J Plant Growth Regul (1987) 6:159–182

Journal of
Plant
Growth
Regulation

Use of Isopentenyladenosine and Dihydrozeatin Riboside Antibodies for the Quantification of Cytokinins

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Received March 27, 1987; accepted June 5, 1987

Abstract. Antisera have been raised in rabbits against dihydrozeatin riboside and isopentenyladenosine, and their cross-reactivity characteristics have been examined in detail. These antisera, together with an antiserum previously raised against zeatin riboside, have been employed in radioimmunoassays. Separative procedures that enable a wide range of naturally occurring cytokinins to be separated prior to analysis by radioimmunoassay have been developed. The accuracy with which the following cytokinins can be quantified by our methods, which employ tritiated cytokinin recovery markers, has been estimated: zeatin riboside, zeatin, dihydrozeatin riboside, dihydrozeatin, O-glucosyl zeatin riboside, O-glucosyl zeatin, O-glucosyl dihydrozeatin riboside, O-glucosyl dihydrozeatin, zeatin-9-glucoside, zeatin-7-glucoside, kinetin, isopentenyladenosine, and isopentenyladenine.

Cytokinins occur in low concentrations in plant tissues, and their quantification is problematic (see Letham 1978, Badenoch-Jones et al. 1984b). For quantifying cytokinins, radioimmunoassay (RIA) has several advantages over alternative methods available, particularly, its sensitivity and simplicity.

In the present study we have raised antisera in rabbits against isopentenyladenosine ([9R]iP) and dihydrozeatin riboside ([diH][9R]Z) and characterized them in detail. Together with an antiserum elicited against zeatin riboside ([9R]Z) (Badenoch-Jones et al. 1984b), we have developed methods whereby use of these three antisera in RIA enables the quantification of a wide range of naturally occurring cytokinins. Zeatin (Z) and [9R]Z were the first natural cytokinins to be isolated and are now known to occur widely in plants (Letham 1978, Letham and Palni 1983). Cytokinin free bases are the likely active forms of cytokinin (Letham and Palni 1983), and hence quantification of the full range

(isopentenyladenine (iP) and dihydrozeatin ((diH)Z), as well as Z) is particularly important. iP is the dominant cytokinin in mosses (Bopp et al. 1986) and slime mold (Tanaka et al. 1978) and in the former it exerts a regulatory role in morphogenesis. ([9R]iP is prominent in certain higher plants (Letham and Palni 1983), and in some (water chestnut and *Azolla*) it appears to be the principal cytokinin (Tsui et al. 1983, Hashizume et al. 1986).

(diH)Z and (diH)[9R]Z, which are resistant to cytokinin oxidase, represent stabilized forms of Z, and these dihydro compounds and their O-glucosides are the dominant cytokinins in some legume tissues (see, e.g., Palmer et al. 1981, Summons et al. 1979A). The O-glucosides of cytokinins are ubiquitous metabolites, which may be storage forms of cytokinin (Letham and Palni 1983), but their exact function is unclear. As with the O-glucosides, the exact function of cytokinin nucleotides is unclear, but isopentenyladenosine 5′-phosphate ([9R-5′P]iP) may be the initial cytokinin formed during cytokinin biosynthesis in plants. These issues will be resolved only when the endogenous levels of these cytokinins can be determined readily in relation to plant development. Methods for separating the above cytokinins, prior to their quantification by RIA, have been developed and are reported herein.

Materials and Methods

Cytokinins for Cross-Reactivity Tests, and Other Chemicals

The sources of many of the chemicals used in this study have been given by Badenoch-Jones et al. (1984b). Sources of additional chemicals are as follows: iP, *cis*-Z, benzylaminopurine riboside ([9R]BAP), and almond β-glucosidase were purchased from Sigma Chemical Company. *cis*-Z was purified by HPLC (Waters μB C-18, 7.8 mm × 30 cm, 30% (v/v) methanol in 0.2 M acetic acid). *cis*[9R]Z was purchased from Calbiochem. Hydroxybenzlaminopurine ((oOH)BAP) and hydroxybenzylaminopurine riboside ((oOH)[9R]BAP) were a generous gift from Dr. R. Horgan, University College of Wales, Aberystwyth. The enantiomers of (diH)Z, (+)-(diH)Z and (−)-(diH)Z), were a generous gift from Dr. J. Corse, Western Regional Research Center, U.S. Department of Agriculture, Berkeley, California, and the corresponding ribosides ((+)-(diH)[9R]Z, (−)-(diH)[9R]Z) were kindly supplied by Dr. S. Matsubara, Laboratory of Applied Biology, Kyoto Prefectural University, Kyoto, Japan. Dihydrolupinic acid ((diH)[9Ala]Z) and dihydrozeatin-9-glucoside ((diH)[9G]Z) were synthesized by the previously published methods of Parker et al. (1978) and Summons et al. (1980), respectively. Zeatin nucleotide ([9R-5′P]Z) was synthesized according to Summons et al. (1983). [^{3}H]sodium borohydride (spec. act. 2442 GBq mmol^{-1}), for the synthesis of the [9R]iP tracer, was purchased from New England Nuclear, Boston. [^{3}H]sodium borohydride (407 GBq mmol^{-1}), for the synthesis of the (diH)[9R]Z tracer, and [^{3}H](diH)Z (1110 GBq mmol^{-1}) (used as a recovery marker) were purchased from Amersham International Ltd, Amersham, U.K. [^{3}H][9R]Z (141 GBq mmol^{-1}) was a generous gift from C. H. Hocart. [^{3}H](diH)[9R]Z (189 GBq mmol^{-1}) (used as a recovery marker) was synthesized by an unpublished method. Silica gel 60

PF_{254} for TLC was purchased from E. Merck (Darmstadt, West Germany). The following dyes were used as markers for TLC: meldola blue (dye A), toluidine blue (dye B), drimarene brillant blue K-BL (dye C), rhodamine B (dye D), and eosin (water-soluble, yellow shade; dye E). The sources of the dyes have been given previously (Badenoch-Jones et al. 1984a), except for eosin, which was purchased from BDH Chemicals, Poole, U.K.

Preparation of Hapten Conjugates

[9R]iP and (diH)[9R]Z were conjugated to bovine serum albumin (BSA) by a modification of the method of Erlanger and Beiser (1964), following the procedures outlined by Weiler (1980).

Synthesis of Tracers

The tracers for the assay, tritiated [9R]iP dialcohol and tritiated (diH)[9R]Z dialcohol, were synthesized according to the method of Randerath and Randerath (1973), except that [^{3}H]sodium borohydride rather than [^{3}H]potassium borohydride was used as the reducing agent. The reaction products were purified by cellulose TLC using acetonitrile-ethyl acetate-n butanol-isopropanol-6M ammonia (4:3:1:2:2:7, v/v/v/v/v) as solvent. Specific activities of the [9R]iP and (diH)[9R]Z tracers were 629 GBq mmol^{-1} and 289 GBq mmol^{-1}, respectively. Both tracers cochromatographed with their respective authentic unlabeled diols and exhibited mass spectra, which would be expected for these diols. The mass spectrum of [9R]iP diol has been recorded previously (Chen et al. 1976); the chemical ionization mass spectrum (CH_4) of (diH)[9R]Z diol was as follows (m/z with relative intensity in parentheses): 386/384 ($M.C_2H_5^+$, 2.5/8.2); 358/356 (27.2/84.8); 338 (7.6); 282 (5.7); 266/264 (10.8/44.9); 250 (28.5); 222 (100); 204 (12.6).

Immunization Schedules

Three New Zealand white rabbits were injected with the [9R]iP-BSA conjugate, and three with the (diH)[9R]Z-BSA conjugate. The procedures for injecting and bleeding the rabbits and for screening and storing the antisera were as described by Badenoch-Jones et al. (1984b), except for the days that the rabbits were injected. Rabbits were injected with the [9R]iP-BSA conjugate on days 0, 30, 58, 86, and 115. Freund's complete adjuvant was used on days 0 and 30, and Freund's incomplete adjuvant was used on the remaining days. Rabbits were injected with the (diH)[9R]Z-BSA conjugate on days 0, 25, 53, 83, 104, 137, 252, 283, and 318, and Freund's complete adjuvant was used on days 0, 25, and 252.

All rabbits developed antibodies against the cytokinin with which they were injected, but for each antigen, the best antiserum, in terms of affinity and titer, was selected for use in further assays. For [9R]iP, this serum was taken on day

123 from one of the three rabbits injected with this antigen, and for (diH)[9R]Z it was taken on day 324 from one of the three rabbits injected with this antigen.

