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Radioimmunoassays for trans-Zeatin and Related Cytokinins*

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Abstract. Radioimmunoassays for the quantitation of trans-zeatin and related cytokinins have been developed. Antisera produced against bovine serum albumin conjugates of trans-zeatinriboside have a high affinity ($K_a = 2.4 \cdot 10^{-11}$ M) for zeatinriboside and for zeatin, but show a negligible cross reaction to isopentenyladenosine (0.1%) and cis-zeatinriboside (0.4%), only a slight cross reaction to dihydrozeatin (1.7%), and no cross reaction at all to other purines, such as adenosine and related compounds, was observed. The assays are sensitive and measuring ranges extend from 0.06–30 pmol (0.02–10 ng) of zeatinriboside. This has been achieved by employing as tracers immunoreactive zeatin derivatives with high-specific activity, (tritiated zeatinriboside-dialcohol: $8.37 \cdot 10^{11}$ Bq mmol^{-1} and zeatinribosyl-[^{125}I]tyramine: ca. $1.9 \cdot 10^{13}$ Bq mmol^{-1}. The detection limit is 40 fmol (15 pg) for the assay employing the tritiated tracer, and assay reproducibility is high (variation coefficients of triplicates less than 5%). Several hundred assays can be completed in one day, and, due to the high specificity of this assay, crude extracts may be used for analysis. The course of zeatin levels in developing fruits of *Lycopersicon esculentum* cv. Moneymaker is given.

Key words: Cytokinin (radioimmunoassay) – *Lycopersicon* – Radioimmunoassay (cytokinins).

Introduction

Cytokinins are considered to be one of the classes of phytohormones which are the most difficult to quantitate. The determination of cytokinins is still largely dependent on bioassays (for a recent review, see Letham 1978), although other techniques, such as GLC (Chapman et al. 1976; Upper et al. 1970), MS (Hashizume et al. 1979; Morris 1977), LC (Carnes et al. 1975; Challice 1975; Thomas et al. 1975), and more recently HPLC (Horgan and Kramers 1979; Kannangara et al. 1978; Holland et al. 1978) have been applied to the separation, identification, and quantitation of cytokinins as well. However, the physico-chemical methods currently available are of low sensitivity and require extraction and purification of almost µg-amounts of cytokinins for analysis. Consequently the sample size must be large and the work-up requires considerable time, with overall recoveries of sometimes not even 10% (Monselise et al. 1978).

By comparison with the physico-chemical assays, several bioassays are more sensitive, but they are unfortunately very time-consuming and sometimes require several weeks before results are obtained. Bioassays in general are subject to different sources of variation and, as a consequence, are rather unprecise.

Radioimmunoassay, an analytical method originally developed to quantitate human hormones at physiological tissue levels (Berson and Yalow 1959), would seem to be ideally suited to the determination of cytokinins in that it can be rendered highly specific and very sensitive.

Immunological assays have been applied to the analysis of purines (see review by Lübke and Nieuweboer 1978) after it had been demonstrated that coupling of nucleosides to proteins gives immunogenic conjugates which can be used to produce nucleoside-specific antisera (Erlanger and Beiser 1964). Antisera have also been produced against isopentenyladenosine (Humayun and Jacob 1974; Khan et al. 1977; Milstone et al. 1978) and benzylaminopurine (Constantinidou et al. 1978) and have been used in the immunological assays for these compounds. Although

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Abbreviations: GC = gas chromatography; LC = liquid chromatography; MS = mass spectroscopy; RIA = radioimmunoassay; TLC = thin layer chromatography; UB = unspecific binding

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in most cases specificity of the antisera was rather poor, it was also demonstrated that more specific isopentenyladenosine-antisera can be obtained (Hamayun and Jacob 1974) which are useful for the radioimmunoassay of this compound (Khan et al. 1977). Antisera against dihydrozeatin have also been reported in abstract form (Brandon et al. 1979) but were found to be unspecific.

The most important cytokinins of higher plants, besides those containing isopentenyladenine, are those of the trans-zeatin type. However, immunological assays for this important plant-growth regulator are not available yet.

