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The immunoassay of gibberellins

I. Radioimmunoassays for the gibberellins A_1, A_3, A_4, A_7, A_9 and A_{20}

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Abstract. Immunological assays for the detection of picogram amounts of gibberellins A_1, A_3, A_4, A_7, A_9 and A_{20} are described. Antisera of high affinity (K_a over 10^{10} l mol^{-1}) were raised in rabbits by immunization against carboxyl-coupled gibberellin-bovine-serum-albumin conjugates and used in combination with tritiated gibberellins or gibberellin derivatives of high specific radioactivity (over 10^{14} Bq mol^{-1}). The assays allow gibberellin quantitation in as little as 1 mg of vegetative plant tissue.

Key words: Gibberellin (radioimmunoassay) – *Hyoscyamus* – Radioimmunoassay (gibberellin).

Introduction

The quantitative determination of gibberellins (GAs) in plant tissues poses considerable problems. Whereas bioassays for this class of plant hormones are error-prone and imprecise, physicochemical techniques are relatively insensitive and require highly pure hormone fractions for analysis (for review see Reeve and Crozier 1980). Sensitive and selective immunological assays were recently introduced for the analysis of plant hormones (for review see Weiler 1982). However, the techniques available for gibberellins so far (Weiler and Wieczorek 1981) only allowed the detection of GA_3 and GA_7. It was necessary, therefore, to develop immunological assays for a broader range of GAs with special emphasis on the design of techniques for GAs available only in small amounts. It is demonstrated in the present paper that radioimmunological assays for a variety of polar as well as apolar GAs can be developed and that by choice of appropriate antisera, a range of gibberellins may be quantitated by a small number of assays. Additionally, sera specific for individual GAs can be raised as well.

Abbreviations: GA = gibberellin; GA_3 = gibberellic acid; TLC = thin-layer chromatography

Material and methods

Chemicals. Bovine serum albumin and gibberellic acid (GA_3) were purchased from Serva, Heidelberg, FRG. Pure GA_1, GA_4, GA_7, GA_9 and GA_{13} were a gift from ICI Corp., London, UK. Other gibberellins were kindly provided by Professor N. Takahashi, Tokyo, Japan and by Professor Jake MacMillan, Bristol, UK. Tritiated sodium borohydride ($1.8 \cdot 10^{15}$ Bq mol^{-1}) and [1,2-^{3}H]GA_1 ($1.4 \cdot 10^{15}$Bq mol^{-1}, radiochemical purity over 95%) were purchased from NEN Chemicals, Dreieich, FRG. All other chemicals and reagents were of the highest purity available.

Extraction of plant material. Three-month-old vegetative plants of *Hyoscyamus niger* L., grown in short days, were used. Apices (consisting of the apical dome plus the youngest developing leaves with a maximum length of 3 mm) were harvested. Ten apices (approx. 500 mg fresh weight) were ground in liquid N_2 and extracted in 3 ml 80% methanol (containing 10 mg l^{-1} butylated hydroxy toluene (2,6-di-tert-butyl-4-methylphenol) at 4° C for 24 h. The supernatant was collected after centrifugation, the solvents removed in vacuo and the residue redissolved in 0.5 ml methanol. Aliquots of the extract were methylated and assayed directly before or after thin-layer chromatography (TLC), or were chromatographed and then the individual fractions were methylated (Weiler and Wieczorek 1981) followed by immunoassay.

Preparation of GA-bovine-serum-albumin immunogens. Gibberellins A_1, A_3 and A_9 were coupled to free amino groups of bovine serum albumin via mixed anhydride intermediates as described (Weiler and Wieczorek 1981). As starting material, between 7 and 25 mg of the respective GAs were used. Coupling ratios in all cases were 3.6–4 mol of GA per mol of protein, as determined by spectroscopic analysis (in concentrated H_2SO_4; GA_1: 385 nm, $\varepsilon = 2.0 \cdot 10^6$ cm^2 mol^{-1}; GA_3: 391 nm, $\varepsilon = 2.4 \cdot 10^6$ cm^2 mol^{-1}; GA_9: 381 nm, $\varepsilon = 2.6 \cdot 10^6$ cm^2 mol^{-1}; molar coupling ratio $= (\varepsilon_{conjugate} - \varepsilon_{BSA}) : \varepsilon_{GA}$).