Radioimmunoassay

The assay was performed as described by Badenoch-Jones et al. (1984b), with minor modifications. The $(NH_4)_2SO_4$ solution was used at 98.8% saturation rather than 91%, since the former concentration resulted in greater precipitation of the antigen-antibody complex. The washing procedure employing 50% saturated $(NH_4)_2SO_4$ was omitted, because it was found that, if care was taken to completely remove the supernatant after centrifugation, nonspecific binding (binding in the absence of antibody) was still less than 1%.

Thin-Layer Chromatography

TLC on silica gel (0.25-mm-thick layer), using butan-1-ol/acetic acid/water (12:3:5, v/v/v) as solvent, was used to separate the following cytokinins or groups of cytokinins: [9Ala]Z; [7G]Z and the O-glucosides; [9G]Z; Z/(diH)Z/[9R]Z/(diH)[9R]Z; and iP and [9R]iP. The cytokinins were located with reference to dyes as follows: [9Ala]Z—dye A; [7G]Z and the O-glucosides—dyes A and B; [9G]Z—dyes B and C; Z/(diH)Z/[9R]Z/(diH)[9R]Z—dyes C and D; and iP and [9R]iP—dye E. Chromatogram zones were packed into columns and eluted exhaustively with methanol/water/acetic acid (50:48:2, v/v/v).

High-Performance Liquid Chromatography

The following groups of cytokinins were separated by HPLC: Z/(diH)Z/[9R]Z/(diH)[9R]Z on a Zorbax C_8 column (9.4 × 250 mm) (DuPont Company) using 25% (v/v) methanol in 0.2 M acetic acid as solvent at a flow rate of 5.0 ml min^{-1}, and iP and [9R]iP on a Techsil 10 C_8 column (8.0 × 250 mm) (HPLC Technology, Cheshire, U.K.) using 30% (v/v) methanol in 0.2 M acetic acid as solvent at a flow rate of 4.0 ml min^{-1}.

The O-glucoside/[7G] Z zone was hydrolyzed with β-glucosidase (Letham et al. 1975) and purified using Baker SPE C18 minicolumns (J. T. Baker Chemical Co., Phillipsburg, NJ) (3ml). Samples were loaded onto the columns in water and eluted with ethanol/water/acetic acid (50:49:1, v/v/v). The resulting cytokinins (Z/(diH)Z/[9R]Z/(diH)[9R]Z/[7G]Z) were subjected to HPLC on the Zorbax C_8 column using 20% (v/v) methanol in 0.2 M acetic acid as solvent.

Periodate Oxidation

Periodate oxidation of [7G]Z and [9G]Z was carried out as described by Letham and Gollnow (1985). Freshly redistilled cyclohexylamine was used.

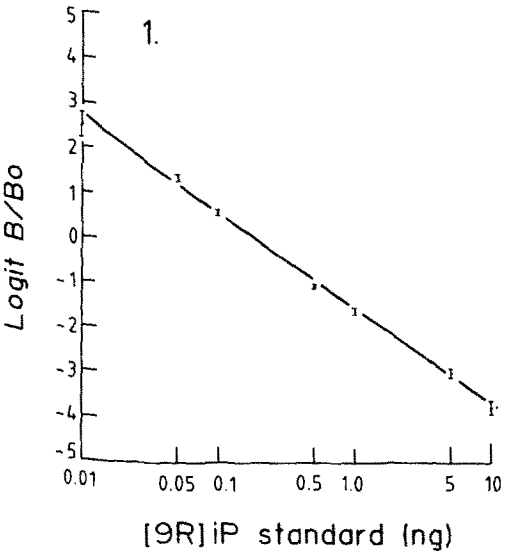

Fig. 1. Logit transformation of the mean standard curve for [9R]iP. Bars indicate SE (n = 18). B, binding of tracer to antibody in the presence of [9R]iP standards; Bo, binding of tracer to antibody in the absence of [9R]iP.

Results

Standard Curves

Standard curves for [9R]iP and (diH)[9R]Z could be linearized over the measuring range by logit transformation of the B/Bo values (Figs. 1, 2). The measuring range extended from ~0.03 to 30 pmol for [9R]iP and from ~0.03 to 150 pmol for (diH)[9R]Z. At the following final dilutions in the assay, [9R]iP—1:4500 and (diH)[9R]Z—1:10,800, the antisera bound approximately 53% and 51%, respectively, of the tracer in the absence of unlabeled riboside. For the anti-[9R]Z serum described in a previous study (Badenoch-Jones et al. 1984b), the final dilution required to give approximately 50% binding of tracer (in the absence of unlabeled [9R]Z), under the current experimental conditions, was 1:15,750. Nonspecific binding (binding in the absence of antibody) was less than 1% in all of the RIAs. Both antisera contained high-affinity antibodies, as indicated by maximum affinity constants (determined from Scatchard plot analysis of standard curve data) of 2.46×10^{-11} M for the anti-[9R]iP serum and 2.58×10^{-11} M for the anti-(diH)[9R]Z serum.

Cross-Reactivity Studies

Antisera specificities were determined by cross-reactivity studies, and the results are shown in Fig. 3 for the anti-[9R]iP serum and in Fig. 4 for the anti-(diH)[9R]Z serum. The slopes of the logit transformation curves for most of the compounds tested were generally similar to, though almost invariably lower than, the slope of the logit-transformed standard curve for the antigen. BAP, (oOH)BAP, [9R]BAP, (oOH)[9R]BAP, and [9R-5'P]Z for the anti-[9R]iP

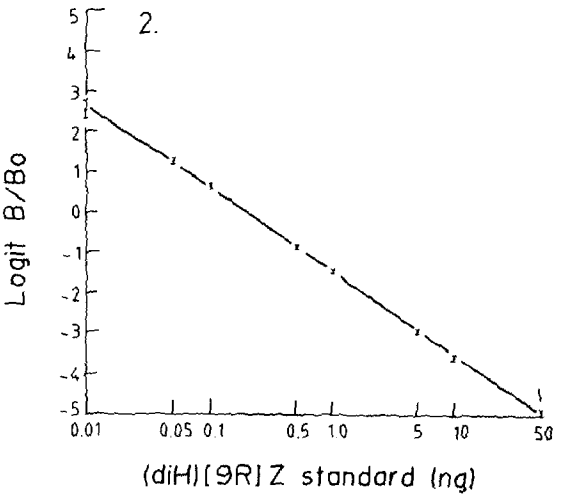

Fig. 2. Logit transformation of the mean standard curve for (diH)[9R]Z. Bars indicate SE (n = 21). B, binding of tracer to antibody in the presence of (diH)[9R]Z standards; Bo, binding of tracer to antibody in the absence of (diH)[9R]Z.

serum, and *cis*-Z, *cis*[9R]Z, [9R-5'P]Z, and [9Ala]Z for the anti-(diH)[9R]Z serum had slopes very significantly lower than that of the corresponding standard. (+)-(diH)[9R]Z and (−)-(diH)[9R]Z each had the same cross-reactivity with the anti-(diH)[9R]Z serum (Fig. 4a), but this cross-reactivity was lower than that of the racemic mixture ((±)-(diH)[9R]Z) against which the antiserum was raised. Similarly, (+)-(diH)Z and (−)-(diH)Z each had the same cross-reactivity with the anti-(diH)[9R]Z serum (Fig. 4c), but this cross-reactivity was lower than that of the racemic mixture ((±)-(diH)Z).

Molar cross-reactivities were calculated and are presented in Tables 1, 2, and 3 for anti-[9R]Z, anti-[9R]iP and anti-(diH)[9R]Z sera, respectively. Results are expressed as the inverse of the molar concentration of the compound at B/Bo = 0.5, relative to the concentration of the riboside antigen required to produce the same effect (multiplied by 100).