Cis-zeatin may occur in the same tissue together with trans-zeatin, although the former has been identified only in t-RNA (see: Letham 1978). A closely related compound, dihydrozeatin, may also occur. In addition, zeatin differs from isopentenyladenin only by the presence of the side-chain hydroxyl group. There is therefore a need for antisera of highest specificity to differentiate between the naturally occurring cytokinins. We report here on the development and characterization of sensitive and specific radioimmunoassays for the quantitation of trans-zeatin and related cytokinins, at the fmol (10^{-15} mol) level, which make use of immunoreactive derivatives of trans-zeatinriboside labeled with either tritium or with iodine-125 as tracers. These assays have been applied to the direct analysis of crude plant extracts.

Materials and Methods

Plant Material. Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were grown in the field. Fruits of different ripening stages were harvested from single plants and worked up as described below.

Chemicals and Immunochemicals. Zeatin, zeatinriboside, isopentenyladenosine, adenosine, guanosine, cytidine, and tyramine were purchased from Sigma, sodium-cyanoborohydride was from Aldrich. Bovine serum was purchased from Mediapharm, bovine serum albumin (purity over 99%) came from Serva, and Freund's complete adjuvant was obtained from Difco. All other chemicals and reagents were of the highest purity available.

Extraction of Plant Material. Tissues were frozen in liquid nitrogen, powdered, and extracted three times (for 1, 2, and 11 days) with 20 ml of 80% methanol per 10 g fresh weight. Extraction was carried out at 4° C in the dark. The methanolic fraction of the combined extracts was evaporated in vacuo to leave an aqueous phase which was cleared by centrifugation (15 min at 10.000 *g*). Aliquots (0.1 ml) of the supernatants were immunoassayed directly.

Preparation of Bovine Serum Albumin Conjugates of trans-Zeatinriboside. The procedure used is a modification of the method described by Erlanger and Beiser (1964) originally used for coupling nucleosides to proteins.

Zeatinriboside (10.3 mg, 29.4 μmol) was suspended in 1 ml methanol and 5 ml 0.01 M $NaJO_4$ solution (50 μmol) was added over a period of 7 min. The clear solution was stirred for another 13 min, and 0.3 ml 0.1 M ethylene glycol (30 μmol) were added. After 5 min, the reaction mixture was added dropwise to a stirred solution of 110 mg bovine serum albumin in 5 ml water (adjusted to pH 9.3 with 5% K_2CO_3). During the addition of the oxidized riboside, the pH was kept constant between 9.2 and 9.4 by further addition of 5% K_2CO_3. After 60 min, solid $NaBH_4$ (5 mg) was added, and after another 40 min this step was repeated. Thereafter, the pH was lowered to 6.5 by the addition of 1 M acetic acid and the solution stirred for another 2 h. The conjugate was purified by dialysis against water (3 days, 4° C), lyophilized, and stored at −18° C. From the ultraviolet spectra, a coupling ratio of 2.6 mol zeatin per mol of protein was calculated.

Synthesis of Tritiated trans-Zeatinriboside-dialcohol of High-Specific Activity. Zeatinriboside was periodate oxidized as described above, but after the addition of ethylene glycol, the reaction mixture was purified by TLC ($CHCl_3$: methanol = 9:1) and the dialdehyde of zeatinriboside ($R_f = 0.1$) was isolated in quantitative yield. The chromatographically pure compound (1.2 mg, 3.4 μmol) was added as an ethanolic solution to solid, tritiated, sodium-borohydride (NEN, spec. act. $1.8 \cdot 10^{12}$ Bq mmol^{-1}, approx. $1.1 \cdot 10^{7}$ Bq, 6.2 μmol). After a reaction period of 10 min at room temperature, the reaction mixture was purified by TLC ($CHCl_3$: metanol = 9:2) and was found to contain only a single labeled compound, namely trans-zeatindialcohol ($R_f = 0.13$); no mono-reduced reaction intermediate ($R_f = 0.3$) was found. The compound was obtained radiochemically pure (>99%) and stored as a methanolic solution at −18° C under N_2. The specific activity of this material was $8.37 \cdot 10^{11}$ mmol^{-1}, as determined by the self-displacement method (Chervu and Murty 1975).