Synthesis of reduced GA-17-norketones. The 17-norketones of GA_3 and GA_9 were synthesized by a modification of the method of Bearder et al. (1975). Gibberellin (20 mg) dissolved in 20 ml tetrahydrofuran was added to an aqueous solution of OsO_4 (5 mg) and $NaIO_4$ (100 mg). The mixture was stirred at room temperature for 16 h. After removal of the solvent in vacuo, phosphate buffer (10 ml, 0.1 M, pH 3) was added to the aqueous residue and the mixture was extracted three times with an equal volume of ethyl acetate. The norketones were purified further by TLC (GA_3-norketone: solvent system ethyl acetate:acetic acid = 100:5 by vol. R_f 0.23, yield 10%; GA_9-norketone: solvent system ethyl acetate:chloroform: acetic acid = 60:40:5 by vol., R_f 0.58, yield 95%). The norketones (2.5 µmol) were dissolved in 50% ethanol (0.1 ml) and reacted with tritiated sodium borohydride (approx. 1.6 µmol) for 2 d at −18° C. The reaction mixtures were then purified by TLC in the above solvent systems (R_f of the reduced GA_3-norketone, 0.12; R_f of the GA_9-derivative, 0.25). The compounds were obtained radiochemically pure and stored as dilute methanolic solutions at −18° C under N_2. The specific activities of the tracers were determined immunologically by the self-displacement method (Chervu and Murty 1975); reduced GA_3-norketone: $2.2 \cdot 10^{14}$ Bq mol^{-1}, reduced GA_9-norketone: $1.8 \cdot 10^{14}$ Bq mol^{-1}.

Immunization and antiserum production. Rabbits (12–16 weeks old) were immunized with buffered solutions of the conjugates (2 mg ml^{-1}) emulsified in an equal volume of Freund's complete adjuvant as previously described (Weiler and Zenk 1976).

Radioimmunoassay. Radioimmunoassays were performed exactly as described (Weiler and Wieczorek 1981; Weiler 1980). Data processing and calculation of the results were performed on a BASIS 108 microcomputer using a second- to fifth-order polynomial regression algorithm for standard curve approximation (Isomess Corp., Straubenhardt, FRG, program written by R. Grugel, Nuclear Interface, Münster, FRG).

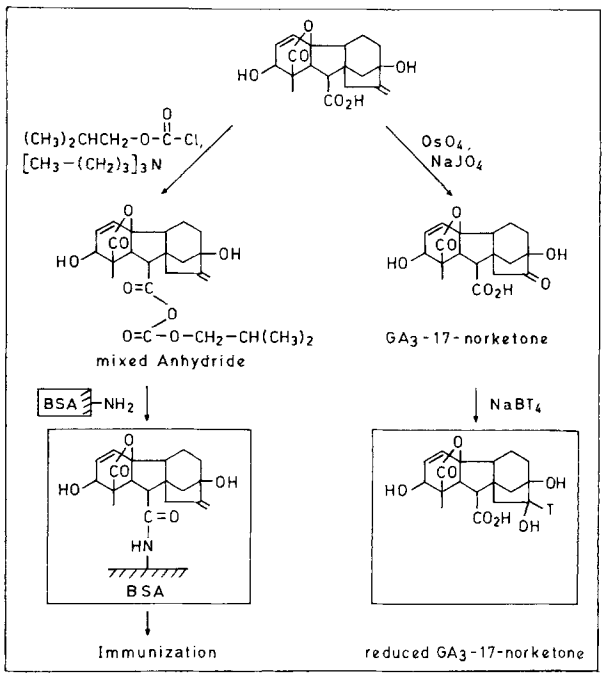

Fig. 1. Synthesis of GA_3-immunogen and of tritiated reduced GA_3-17-norketone (reactions similar for other GAs). *BSA*, bovine serum albumin