No cross-reactivity was found for AMP or [7G]Z when tested against either of the antisera in amounts up to 5000 ng or 500 ng per assay tube, respectively. Very slight activity was detected for [7G]Z (1000 ng per assay tube) against the anti-(diH)[9R]Z serum. No cross-reactivity was found for adenosine or adenine when tested against the anti-(diH)[9R]Z serum in amounts up to 1000 ng per assay tube. No cross-reactivity was observed for O-glucosyl zeatin riboside ((OG)[9R]Z), O-glucosyl dihydrozeatin riboside ((diH OG)[9R]Z), or O-glucosyl zeatin ((OG)Z) when tested against the anti-[9R]iP serum in amounts up to 500 ng per assay tube. Slight activity was observed for the latter two O-glucosides at 1000 ng per assay tube. Small (but measurable) cross-reactivity was found for O-glucosyl dihydrozeatin ((diH OG)Z) against the anti-[9R]iP serum (Fig. 3c). No cross-reactivity was found for (OG)Z when tested against the anti-(diH)[9R]Z serum in amounts up to 250 ng per assay tube, and only very slight activity was observed at 500 ng per assay tube. The other O-glucosides were tested at higher concentrations and were found to exhibit small, but measurable, cross-reactivity against this antiserum (Fig. 4a; Table 3).

Data have been published for the anti-[9R]Z serum raised in our laboratory

(Badenoch-Jones et al. 1984b), but further cross-reactivity tests have been done, and the data are shown in Table 1. Also shown in Tables 1–3 are molar cross-reactivities published in the literature for anti-[9R]Z, anti-[9R]iP, and anti-(diH)[9R]Z sera.

Comparison of Cross-Reactivities of Antisera from Different Rabbits Injected with the Same Antigen

For both of the antigens, antisera obtained from different rabbits had similar cross-reactivity characteristics. For example, anti-(diH)[9R]Z serum from a second rabbit had cross-reactivities of 33.7%, 32.9%, and 7.2% against (diH)Z, (diH)[9G]Z, and (diH)[9Ala]Z, respectively; corresponding values for the selected antiserum were 32.4%, 21.9%, and 10.4%. Anti-[9R]iP serum from a second rabbit had cross-reactivities of 61.9%, 11.0%, and 3.7% against [9R-5'P]iP, iP, and [9R]Z, respectively; corresponding values for the selected antiserum were 37.9%, 19.4%, and 2.6%, respectively.

Effect of [^{3}H][9R]Z Recovery Marker on the Radioimmunoassay

When performing RIA in our laboratory, we have employed high-specific-activity [^{3}H][9R]Z, [^{3}H](diH)[9R]Z, and/or [^{3}H](diH)Z as recovery markers to account for losses of cytokinins during sample purification prior to RIA. To investigate the effect of such markers on the RIA, graded amounts (0 to 77.4 Bq (4608 dpm)) of [^{3}H][9R]Z were added to six sets of [9R]Z standards. Analysis of these spiked samples by RIA employing the anti-[9R]Z serum (Fig. 5) indicated that, provided the appropriate blanks are used (i.e., the tubes to which no antiserum and no standard were added were also spiked), addition of [^{3}H][9R]Z to the assay (up to 77.4 Bq) did not alter the shape of the standard curve. When ~33.6 Bq (2000 dpm) [^{3}H][9R]Z (or more) was added, and if the appropriate blanks were not used, the standard curves were affected at the top end (most notably, the 10- and 50-ng points) in a direction that would result in the underestimation of sample [9R]Z concentration.

Quantification of the O-Glucosides Following Hydrolysis with β-Glucosidase

(OG)[9R]Z and (diH OG)[9R]Z (60-ng equivalents of [9R]Z or (diH)[9R]Z, respectively) and (OG)Z and (diH) OG)Z (120-ng equivalents of Z or (diH)Z, respectively) were each subjected to TLC as described in Materials and Methods. The O-glucoside zone was located with reference to dyes, eluted, and hydrolyzed with β-glucosidase. Samples were evaporated, made up in 60 μl of RIA buffer, then analyzed over a range of dilutions by RIA using either the anti-[9R]Z serum (for (OG)[9R]Z and (OG)Z) or the anti-(diH)[9R]Z serum (for (diH OG)[9R]Z and (diH OG)Z). The estimated amounts of cytokinin recovered were 36.1 ng [9R]Z for (OG)[9R]Z (60.1% recovery), 89.9 ng Z for (OG)Z (74.9% recovery), 27.7 ng (diH)[9R]Z for (diH OG)[9R]Z (46.1% recovery), and 58.1 ng (diH)Z for (diH OG)Z (48.5% recovery).

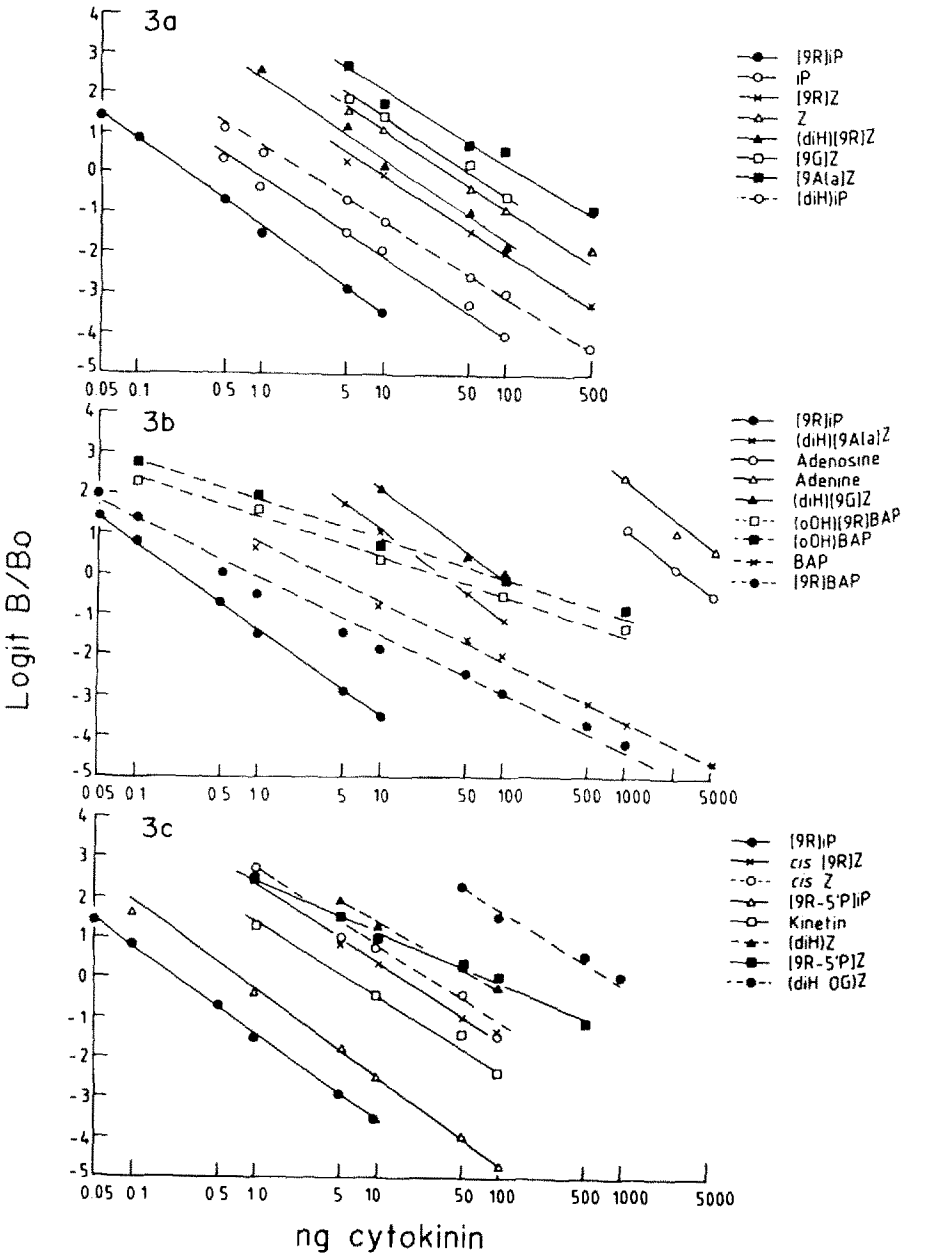

Fig. 3. Logit transformation curves for [9R]iP and compounds cross-reacting with the anti-[9R]iP serum. Results are for representative experiments. B, binding of tracer to antibody in the presence of compound; Bo, binding of tracer to antibody in the absence of compound.