Synthesis of Zeatinribosyl-[^{125}I]tyramine. Reductive coupling of tyramine to zeatinriboside-dialdehyde was performed by a modification of the procedure described by Röder and Focken (1978) for cardiac glycosides. Zeatinriboside-dialdehyde (6 μmol) prepared as described above was dissolved in 0.5 ml dry methanol, and tyramine hydrochloride (9.4 mg, 54 μmol) was added with stirring. To this solution was added 0.342 mg $NaCNBH_3$ dissolved in 0.1 ml dry methanol. The mixture was stirred at room temperature for 2 days, after which the dialdehyde was converted completely and two products were formed (product A, ca. 85%, $R_f = 0.23$, product B, ca. 15%, $R_f = 0.29$, plates were developed 2 times in $CHCl_3$: methanol = 9:1). Iodination of both fractions yielded highly immunoreactive tracers, but further work was performed only with product B. Upon iodination, this compound gave two products (mono- and diiodo-derivative), whereas product A gave at least 3 labeled, immunoreactive derivatives. It is thus assumed that product B is the cyclic mono-tyramyl-derivative, and product A is the di-tyramyl-derivative of zeatinriboside-dialdehyde. Iodination was performed as described previously (Weiler and Zenk 1979), but with the modification that 0.2 M phosphate buffer pH 7.5 was used for all reagents. The monoiodinated zeatinribosyl-[^{125}I]tyramine ($R_f = 0.35$ in the above solvent system) could be readily separated from the diiodinated compound ($R_f = 0.47$), and after a single TLC purification step it exhibited immunoreactivities of 80% (tracer binding to excess of antiserum, corrected for unspecific binding).

Immunization and Antiserum Production. Randomly bred rabbits (12–16 weeks old) were immunized as described before (Weiler and Zenk 1976), and blood was collected from the marginal ear veins. The antisera were stored at −18° C and were found to be stable under these storage conditions.

Radioimmunoassay. Radioimmunoassays were performed using semi-automated equipment, as described before (Weiler and Zenk 1976, Arens et al. 1978, Weiler 1979). Briefly, assays were performed as follows: Standards and samples were processed in triplicate. The incubation mixture contained per tube: 0.5 ml buffer (0.01 M phosphate, 0.15 M sodium chloride, pH 7.4), 0.1 ml dilute bovine serum, 0.1 ml dilute tracer solution, 0.1 ml standard or sample, and 0.1 ml dilute antiserum (or water for determination of unspecific binding). After mixing the tubes were incubated for 60 min at room temperature, and 1.2 ml 91% saturated $(NH_4)_2SO_4$ solution was added to precipitate the immunoglobulins. After 30 min at room temperature, the tubes were centrifuged and the pellets were either dissolved in 0.5 ml water and counted directly (^{125}I-assay) or washed with 1 ml half-saturated $(NH_4)_2SO_4$, recentrifuged, and dissolved in 0.2 ml water, followed by the addition of 1 ml scintillator (Minisolve). After thorough mixing the tubes were counted for radioactivity (^{3}H-assay). The results were computed in a programmed calculator (Hewlett-Packard) using the spline approximation method.

Results

1. Antiserum Characteristics and Tracer Stability. Although all animals developed antibodies against zeatin, serum from only one animal was selected. After a preliminary binding and specificity study of the individual serum fractions obtained, those exhibiting the best properties were pooled and used for the present study.

In the range of pH 5.5 to pH 9.5, binding of the zeatin tracers to the antibodies was not pH-dependent. The ammonium sulfate technique for separating antibody-bound from free antigen, which had given good results in previous work (Weiler and Zenk 1976, Weiler 1979), also proved to be useful for this assay. Maximum precipitation of antigen-antibody complexes occurred at 52% final saturation, and unspecific binding (UB, binding in the absence of antibody) was below 1% for the assay employing the tritiated tracer, However, UB values were much higher with zeatinribosyl-[^{125}I]tyramine (Table 2).

From Scatchard plots (Scatchard 1949) of standard curve data, maximum affinity constants of the antibodies of $2.4–2.7 \cdot 10^{-11}$ mol l^{-1} were calculated, demonstrating the presence of very high-affinity antibodies in the serum. At a final dilution of 1:13.500, the serum bound 30% of an added 0.67 pmol of tritiated tracer ($8.37 \cdot 10^{11}$ Bq $mmol^{-1}$) and at a final dilution of 1:31.500, 30% of an added 0.012 pmol of zeatinribosyl-[^{125}I]tyramine (approx. $1.9 \cdot 10^{13}$ Bq $mmol^{-1}$) was bound under standard assay conditions. Counting rates thus achieved allowed for the use of counting periods of 2–4 min per sample.

