 min) and i^6Ado (t_R = 27.6 min). The latter peak was broad and clearly contained other species capable of cross-reacting with the antibody. Calculation of the amounts of each cytokinin present gave values in the range 40–60 ng l^{-1} in reasonable agreement with previous estimates. Material apparently cross-reacting with zeatin was also seen in the very early fractions eluting prior to the void volume of the column. This was found to be an artifact caused by adsorption of [^{3}H]zeatin dialcohol to the pipet tip used in the assay.

Application of the technique to a plant sample is illustrated in Fig. 4. The cytokinins from the crown gall shoot teratoma line BT37 incited on tobacco by *A. tumefaciens* strain T37 were extracted, put through a series of rapid column chroma-

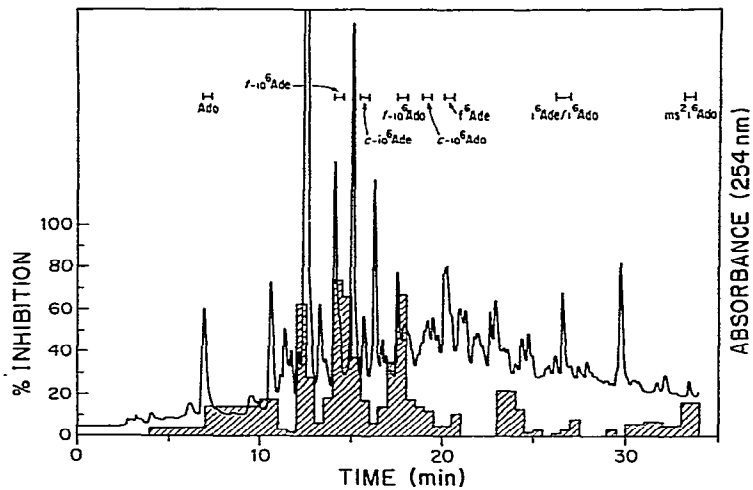

Fig. 4. Cytokinin-like substances in an extract of *Nicotiana* crown gall tissue. Chromatographic conditions as described in the text. Sample equivalent to 1 g fresh weight tumor tissue. Histogram: Assay with anti-ribosylzeatin antiserum.

tographic procedures to remove the bulk of the impurities[7] and then fractionated by HPLC as previously described. RIA of the column eluate with ribosylzeatin antiserum revealed the presence of at least four major peaks of cross-reacting material. The first (t_R = 12–12.5 min) had a retention time earlier than any known standard and remains to be identified. A broad peak (t_R = 14–14.5 min) centered on the elution volume of authentic *trans*-zeatin. It contained two (or more) species and was followed by another peak having the retention time (17.5–18.0 min) of authentic *t*-io^{6}Ado. Other, much smaller, peaks could also be discerned in the eluate. Estimates of the level of zeatin gave a value of 11 ng g^{-1}, comparable to 9 ng g^{-1} reported by Horgan[10] for *Vinca rosea* unorganized crown gall callus. The level of ribosylzeatin (11 ng g^{-1}) was much lower than that reported for the *Vinca* tumor[10].

DISCUSSION

The extension of conventional radioimmunoassay by using it in conjunction with HPLC allows rapid, specific and sensitive detection, identification and quantitation of cytokinins. Because of the inherent sensitivity and rapidity of the method, identification and analysis of specific hormones in gram and milligram quantities of plant tissue becomes possible.

Radioimmunoassay has had previous application to plant hormone analysis. Pengelly and Meins[11] described the analysis of indole-3-acetic acid, Weiler[12] reported a procedure for abscisic acid, and Khan *et al.*[13], Constantinidou *et al.*[14], Weiler[3], and Vold and Leonard[15] have all described RIA of both natural and synthetic cytokinins. In all cases, high affinity antisera were obtained by using appropriate hormone–protein conjugates. In this work, affinity and specificity were found to be high and in the case of *cis*-zeatin surprisingly stringent. Similar results were reported by Vold and Leonard[15] and by Weiler[3].

A comparison of the relative molar cross-reactivities of a number of cytokinins suggests that the primary antigenic determinants are located in the isoprenoid side chain and in the purine nucleus. However, it is clear that the ribose moiety also plays a definite part in recognition. The presence of the isoprenoid double bond is not critical since zeatin and dihydrozeatin compete quite effectively for the same antiserum. It is therefore remarkable that *cis*-zeatin does not compete with the *trans*-isomer. Restricted rotation about the exocyclic N–C bond may provide the chemical basis for such specificity.

The significant cross-reaction between *trans*-zeatin and dihydrozeatin is, in fact, an advantage if RIA is to be combined with HPLC. Both sets of cytokinins can be separated chromatographically and a single antiserum can be used for detection. This also emphasizes the major benefit of combining the two analytical techniques. As is evident from Fig. 4, it is now possible to separate and identify individual zeatin-like cytokinins present in a complex natural mixture and yet at the same time retain the inherent sensitivity of the RIA approach. Zeatin and ribosylzeatin have been shown to be present in tobacco crown gall line[16]. Two of the four peaks of Fig. 4 are thus accounted for. The nature of the others remains to be determined. One possible candidate is dihydrozeatin which has a retention time intermediate between those of the *trans*- and *cis*-zeatin.

Although in its present state of development, the assay has low precision ($\pm 20\%$) below 100 pg, good quantitative agreement was obtained between cytokinin levels measured in *A. tumefaciens* culture filtrates and those obtained previously by other methods. Currently, studies are directed towards improving the assay precision and extending it to include other cytokinin derivatives such as the side chain glucosides.

ACKNOWLEDGEMENTS

This work was supported in part by grant PCM 78-22963 from the National Science Foundation. We wish to thank D. A. Regier for assistance with antisera production.

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