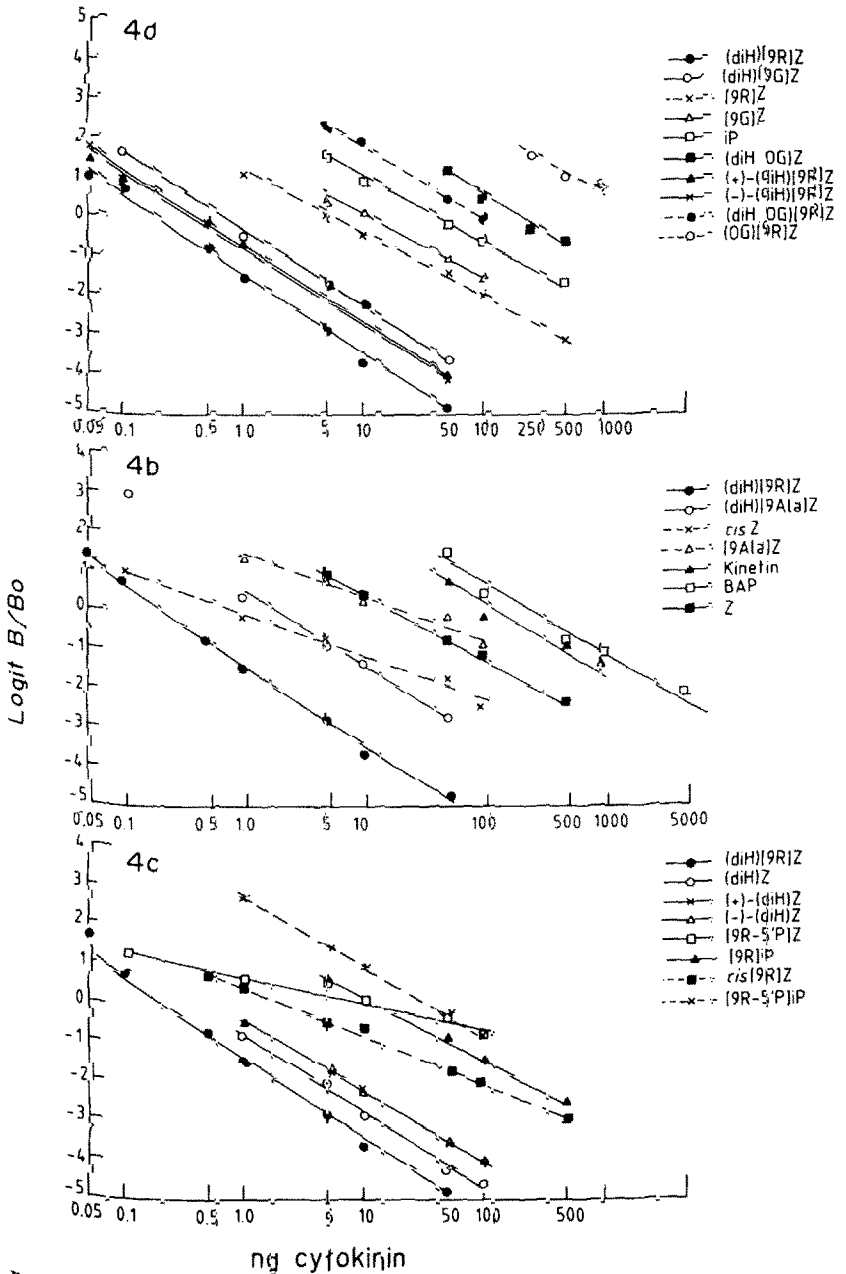

Fig. 4. Logit transformation curves for (diH)[9R]Z and compounds cross-reacting with the anti-(diH)[9R]Z serum. Results are for representative experiments. B, binding of tracer to antibody in the presence of compound; Bo, binding of tracer to antibody in absence of compound.

Table 1. Molar cross-reactivities of various purines with antibodies against [9R]Z. All studies except that of Eberle et al. (1986) refer to polyclonal antisera raised in rabbits.

Compound	(1)*	(2)*	(3)*	(4)*	(5)*	(6)*	(7)*	(8)*	(9)*	(10)*	(11)*	(12)*	(13)*[b] (i)	(ii)	(iii)
[9R]Z	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
(diH)[9R]Z	—[c]	—	—	1.9	—	—	—	—	—	0.56	—	—	2.6	2.4	1.2
Z	44	60	—	42	33.8	40.5	80	45	45	60.0	46	40.5	36.2	52.3	47.3
(diH)Z	1.72	33	—	1.3	2.14	0.3	18	1.3	31	1.2	1.0	0.3	3.0	2.9	2.3
[9G]Z	—	—	—	46	—	—	—	100	—	—	—	—	—	—	—
(diH)[9G]Z	—	—	—	2.8[a]	—	—	—	—	—	—	—	—	—	—	—
[9Ala]Z	8.4	—	—	21.9	—	—	—	—	—	—	—	—	—	—	—
(diH)[9Ala]Z	—	—	—	3.4[a]	—	—	—	—	—	—	—	—	—	—	—
cis[9R]Z	—	—	—	0.77[a]	0.78	—	0.6	0.9	—	—	1.4	—	5.3	1.9	0.8
cis-Z	—	—	—	2.1[a]	—	—	—	—	—	—	—	—	0.9	2.0	0.4
[7G]Z	—	—	—	0	—	—	—	3.0	—	—	—	—	—	—	—
[9R]iP	0.1	<0.5	0.26	0.2	0.84	0.4	5	0.44	0.15	0.47	0.1	0.4	1.8	3.1	0.5
iP	—	—	—	0.23[a]	0.23	0	9	0.27	—	0.21	0.4	0	0.6	0.5	0.9
(OG)[9R]Z	—	—	—	0	—	—	—	—	—	—	—	—	3.6	5.4	0.8
(diH OG)[9R]Z	—	—	—	0	—	—	—	—	—	—	—	—	3.2	0.04	0.07
(OG)Z	—	—	—	0	—	—	—	1.8	—	—	—	—	3.3	0.5	7.7
(diH OG)Z	—	—	—	0	—	—	—	0	—	—	—	—	0.2	0.02	0.8
[9R-5′P]Z	—	—	—	104	—	—	—	100	—	—	—	—	97.8	95.2	95.2
[9R-5′P]iP	—	—	—	0.49[a]	—	—	—	—	—	—	—	—	—	—	—
AMP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Adenosine	0	<0.1	—	0	<0.004	0	<0.05	0.002	—	0	0	0	<0.001	<0.001	<0.002
Adenine	0	—	—	0	<0.002	—	<0.005	0.006	0	0	—	—	0.08	<0.001	<0.006
K	0.03	<0.1	—	0.08	0.09	0	4	0.15	0.014	0.14	—	0	1.2	1.4	0.06
BAP	0.26	—	—	0.39	0.12	0	3	—	0.04	0.17	—	0	—	—	—

*(1) Weiler (1980); (2) MacDonald *et al.* (1981); (3) Vold and Leonard (1981); (4) Badenoch-Jones *et al.* (1984[b]); (5) Hansen *et al.* (1984); (6) Van Onckelen *et al.* (1984); (7) Barthe and Stewart (1985); (8) Turnbull and Hanke (1985); (9) Cahill *et al.* (1986); (10) Hofman *et al.* (1986[a]); (11) Maldiney *et al.* (1986); (12) Vonk *et al.* (1986); (13) Eberle *et al.* (1986).

[a]Values determined in the current study.

[b]Monoclonal antibodies: i, 118-1-D1; ii, 118-III-B5; iii, 13-1-B3.

[c]Not determined.

Table 2. Molar cross-reactivities of various purines with anti-[9R]iP serum. Data from the literature are also given.