The tritiated zeatin derivative showed no detectable changes in chromatographic purity or immunoreactivity over a period of at least one year. Zeatingribosyl-[^{125}I]tyramine can be used for two to three months without significant changes in immunological properties.

Table 1. Cross-reactivities of zeatin antiserum (on molar basis, determined as described (Weiler 1979); n.d. = not determined)

Compound		Cross-reactivity (%)	
		^{3}H-assay	^{125}I-assay
I	trans-zeatinriboside	100	100
II	trans-zeatin	44	45
III	isopentenyladenosine	0.10	0.16
IV	cis-zeatinriboside	0.40	n.d.
V	dihydrozeatin	1.72	n.d.
VI	6-furfurylaminopurine	0.03	0.01
VII	6-benzylaminopurine	0.26	0.11
VII a	6-benzylaminopurine-7-glucoside	0	n.d.
VII b	6-benzylaminopurine-3-glucoside	0.24	n.d.
VII c	6-benzylaminopurine-9-glucoside	0.29	n.d.
VIII	6-n-hexylaminopurine	0.01	0.01
IX	adenine	0	0
X	adenosine	0	0
XI	4-(2-ethylhexylamino)-2-methyl-pyrrolo [2,3-d] pyrimidine	0	n.d.
XII	4-allylamino-2-methylpyrrolo [2,3-d] pyrimidine	0	n.d.
XIV	4-(1-hydroxymethylamino)-2-methyl-pyrrolo [2,3-d] pyrimidine	0	n.d.
XV	4-(2-hydroxymethylamino)-2-methyl-pyrrolo [2,3-d] pyrimidine	0	n.d.
XVI	guanosine	0	0
XVII	cytidine	0	0
XVIII	triacanthine	0.01	0.01

Assay Specificity. Antiserum specificity was determined by cross-reactivity studies. Cross reactivity was determined as described previously (Weiler 1979), and the results are presented in Table 1 (for formulas see Figure 1). For a number of compounds, the influence of the nature of the tracer used on assay specificity was also determined, and it can be clearly seen from Table 1 that cross reactivities are practically identical in both assays, i.e., they are a function of the antiserum and not of the tracer used.

Assay Sensitivity. Typical standard curves constructed with zeatinriboside are shown in Fig. 2 and some characteristic data of assay sensitivity are included in Table 2. Curves can be linearized over the whole measuring range by logit transformation of the B/B_0 values. The assays employing iodinated and tritiated tracer have practically the same measuring range, which extends from below 0.1 pmol up to 30 pmol per assay. However, due to the better precision of the tritium assay in the lower measuring range, the detection limit of this assay is lower (40 fmol, 14 pg) as compared to 140 fmol, 50 pg.