Results

Basic assay parameters. Each of the three conjugates was administered to four to six rabbits and the resulting serum fractions subjected to a preliminary check of antibody titer and selectivity. Appro-

Table 1. General assay parameters of GA-radioimmunoassays. K_a = Average affinity constant, derived from Scatchard plots. CV = average variation coefficient for sample triplicates throughout measuring range. Titer = final dilution of antiserum binding 30% of the tracer added

	Serum raised against					
	GA_1				GA_3	GA_9
	Analysis for					
	GA_1	GA_4	GA_7	GA_{20}	GA_3	GA_9
K_a (10^{10} l mol^{-1})	2.3	3.1	2.6	3.3	1.7	1.9
Titer	900	900	900	900	900	450
Specific activity of tracer (10^{14} Bq mol^{-1})	18	18	18	18	2.2	1.8
Amount of tracer per assay (pmol)	0.55	0.55	0.55	0.55	1.54	1.31
Measuring range (pmol)	0.07–5	0.15–5	0.1–5	0.25–10	0.1–5	0.1–5
Detection limit (fmol)	70	150	100	250	100	100
(pg)	24	45	33	83	35	32
Unspecific binding (%)	0.7	0.7	0.7	0.7	0.5	0.5
CV (%)	4.4	8.3	6.9	n.d.[a]	4.6	4.5

[a] Not determined

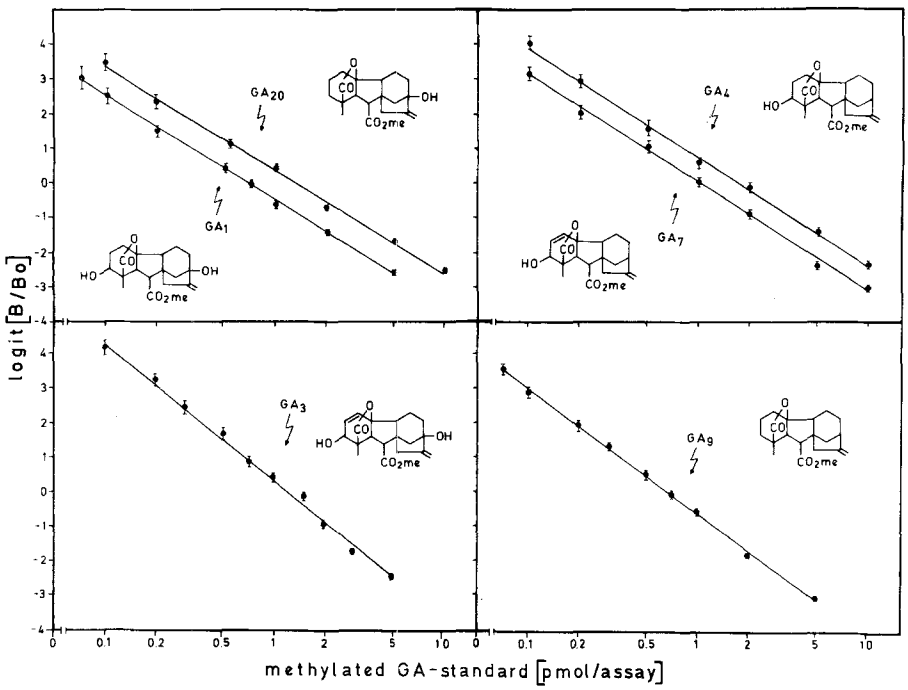

Fig. 2. Logit-log plot of GA standard curves. The GAs are analyzed as methyl esters. The bars represent ± SD of triplicate standards. Logit $[B/B_0]=\ln [B/B_0/(100-B/B_0)]$ with B = tracer binding in presence of unlabeled GA; B_0 = tracer binding in absence of unlabeled GA

priate fractions were pooled and characterized in detail. The structures of the immunogens and tracers used in this study are presented in Fig. 1. The antiserum raised against GA_1 was used in combination with [1,2-^{3}H]GA_1, the sera raised against GA_3 and GA_9 were used together with the respective tritiated reduced 17-norketone. Serum selection was carried out in order to provide an antiserum reacting with a range of physiologically active GAs as well as to demonstrate that selective assays for specific GAs can be raised. As in our earlier work (Weiler and Wieczorek 1981), assay sensitivity was optimum with GA methyl esters as standards and methylated samples.