Compound	Present study	(1)*	(2)*	(3)*	(4)*	(5)*	(6)*	(7)*	(8)*	(9)*	(10)*
[9R]Z	2.6	—[a]	—	<5	1.8	<1.5	<0.5	3	0.26	0.3	0.3
(diH)[9R]Z	1.61	—	—	—	—	—	—	—	—	0.3	—
Z	0.43	—	1.3	<5	0.9	<1.0	<0.5	0.1	0.031	0.3	0
(diH)Z	0.21	—	—	—	0.1	—	—	4	0.017	0.2	0
[9G]Z	0.45	—	—	—	—	—	—	—	—	—	—
(diH)[9G]Z	0.25	—	—	—	—	—	—	—	—	—	—
[9Ala]Z	0.13	—	—	—	0.1	—	—	—	—	—	—
(diH)[9Ala]Z	0.67	—	—	—	—	—	—	—	—	—	—
cis[9R]Z	1.47	—	—	—	1.4	—	—	—	—	—	—
cis-Z	0.69	—	—	—	—	—	—	<0.1	—	—	—
[7G]Z	0	—	—	—	—	—	—	—	—	—	—
[9R]iP	100	100	100	100	100	100	100	100	100	100	100
iP	19.4	26.9	98	100	56	67	17.4	49	64	40	38.2
(diH)iP	5.7	—	—	—	—	—	—	—	—	—	—
(OG)[9R]Z	0	—	—	—	—	—	—	—	—	—	—
(diH OG)[9R]Z	0	—	—	—	—	—	—	—	—	—	—
(OG)Z	0	—	—	—	—	—	—	—	—	—	—
(diH OG)Z	<0.03	—	—	—	—	—	—	—	—	—	—
[9R-5′P]Z	0.39	—	—	—	—	—	—	—	—	—	—
[9R-5′P]iP	37.9	87.5	—	37	—	—	—	—	—	—	—
AMP	0	—	—	—	—	—	—	—	—	—	—
Adenosine	0.0057	0.0035	0	<1	0	0	—	0.03	—	0	0
Adenine	<0.002	0.0002	0	—	0	0	—	—	0	—	0
K	2.6	2.3	8	<5	7.4	—	—	31	9	0.5	4.8
BAP	5.0	—	25	—	21.5	118	—	23	14	3.8	9.2
[9R]BAP	30.0	—	—	—	—	—	—	100	—	—	—
(oOH)BAP	0.17	—	—	—	—	—	—	—	—	—	—
(oOH)[9R]BAP	1.14	—	—	—	—	—	—	—	—	—	—

*(1) Milstone *et al.* (1978); (2) De Greef *et al.* (1980); (3) MacDonald *et al.* (1981); (4) Weiler and Spanier (1981); (5) Ernst *et al.* (1983a); (6) Van Onckelen *et al.* (1984); (7) Barthe and Stewart (1985); (8) Cahill *et al.* (1986); (9) Hofman *et al.* (1986a); (10) Vonk *et al.* (1986).
[a]Not determined.

Table 3. Molar cross-reactivities of various purines with anti-(diH)[9R]Z sera. The study of Eberle *et al.* (1986) refers to a monoclonal antibody they designated as J23-II-B1.

Compound	Present study	(1)*	(2)*	(3)*
[9R]Z	3.6	—[a]	1.7	16.1
(diH)[9R]Z	100	100	100	100
(+)-(diH)[9R]Z	52.3	—	—	—
(−)-(diH)[9R]Z	46.8	—	—	—
Z	0.69	—	1.7	1.3
(diH)Z	32.4	10	67.4	59.7
(+)-(diH)Z	23.4	—	—	—
(−)-(diH)Z	23.0	—	—	—
[9G]Z	1.64	—	—	—
(diH)[9G]Z	21.9	—	—	—
[9Ala]Z	0.82	—	—	—
(diH)[9Ala]Z	10.4	—	—	—
cis[9R]Z	6.9	—	10.7	—
cis-Z	14.2	—	1.0	—
[7G]Z	0	—	—	—
[9R]iP	1.63	—	1.0	3.8
iP	0.14	—	0.6	2.0
(OG)[9R]Z	<0.01	—	1.4	—
(diH OG)[9R]Z	0.29	—	0.7	—
(OG)Z	0	—	0.3	—
(diH OG)Z	0.10	—	9.7	—
[9R-5'P]Z	2.2	—	2.4	—
[9R-5'P]iP	0.69	—	—	—
AMP	0	—	—	—
Adenosine	0	<0.0001	<0.001	0
Adenine	0	—	<0.001	0
K	0.10	0.02	0.6	36.4
BAP	0.05	—	—	63.9

*(1) Zavala and Brandon (1983); (2) Eberle *et al.* (1986); (3) Hofman *et al.* (1985).
[a]Not determined.

Quantification of [7G]Z and [9G]Z Following Periodate Oxidation

[7G]Z (0- and 120-ng equivalents of Z) and [9G]Z (0- and 120-ng equivalents of Z) were periodate-oxidized as described in Materials and Methods. The oxidation product was made up in 60 µl of RIA buffer and assayed over a range of dilutions by RIA employing the anti-[9R]Z serum. The estimated amount of Z recovered was 137.4 ng (114.5% recovery) for [7G]Z and 119.4 ng (99.5% recovery) for [9G]Z.

Separation and Quantification of the Major Naturally Occurring Cytokinins

A mixture of cytokinins comprising the following authentic standards—[9R]Z, Z, (diH)[9R]Z, (diH)Z, (OG)[9R]Z, (OG)Z, (diH OG)[9R]Z, (diH OG)Z, [9G]Z, [7G]Z, [9Ala]Z, [9R]iP and iP—and [^{3}H](diH)Z and [^{3}H](diH)[9R]Z as recovery markers was subjected to TLC and HPLC as described in Materials and Methods. Following separation of all these cytokinins and β-glucosidase hydrolysis of the O-glucosides and periodate oxidation of [7G]Z, each cyto-

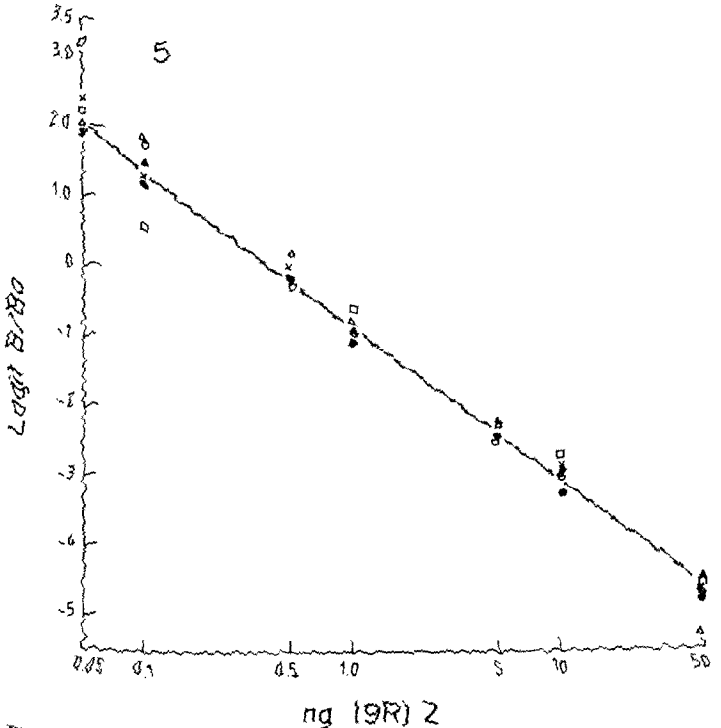

Fig. 5. Logit transformation curve for [9R]Z. [9R]Z standards were in the presence of graded amounts (0 to 4608 dpm) of [^{3}H][9R]Z recovery marker (14) GBq mmol^{-1}); B, binding of tracer to antibody in the presence of [9R]Z; Bo, binding of tracer to antibody in the absence of [9R]Z. Added [^{3}H][9R]Z recovery marker (dpm) was ●, 0; ▲, 500; ○, 1007; X, 2200; □, 2828; △, 4608. In calculating B and Bo, appropriate blank values were used (i.e., blank tubes contained no antiserum, no [9R]Z, but added [^{3}H][9R]Z).

kinin was assayed using the appropriate RIA. Each cytokinin was detected at its expected elution time or R_f, and recoveries (calculated as a percentage of the amount of each cytokinin added to the initial mixture) were as follows: [9R]Z (83.6%), Z (66.2%), (diH)[9R]Z (72.3%), (diH)Z (61.6%), (OG)[9R]Z (24.8%), (OG)Z (32.3%), (diH OG)[9R]Z (50.5%), (diH OG)Z (50.4%), [9G]Z (88.4%), [7G]Z (12.9%), [9Ala]Z (68.1%), [9R]iP (49.5%), and iP (34.3%). Recoveries of the recovery markers (calculated as a percentage of each radioactive recovery marker added to the initial mixture) were as follows: [^{3}H](diH)Z (55.0%) and [^{3}H](diH)[9R]Z (82.1%).

Discussion

Specificities of Antisera

We have raised antisera in rabbits against [9R]iP and (diH)[9R]Z, characterized them, and employed them in RIAs. The extensive cross-reactivity data on these antisera (and on an anti-[9R]Z serum raised previously; Badenoch-Jones

et al. 1984b) can be compared with data in the literature for similar antisera (Tables 1–3). All the cytokinin antisera show marked selectivity for the N^6-prenyl side chain. In most studies so far, cytokinin ribosides have been used for immunogen synthesis, with the ribose moiety forming the bridge to the protein in the immunogen, and the antisera show only weak specificity for the ribose moiety. Thus, the free bases, the nucleotides, and other 9-substituted Z or (diH)Z cytokinins usually show moderate to strong cross-reactivity but generally less than the cross-reactivity of the riboside. In contrast, when antibodies were raised against a cytokinin nucleotide ([9R-5'P]iP (Milstone et al. 1978), the phosphate group played an important role in antibody recognition.