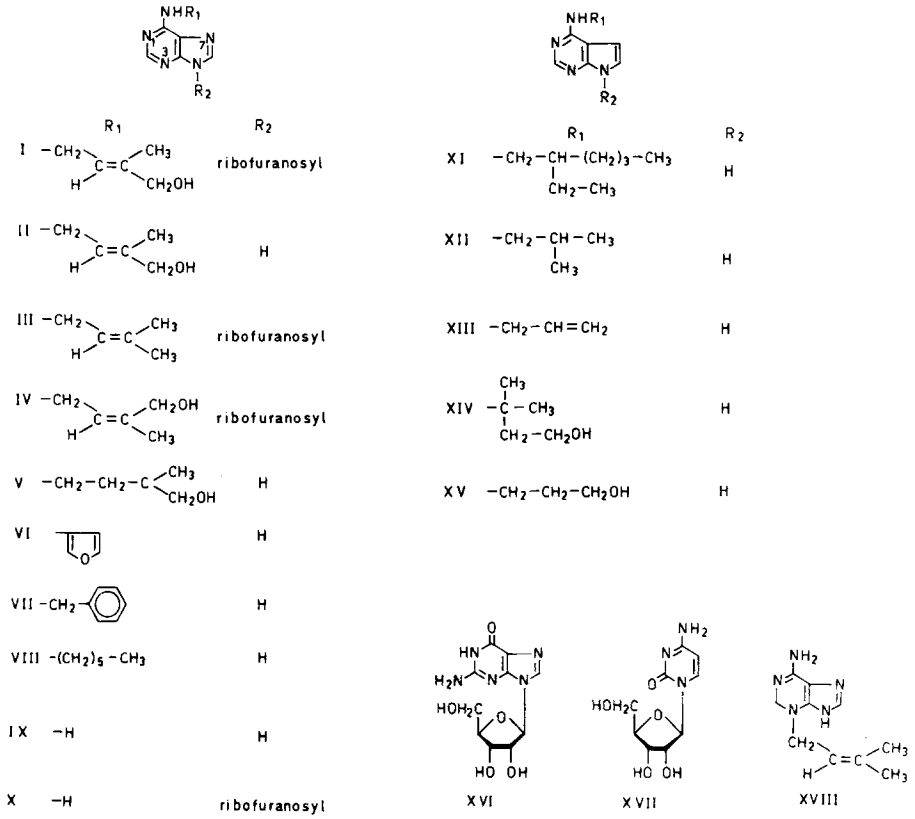

Fig. 1. Compounds assayed for cross-reactivity (cf. Table 2)

Table 2. Assay parameters of zeatin radioimmunoassays with zeatinriboside as standard

	Radiotracer	
	[^{3}H]Zeatinriboside-di-alcohol	Zeatinribosyl-[^{125}I]tyramine
maximum affinity constant of serum	$2.4 \cdot 10^{-11}$ M	$2.7 \cdot 10^{-11}$ M
serum titer (B=30%)	1:13.500	1:31.500
amount of tracer per assay	0.67 pmol	0.012 pmol
specific radioactivity of tracer	$8.37 \cdot 10^{11}$ Bq mmol^{-1}	ca. $1.9 \cdot 10^{13}$ Bq mmol^{-1}
unspecific binding	1%	12%
detection limit	40 fmol, 15 pg	140 fmol, 50 pg
linear range of logit/log plot	0.06–30 pmol	0.03–30 pmol
average recovery throughout measuring range	99.5%	96.3%
average coefficients of variation for triplicate samples throughout measuring range	4.2%	9.6%

The striking fact that, despite its higher specific radioactivity, zeatinribosyl-[^{125}I]tyramine results in the same assay sensitivity can be explained by higher affinity of the antibodies for this tracer, the structure of which closely resembles the structure of zeatinriboside linked to aminogroups in the protein. Thus, it is reasonable to assume that in competition with unchanged zeatinriboside, it is less readily displaced from the antibodies, resulting in a loss in assay sensitivity.

Standard curves for zeatin and zeatinriboside are parallel, but since cross reactivities are not identical and the latter compound is used routinely as the standard, values are given as zeatinriboside equivalents when crude extracts are analyzed. In fractionated extracts, zeatin and its derivatives may be quantitated individually by reading from one standard curve and correcting the value found for the compound's cross reactivity.

Assay Accuracy. Besides cross-reactivity studies, several other checks for assay validity were carried out. Dilution curves of plant extracts paralleled the standard curve over the whole measuring range. The addi-