The general parameters valid for the optimized assays are summarized in Table 1. As shown, the antisera raised were of high affinity and titer. It was possible to select an antiserum which allowed for the analysis of GA_1, GA_3, GA_4 and GA_7 as well as GA_{20} with high sensitivity. Separate quantitation of these GAs with the same antiserum was thus possible in conjunction with suitable separation methods for these GAs. Furthermore, all assays yielded highly reproducible results as shown by the low variation coefficients of sample replicates. The standard curves for all assays could be expressed as straight lines, thus facilitating manual as well as computerized data processing (Fig. 2).

Assay selectivities. A range of GAs and related compounds were tested for their ability to compete

Table 2. Specificity of GA-antisera (cross-reactions in % on molar basis, determined as described in Weiler and Zenk 1976). For structures, see Fig. 3. All compounds were treated with diazomethane prior to analysis. n.d. = not determined

		Antiserum raised against		
		GA_1	GA_3	GA_9
I	GA_1	100	11	<0.1
II	GA_3	70	100	<0.1
III	GA_4	40	9	3
IV	GA_5	29	0.2	<0.1
V	GA_7	70	35	1.1
VI	GA_8	11	<0.1	<0.1
VII	GA_9	15	<0.1	100
VIII	GA_{12}	<0.1	<0.1	<0.1
IX	GA_{13}	<0.1	<0.1	<0.1
X	GA_{16}	0.1	<0.1	<0.1
XI	GA_{19}	<0.1	<0.1	<0.1
XII	GA_{20}	55	22	<0.1
XIII	GA_{24}	<0.1	<0.1	<0.1
XIV	GA_{32}	<0.1	<0.1	<0.1
XV	GA_{34}	<0.1	<0.1	<0.1
XVI	GA_{42}	<0.1	<0.1	<0.1
XVII	Iso-GA_3	10	<0.1	<0.1
XVIII	Iso-GA_7	3	<0.1	<0.1
XIX	allo-Gibberic acid	<0.1	0.1	n.d.
XX	epiallo-Gibberic acid	<0.1	<0.1	<0.1
XXI	Gibberic acid	<0.1	<0.1	n.d.
XXII	epi-Gibberic acid	<0.1	<0.1	<0.1
XXIII	Steviol	0	0	0
XXIV	Helminthosporic acid	0	0	0
XXV	Gibberethione	0	0	0
XXVI	GA_8-glucoside	0.1	<0.1	n.d.