Weiler (1982) and Badenoch-Jones et al. (1984b) pointed out that the cross-reactivities of various purines to anti-[9R]Z sera raised in different laboratories are very similar. Since then, other workers have raised and characterized such sera (Hansen et al. 1984, Van Onckelen et al. 1984, Barthe and Stewart 1985, Turnbull and Hanke 1985, Vonk et al. 1986), and these antisera also have similar cross-reactivity characteristics to those described previously, particularly the antiserum raised by Hansen et al. (1984) (Table 1). The antiserum elicited by Van Onckelen et al. (1984) was unusual in having a high (63%) cross-reactivity (presumably molar cross-reactivity) with (OG)[9R]Z. The antiserum raised by Turnbull and Hanke (1985) differed slightly from that raised by Badenoch-Jones et al. (1984b) in having higher molar cross-reactivity with [9G]Z (100% and 46%, respectively) and in having detectable cross-reactivity with [7G]Z (3%) and with (OG)Z (1.8%).

Other workers have not determined the cross-reactivities of their antisera with [9G]Z or [7G]Z, but the monoclonal antibodies developed by Eberle et al. (1986) all showed low, but detectable, cross-reactivities with the four O-glucosides. The antiserum raised by Barthe and Stewart (1985) differed slightly from those raised by other workers in having higher cross-reactivities with Z, [9R]iP, iP, BAP, and kinetin (K); like the antisera raised by MacDonald et al. (1981) and Cahill et al. (1986), it had moderate cross-reactivity with (diH)Z.

As with anti-[9R]Z sera, the cross-reactivities of various purines to anti-[9R]iP sera raised in different laboratories are generally similar (Table 2). All antisera (when tested) showed moderate or strong cross-reactivity with iP, [9R-5'P]iP, BAP, and [9R]BAP, but the exact molar cross-reactivities against each of these compounds varied to some extent—for example, for iP, from 17.4% to 100%; for BAP, from 3.8% to 118%; and for [9R-5'P]iP, from 37% to 88%. The antiserum raised by Barthe and Stewart (1985) was unusual with respect to its high cross-reactivity against K.

Some details of the cross-reactivity characteristics of anti-(diH)[9R]Z sera have been published by Zavala and Brandon (1983) and Hofman et al. (1985), and extensive data have been published on a (diH)[9R]Z monoclonal antibody (Eberle et al. 1986), but other results have only been published in preliminary form (Brandon et al. 1980, Sayavedra-Solo et al. 1983, Griggs et al. 1985) (Table 3). Thus, for anti-(diH)[9R]Z sera, there are only limited data for making comparisons. However, it would also appear that cross-reactivities of various purines to the anti-(diH)[9R]Z sera raised in different laboratories are similar,

although the very high cross-reactivity of the antiserum raised by Hofman et al. (1986b) with BAP and K is rather surprising.

For both [9R]iP and (diH)[9R]Z, cross-reactivity characteristics of antisera from different rabbits immunized with the same antigen were very similar. This finding is in agreement with the observation noted above that for a given cytokinin antigen, the cross-reactivity characteristics of antisera raised in different laboratories are similar. This suggests that if one specifically wishes to obtain any cytokinin antibody with cross-reactivity characteristics that are different from those of cytokinin antisera already raised and characterized—for example, an antibody that is highly specific for a single cytokinin—then it will be necessary to use an alternative to polyclonal antisera elicited in rabbits.

The development of an antibody that is specific for a single cytokinin might eliminate the need for many of the sample purification steps currently required prior to analysis by RIA. However, it would appear that little success has been achieved in developing monoclonal antibodies that are essentially monospecific. Woodsworth et al. (1983) developed monoclonal antibodies against [9R]iP and [9R]Z with the specific aim of obtaining antibodies with increased specificity. Clone JEL 75 showed the greatest specificity for [9R]Z; however, it cross-reacted measurably (6–7%) with [9R]iP, methylthio[9R]Z, *cis*-methylthio[9R]Z, and adenosine. Clone JEL 77 showed the greatest specificity for [9R]iP, and its cross-reactivity with five other cytokinins was less than 5%. However, before this antibody can be considered specific for [9R]iP, much more extensive and more sensitive data are required on its cross-reactivity characteristics.

Trione et al. (1985) characterized seven monoclonal antibodies developed against [9R]Z. Cross-reactivities against *cis*[9R]Z, (diH)[9R]Z, Z, and [9R]iP were measured, and each antibody cross-reacted measurably (about 10–200%) with two or three of these cytokinins. Eberle et al. (1986) extensively characterized three monoclonal antibodies developed against [9R]Z and one developed against (diH)[9R]Z. All antibodies cross-reacted strongly with their corresponding bases. In fact, cross-reactivity data for the monoclonal antisera were very similar to those for polyclonal sera (see Tables 1, 3). Monoclonal antibodies, however, do have an advantage over polyclonal antibodies by providing an indefinite supply of a standardizable reagent. They may suffer from the disadvantage that assays employing them have a narrower measuring range than those employing polyclonal antisera (Trione et al. 1985).

In the present study, racemic (±)-(diH)[9R]Z was conjugated to BSA prior to immunizing the rabbits to elicit the anti-(diH)[9R]Z sera and was also used for the synthesis of the tracer. Cross-reactivity data for the selected anti-(diH)[9R]Z serum revealed that it had equal affinity for (+)-(diH)[9R]Z and (−)-(diH)[9R]Z and equal affinity for (+)-(diH)Z and (−)-(diH)Z. However, it had less affinity, by factors of 49.5% and 71.6%, respectively, for the separate enantiomers of (diH)[9R]Z than for the racemic (±)-(diH)[9R]Z, and for the separate enantiomers of (diH)Z than for racemic (±)-(diH)Z.

These results suggest that two distinct populations of antibodies were raised, with equal affinity for their corresponding haptens, but with less affinity (but

both to the same extent) for the opposite enantiomer. It is of interest that when antisera have been raised against racemic (±) abscisic acid, it would also appear that two distinct populations of antibodies were produced (Walton et al. 1979, Rosher et al. 1985), but in this case, antibodies with different affinities for the (+) and (−) enantiomers were produced. Because of the more ready availability of racemic (±)-(diH)[9R]Z than the naturally occurring (−)-(diH)[9R]Z enantiomer (Fujii and Ogawa 1972), we routinely use (±)-(diH)[9R]Z as the standard and apply the appropriate correction factors —2.02 and 1.40, respectively—when calculating the amounts of (diH)[9R]Z and (diH)Z in biological samples, in order to avoid underestimation of these two cytokinins.

Quantification of Cytokinins by RIA and Associated Separative Procedures

Using the two RIAs developed in this study, and an RIA employing anti-[9R]Z serum, it is possible to quantify by RIA a wide range of naturally occurring cytokinins: [9R]Z, Z, (diH)[9R]Z, (diH)Z, [9G]Z, (diH)[9G]Z, [9Ala]Z, (diH)[9Ala]Z, [9R]iP, iP, [9R-5′P]Z, and [9R-5′P]iP, and, most likely, [9R-5′P](diH)Z. Although not cross-reactive with the antisera, the O-glucosides ((OG)[9R]Z, (OG)Z, (diH OG)[9R]Z, and (diH OG)Z) can be quantified following β-glucosidase hydrolysis to their respective bases or ribosides as suggested previously (Badenoch-Jones et al. 1984b), and [7G]Z can be quantified following periodate oxidation. The importance of being able to quantify a wide range of naturally occurring cytokinins cannot be overemphasized. Thus, whereas previous studies employing bioassays have often placed emphasis on Z and [9R]Z, it is becoming increasingly apparent that other cytokinins may be quantitatively important (see Introduction), depending on the plant species, the plant tissue, and the stage of growth.