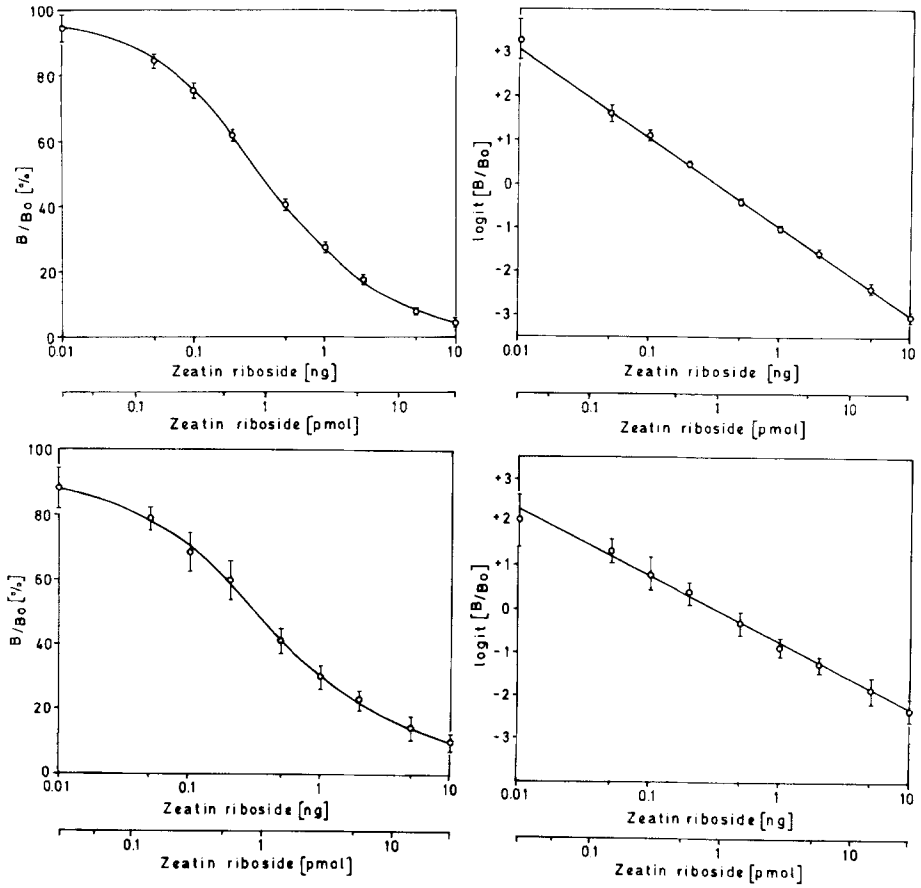

Fig. 2. Typical standard curves obtained with zeatinriboside as standard and tritiated zeatinriboside-dialcohol (spec. act. $8.37 \cdot 10^{11}$ Bq mmol^{-1}), above, or zeatinribosyl-[^{125}I]tyramine (spec. act. ca. $1.9 \cdot 10^{13}$ Bq mmol^{-1}), below. Bars indicate the standard deviation of triplicate samples. B = binding of tracer to antibody in the presence of zeatinriboside standards, B_0 = binding in the absence of zeatinriboside

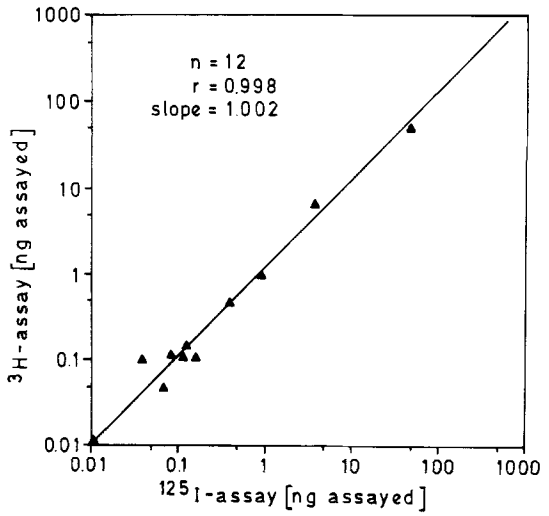

Fig. 3. Correlation of radioimmunoassays performed with tritiated zeatin-riboside-dialcohol and zeatinribosyl [^{125}I]tyramine as tracers

tion of increasing standard amounts of zeatinriboside to those extracts resulted in a linear relationship when the added-versus-found standard was plotted. Average recoveries of zeatinriboside were 96.3% and 99.5% for the assays employing iodinated and tritiated tracer, respectively.

Both assays give essentially identical results, as is shown in Figure 3, for a number of plant extracts. The correlation of both methods is 0.998 and the slope of the regression line is 1.002. Triplicate determinations have coefficients with a variation of 4.2% for the assay employing tritiated tracer and 9.6% for the assay using zeatinribosyl-[^{125}I]tyramine (average value throughout measuring range). The higher precision of the tritium assay can be traced back mainly to the much lower and very constant unspecific binding of the tracer (0.5–0.6%). In contrast, zeatinribosyl-[^{125}I]tyramine is, to an extent of 12% of the total activity, added unspecifically bound, and the variability of UB values is higher. For these reasons, the tritium-labeled zeatin tracer was used for further work described here.