We have previously emphasized the need for the separation of cytokinins prior to analysis by RIA (Badenoch-Jones et al. 1984b), and this requirement is now generally well recognized (see Ernst 1986). In this study we have presented methods by which the wide range of cytokinins that might be found in many biological samples can be separated appropriately prior to analysis by RIA. Further work will be required to assess the feasibility of purifying biological samples by the methods proposed in this study. We present a working model that can be readily modified, or combined with other methods such as immunoaffinity chromatography (Morris et al. 1982). In our model, summarized in Fig. 6, it is envisaged that samples would first be purified by ion exchange chromatography on cellulose phosphate (Badenoch-Jones et al. 1984a), which would separate nucleotides (acidic wash) from bases, ribosides, and conjugates (basic eluate). This procedure is known to give almost quantitative recoveries of cytokinin ribosides (Summons et al. 1979b).

Using a mixture of authentic standards, we have shown that the basic eluate can then be subjected to TLC, using butanol/acetic acid/water as solvent, for separation of the following cytokinins or groups of cytokinins: [9Ala]Z; [7G]Z and the O-glucosides; [9G]Z; Z, (diH)Z, [9R]Z, and (diH)[9R]Z; and iP and [9R]iP. [9Ala]Z and [9G]Z can be assayed directly by RIA employing the anti-[9R]Z serum. iP and [9R]iP can be separated from each other by HPLC on

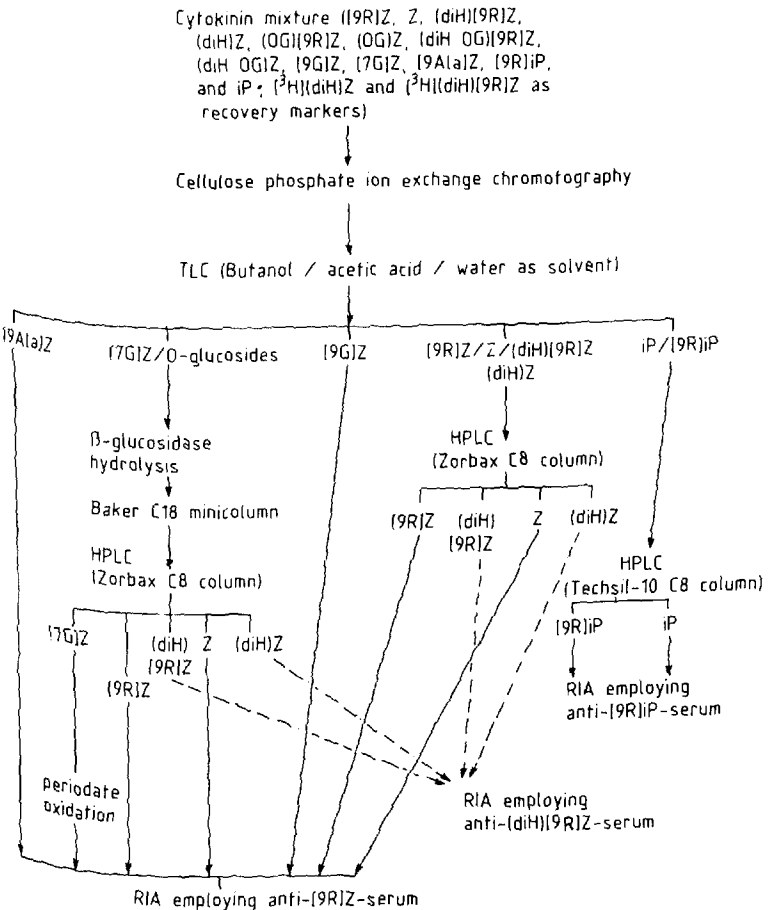

Fig. 6. Model scheme for the separation of cytokinins prior to RIA.

a Techsil 10 C_8 column and then assayed by RIA employing the anti-[9R]iP serum. The Z/(diH)Z/[9R]Z/(diH)[9R]Z mixture can be separated into its four component cytokinins by HPLC on a Zorbax C_8 column (Badenoch-Jones et al. 1984b). Z and [9R]Z can be assayed by RIA employing the anti-[9R]Z serum, and (diH)Z and (diH)[9R]Z can be assayed by RIA employing the anti-(diH)[9R]Z serum. The [7G]Z/O-glucoside mixture can be subjected to β-glucosidase digestion to hydrolyze the O-glucosides to their component bases or ribosides, and the cytokinins of the resultant mixture ([7G]Z/Z/(diH)Z/[9R]Z/(diH)[9R]Z) can be separated from each other by HPLC on a Zorbax C_8 column. Z and [9R]Z can then be assayed by RIA employing the anti-[9R]Z serum, and (diH)Z and (diH)[9R]Z can be assayed by RIA employing the anti-(diH)[9R]Z serum. [7G]Z requires periodate oxidation prior to analysis by RIA using the anti-[9R]Z serum.

With both labeled and unlabeled cytokinins, our recovery data suggest a better recovery of the Z/(diH)Z ribosides than the bases. Correcting the recovery of bases using the recovery of the [3H](diH)Z marker (57.0%) and the

recovery of ribosides using the recovery of the [^{3}H](diH)[9R]Z marker (82.1%) gives the following corrected recoveries: [9R]Z (101.8%), Z (116.1%), (diH)[9R]Z (88.1%), (diH)Z (108.1%), (OG)[9R]Z (30.2%), (OG)Z (56.7%), (OG diH)[9R]Z (61.5%), (OG diH)Z (88.4%), [9R]iP (60.3%), and iP (60.2%). These values give an indication of the accuracy with which we can estimate these cytokinins in biological samples using the techniques we have described. Clearly, in the absence of high-specific-activity O-glucoside and [9R]iP and iP recovery markers, the accuracy with which these cytokinins can be quantified is somewhat limited, and recovery corrections based on recovery of zeatin base/riboside markers may underestimate these compounds.

For the O-glucosides, recovery could be more accurately estimated if tritiated base and riboside recovery markers were added prior to the β-glucosidase hydrolysis step. In the absence of high-specific-activity recovery markers for [9G]Z and [9Ala]Z, the results from this study suggest that the (diH)[9R]Z marker might provide a suitable estimate of recovery for [9G]Z and the mean recovery of the (diH)Z and (diH)[9R]Z markers might provide a suitable estimate of the recovery of [9Ala]Z, giving corrected recoveries of 107.7% and 97.9%, respectively. The recovery of [7G]Z, as expected, was low, and even using the recovery of the [^{3}H](diH)Z marker as a correction factor only gives a corrected recovery of 22.6%. Thus, in the absence of a high-specific-activity [7G]Z recovery marker, recovery of [7G]Z will almost certainly be underestimated. It is expected, however, that in the near future it will be possible to purchase high-specific-activity recovery markers for a wider range of cytokinins than is currently available.

In this study we have not examined the nucleotides in detail. However, our previous study (Badenoch-Jones et al. 1984b) indicated that it is possible to estimate [9R-5'P]Z from the cellulose phosphate wash fraction after further purification by DEAE cellulose chromatography. This quantification is possible because of the high cross-reactivity of [9R-5'P]Z with the anti-[9R]Z serum (104%). Data from this study indicate that [9R-5'P]iP has sufficient cross-reactivity with the anti-[9R]iP serum (37.9%) to enable its quantification by RIA. When this work was carried out, a [9R-5'P](diH)Z standard was not available, although it has now become possible to purchase this compound (Apex Organics, Osney Mead, Oxford, U.K.). It is probable that [9R-5'P](diH)Z also shows sufficient cross-reactivity with the anti-(diH)[9R]Z serum to enable its quantification by RIA. [9R-5'P]iP has low cross-reactivity with the anti-[9R]Z and anti-(diH)[9R]Z sera (0.49% and 0.69%, respectively) and [9R-5'P]Z has low cross-reactivity with the anti-[9R]iP and anti-(diH)[9R]Z sera (0.39% and 2.23%, respectively). The cross-reactivities of [9R-5'P](diH)Z with the anti-[9R]Z and anti-[9R]iP sera are unknown, but they are probably also low. Thus, it may be possible to quantify each nucleotide by RIA analysis of the DEAE eluate fractions with its corresponding antiserum, possibly without the need to correct for the presence of the other cytokinin nucleotides in the same sample. Further work is required before it is known whether DEAE cellulose chromatography is sufficient purification prior to nucleotide analysis.

Palmer and Wong (1985) have reported the occurrence of O-glucoside nu-

cleotides that have also been detected in mistletoe xylem saps (Hall et al. 1987). Clearly, these nucleotides could be quantified by RIA following β-glucosidase digestion of an aliquot of the nucleotide fraction.

Unlike the anti-[9R]Z serum, the anti-(diH)[9R]Z serum shows quite strong cross-reactivity with *cis*-Z (14.2%) and, to a lesser extent, with *cis*[9R]Z (6.9%). Hence, it would be desirable to separate the *cis* and dihydro compounds prior to RIA. The chromatographic properties of *cis*-Z and *cis*[9R]Z are described in an accompanying study (Badenoch-Jones et al., submitted).

[9G]Z and (diH)[9G]Z cross-reacted moderately with their corresponding antisera (46% and 21.9%, respectively) and similarly for [9Ala]Z and (diH)[9Ala]Z (22% and 10.4%, respectively) (see Tables 1, 3; Bandenoch-Jones et al. 1984b). Our data indicate that it would be possible to quantify (diH)[9G]Z and (diH)[9Ala]Z using RIA employing the anti-(diH)[9R]Z serum. However, since the cross-reactivities of these compounds with this antiserum are relatively low, the sensitivity of the assay for these compounds would also be relatively low; about 0.1 ng (diH)[9G]Z and 0.5 ng of (diH)[9Ala]Z would be required in order to be detected with any accuracy. (diH)[9G]Z could be hydrolyzed to (diH)Z (by periodate oxidation as described in Materials and Methods for [7G]Z and [9G]Z) prior to quantification by RIA, but the rise in sensitivity would only be about 50% (calcuated from the cross-reactivities of (diH)[9G]Z and (diH)Z with the anti-(diH)[9R]Z serum). (diH)[9Ala]Z would cochromatograph with [9Ala]Z, and (diH)[9G]Z would cochromatograph with [9G]Z following TLC with butanol/acetic acid/water as solvent. For quantifying both [9G]Z and [9Ala]Z, it might be considered preferable to separate them from their dihydro forms, because for both cytokinins there is only about a 10-fold difference in cross-reactivity between the dihydro and unsaturated forms in both RIAs (employing the anti[9R]Z serum or the anti-(diH)[9R]Z serum). These separations can be achieved readily by HPLC (unpublished data).

There is evidence that [9R]BAP occurs naturally in a cell culture of anise (Ernst et al. 1983b) and in tomato (Letham, unpublished data), and that (oOH)[9R]BAP occurs naturally in *Populus robusta* (Horgan et al. 1975). Thus, the need to detect and quantify these cytokinins by RIA requires consideration. In some experiments, it may also be of interest to quantify synthetic BAP (e.g., in culture media). Some workers have elicited antibodies against [9R]BAP (Constantinidou et al. 1978). We did not produce a separate antibody against [9R]BAP, because our anti-[9R]iP serum cross-reacts strongly with [9R]BAP (30% cross-reactivity) and, to a lesser extent, with BAP (5.0% cross-reactivity). With this antiserum it is possible to detect 0.1 ng of [9R]BAP. Because of the shallower slopes of the logit transformation curves for [9R]BAP, BAP and in particular, (oOH)[9R]BAP, compared with the curve for standard [9R]iP, the molar cross-reactivity values determined at 50% binding do not adequately reflect (underestimate) the potential for detecting low levels of these compounds. Thus, even though the cross-reactivity of (oOH)[9R]BAP with the anti-[9R]iP serum is only 1.1%, it is possible to detect this compound at the 0.1-ng level.

Since RIA employing the anti-[9R]iP serum has the potential to detect low

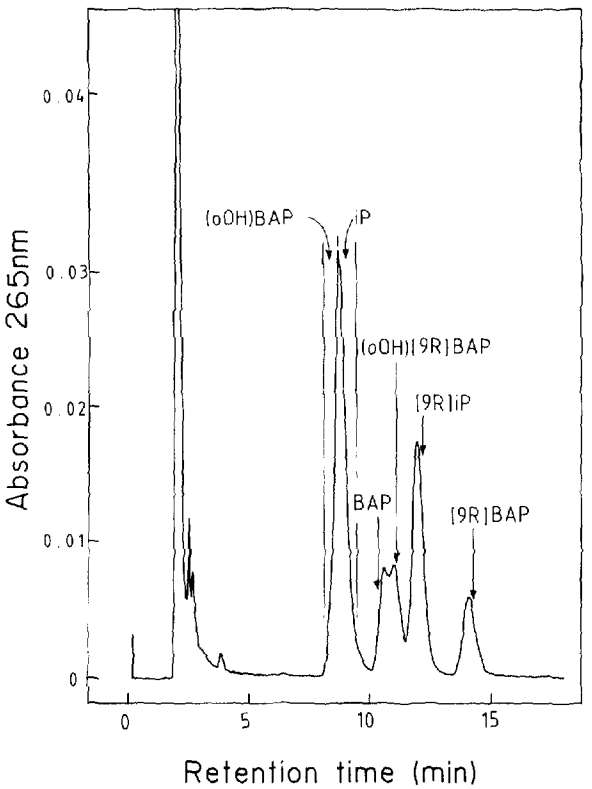

Fig. 7. The elution profile of a mixture of cytokinin standards on a Techsil 10 C_8 column (solvent, 30% (v/v) methanol in 0.2 M acetic acid; flow rate, 4.0 ml min^{-1}), as monitored by UV absorbance (λ = 265 nm).

levels of [9R]BAP and (oOH)[9R]BAP, the possible occurrence of these compounds in biological samples must be borne in mind when one is interpreting the results of RIA (employing the anti-[9R]iP serum) analysis of HPLC fractions off the Zorbax column. The elution times of [9R]BAP and (oOH)[9R]BAP (and also of BAP and (oOH)BAP) in relation to those of [9R]iP and iP on the Techsil 10 C_8 column are shown in Fig. 7. Hofman et al. (1986b) have used RIA employing an anti-(diH)[9R]Z serum for quantifying BAP and also K. A comparison of the cross-reactivity properties of our anti-(diH)[9R]Z and anti-[9R]iP sera (Tables 2, 3) reveals that the anti-[9R]iP serum is the more appropriate of our antisera to use for quantifying BAP, (oOH)BAP, and their ribosides, and also K. Anti-[9R]iP sera raised by other workers (Ernst et al. 1983a, Barthe and Stewart 1985) also cross-reacted strongly with BAP and [9R]BAP (Table 2).

The RIAs developed in this study were of similar sensitivity, and covered similar measuring ranges, as those reported by other workers. Enzyme-linked immunosorbent assays (ELISAs) (Hansen et al. 1984, Barthe and Stewart 1985, Vonk et al. 1986) were generally slightly less sensitive than RIA, but they may have advantages of convenience, safety, and cost of equipment and materials.

Several deficiencies of the RIA employing the anti-[9R]iP serum developed in the present study are apparent. First, for our antiserum, the sensitivity for quantifying the base is only about one-fifth that for quantifying the riboside. Fortunately, however, of all our cytokinin RIAs, that employing the anti-[9R]iP serum is the most sensitive, and it is still possible to detect as little as 0.3 pmol of iP. Second, the anti-[9R]iP serum had the lowest titer of our four antisera, the final dilution in the assay being 1:4500. Apart from the obvious fact that the lower the titer, the fewer the assays that can be performed with a given volume of serum, there is also a greater probability of interference in the assay from other compounds when the antiserum titer is low. In addition, there are many compounds within plant (and animal) cells with isopentenyl moieties. These compounds resemble iP and [9R]iP, and hence the problem of cross-reacting contaminants is likely to be greater in the RIA employing the anti-[9R]iP serum than in the other cytokinin RIAs. Data from the present study and from that of Barthe and Stewart (1985) also suggest that anti-[9R]iP sera show low, but readily measurable, cross-reactivity with a wider range of compounds than do other cytokinin antisera.

We have previously indicated the importance of using high-specific-activity recovery markers for assessing losses of cytokinins during sample purification prior to RIA (Badenoch-Jones et al. 1984b). In this study we have successfully employed two recovery markers simultaneously. About 42 Bq (2500 dpm) of each of the markers was incorporated into the original cytokinin mixture. With a recovery of about 75% at the end of the purification procedure, counting aliquots of about 5–10% of the total sample (i.e., about 94–188 dpm) gave counts well over three times background level. Data in Fig. 5 reveal that such low levels of recovery marker in the sample to be assayed (about 280–470 dpm, as about 15–25% aliquots of the total sample are assayed in RIA) have no effect on the RIA. Even considerably higher amounts of recovery marker would not affect the RIA, provided the appropriate blanks are used in the assay. Also, the mass of cytokinin in the sample that is present as recovery marker would be about 1000-fold to 10,000-fold less than the 0.05 ng detectable in the assay.

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