Quantitation of Zeatin in Developing Tomato Fruits. To demonstrate the applicability of this radioimmunoassay, the levels of zeatin during development of tomato fruits (*Lycopersicon esculentum* cv. Moneymaker) were determined. This variety has been shown to contain mainly, if not exclusively, zeatin in the fruits, although at exceedingly low levels – in the parts per billion range – which did not allow for

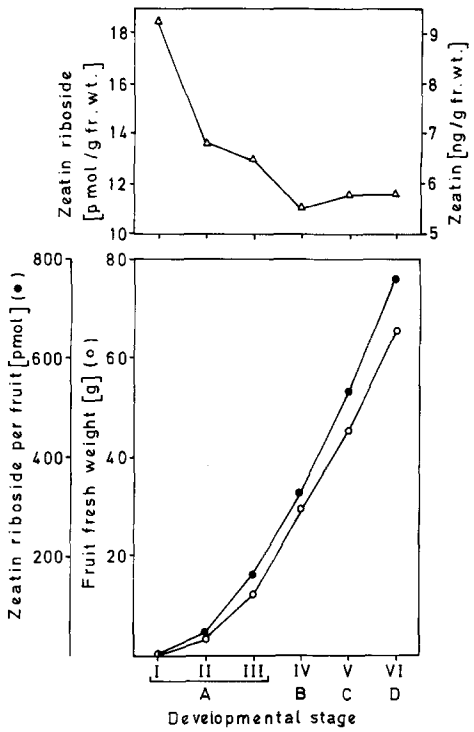

Fig. 4. Course of zeatin content in developing fruits of *Lycopersicon esculentum* cv. Moneymaker. Developmental stages are as follows: A I–III: fruits green, B(IV): fruits slightly pink with chlorophyll still present in large amounts, C(V): fruits orange, with traces of chlorophyll left, D(VI): fruits deeply red

precise quantitation (Monselise et al. 1978), even when hundreds of fruits were worked up.

For the experiment shown in Fig. 4, fruits from a single plant were harvested, extracted, and, after evaporation of the organic solvent, the aqueous extract was immunoassayed directly without further purification. Due to the high specificity and sensitivity of the RIA, precise quantitation of the zeatin levels present in these extracts were possible.

Zeatin levels were highest (9.3 ng g^{-1} fresh weight) in youngest fruits and lowest (5.3 ng g^{-1} fresh weight) in fruits which showed the first appearance of carotinoids (stage IV, pink), remaining at a constantly low level during further ripening of the fruits.

The course of zeatin levels found here is in full agreement with reports by others using different varieties with considerably higher absolute cytokinin levels (Davey and van Staden 1978), thus demonstrating further the validity of the immunodetermination of zeatin.

Discussion

There is unanimous agreement in the literature that the lack of a precise analytical method which is both rapid, sensitive, and at the same time very specific is to a large extent responsible for the sparse information available so far on the function of cytokinins in various physiological processes of higher plants.

It is demonstrated here that it is possible to determine fmol quantities of zeatin and related zeatin derivatives, the major cytokinins of higher plants, by highly specific radioimmunoassays. With a detection limit of 15 pg zeatinriboside, RIA is more sensitive than even the most sensitive bioassays, e.g., the tobacco stem pith callus assay (Murashige and Skoog 1962). This is due to the fact that antisera of high titers could be produced and immunoreactive zeatin derivatives with high-specific radioactivity could be synthesized. Both iodine- and tritium-labeled tracers give essentially comparable assay sensitivity and specificity and both methods correlate closely ($r = 0.998$).

With coefficients of variation of triplicate determinations of less than 5% (tritium assay) or less than 10% (iodine assay), RIA is also highly accurate. This is due in part to the fact that the assay can be applied to the analysis of crude extracts, thus, no purification losses occur and recoveries are higher than 96–99%.

Besides sensitivity, assay specificity is of utmost importance. It is well known that antibody specificity can be partly influenced by the choice of the site at which the hapten is coupled to the protein to yield the immunogenic conjugate. Usually, antibody specificity is directed mainly to the part of the hapten opposite the site of coupling (e.g., Erlanger et al. 1957; Bauminger et al. 1974; Rao et al. 1976). Therefore, immunization was carried out with conjugates in which zeatin was coupled through its ribose moiety using Erlanger and Beiser's (1964) procedure. This leaves the N^6-side chain, i.e., the structural feature which distinguishes trans-zeatin from the other cytokinins, exposed in the coupling product.

From the cross-reactivity data (Table 1), it can be deduced that the nature and correct stereochemistry of the side chain of zeatin is one of the crucial requirements for immunoreactivity of the compound. Thus, isopentenyladenosine (III), lacking only the hydroxyl group in the side chain, shows almost zero cross reactivity (0.1%), and reduction of the double bond in dihydrozeatin (V) or isomerization of the hydroxy group in cis-zeatinriboside (IV) also results in almost complete loss in immunoreactivity. In practical terms, this means that trace amounts of zeatin can be quantitated in the presence of excessive amounts of other cytokinins, e.g., a ten-fold excess of isopentenyladenosine will increase the immunoassayed value for zeatinriboside by only 1% which is still within the limit of experimental error.

The importance of the N^6-side chain for immunoreactivity is further stressed by the lack of reactivity

of synthetic cytokinins such as kinetin, and 6-benzylaminopurine and its glucosides, and a number of related 2-methylpyrrolo [2,3-d]pyrimidines also did not cross react at all. In addition, related compounds which might be present in high amounts in plant tissues, such as adenine, adenosine, and other nucleosides, show no cross reactivity in the assay as does triacanthine (XVIII), an adenine-derived alkaloid known to occur in a few plants at relatively high levels (Leonard and Deyrup 1962).

Taken together, the exclusive immunoreactivity of trans-zeatin in this assay becomes evident. Both free zeatin as well as zeatinriboside react strongly with the antiserum although not to the same extent. Besides these compounds, several other derivatives of zeatin are known to occur in plants, the most prominent being zeatinriboside-5′-monophosphate, O-β-D-glucopyranosylzeatin, and 9- and 7-glucosides of zeatin. These compounds have not been available for cross-reactivity studies but from the data given, it can be derived that it is highly improbable that O-β-D-glucopyranosylzeatin is immunoreactive; whereas zeatinriboside-5′-monophosphate must be highly immunoreactive. In addition, both the 7- and especially the 9-glucosides would also be expected to be reactive. Other 9-substituted derivatives of zeatin, such as lupinic acid, reported to be one of the major metabolites of zeatin in lupin seedlings (Parker et al. 1978), would also most likely be reactive in the assay.

Thus it can be concluded that the radioimmunoassay reported here allows for the specific quantitation of zeatin (total zeatin except for its O-glucoside) in crude plant extracts. Other cytokinins and nucleosides or related compounds do not interfere in the assay at all.

However, this assay can also be used efficiently to quantitate trans-zeatin and its derivatives separately. In this case, only a very preliminary purification of extracts would be necessary. This can be achieved by a single TLC step of the crude extracts to separate the zeatin-type cytokinins from each other. The corresponding fractions can be eluted and assayed directly, since any other contamination, even when very heavy, does not affect the assay. By correction for the individual cross reactivities, fully quantitative results can be obtained.

To demonstrate the applicability of the assay, the course of zeatin levels during development of *Lycopersicon esculentum* cv. Moneymaker fruits was followed. This variety is reported to contain solely zeatin in the fruits of various ripening stages (Monselise et al. 1978) but at levels too low to allow for precise quantitation. This has been possible with our method using crude aqueous fruit extracts, and a gradual decrease in zeatin levels during fruit development and ripening could be detected (Fig. 4). Zeatin levels were lowest in the pink stage of the fruit (stage IV) and remained low during the ripening process. This has also been found by Davey and van Staden (1978), who report exactly the same course of zeatin content in different tomato varieties, although at much higher levels.

The role of cytokinins during the development and ripening of tomatoes is not at all clear. Low levels of the cytokinin seem to be associated with the ripening process, and exogenously applied cytokinin may delay chlorophyll loss and carotenoid accumulation in the fruits of this species (see Review by McGlasson et al. 1978). However, a more pronounced function of cytokinins may be the stimulation of cell division during early phases of fruit growth. This is in accord with our finding that the highest levels of zeatin were found in the youngest fruits at a stage (I) in which fruit growth is mainly due to cell division, and that the most pronounced drop in zeatin levels occurs at a time when fruit growth becomes more and more due to cell enlargement (stage II).

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Note added in proof: The cross-reactivities of lupinic acid and 2-methylthio-trans-zeatinriboside in the ^{3}H-assay are 8.4% and 8.9%, respectively.