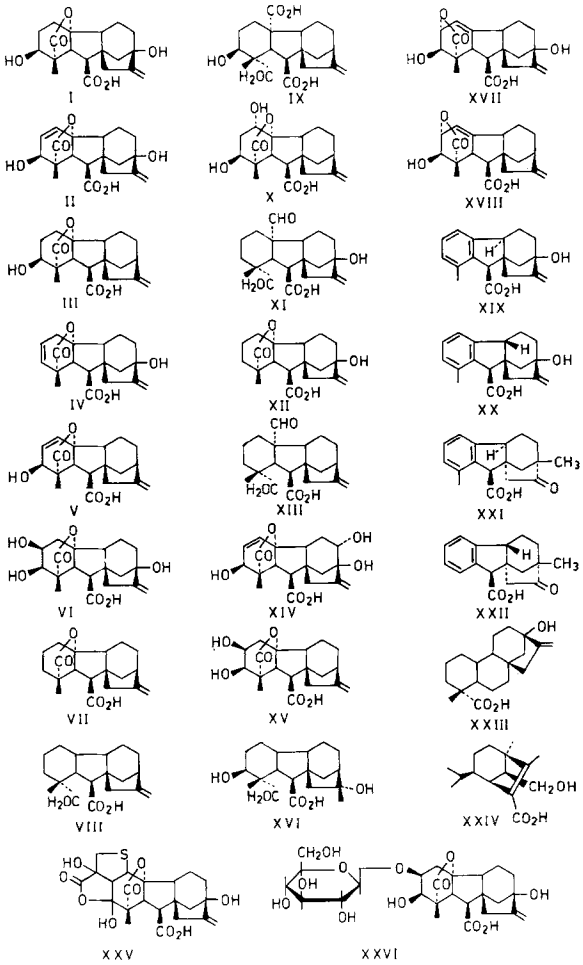

Fig. 3. Structural formula of GAs and related compounds used in this study (compare Table 2)

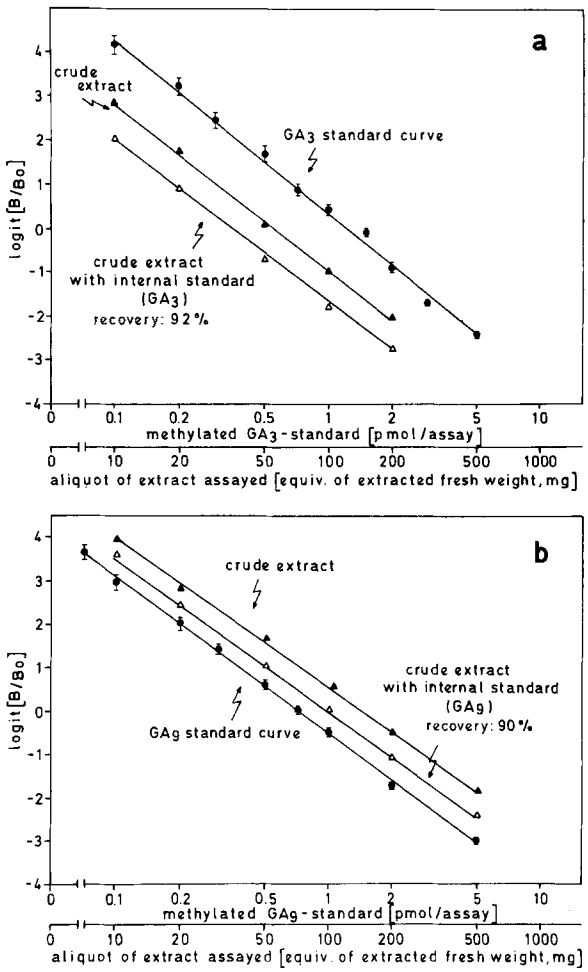

Fig. 4a, b. Extract dilution analysis. Aliquots of crude methanolic extracts of *Hyoscyamus niger* L. apices were assayed. Immunoreactive material was detected both in the assay for GA_3 (**a**) and in the assay for GA_9 (**b**). Extract dilution curves strictly parallel the standard curves. From the analysis, the level of GA_9 in this tissue was determined as 9.1 pmol g^{-1} FW, that of GA_3 as 25.4 pmol g^{-1} FW (see also Figs. 5, 6)

with the respective tracer molecules for antibody binding sites (cross reaction). The results are given in Table 2 (for structures, see Fig. 3). All sera selected for further application were highly specific in that they did not exhibit any cross reactivities against non-GAs and in that they were reactive with only a narrow range of GAs if not with a single one. This selectivity range provided the basis for the application of the assays in extracts of low purity.

Assay performance. Because the immunoassays were more sensitive by several orders of magnitude than other methods of GA quantitation, samples analyzed by radioimmunoassay could not be reassayed by these other methods. Therefore, a range of internal performance controls had to be carried out to gain information on assay accuracy and reliability. Recovery of gibberellins added to crude extracts prior to methylation and analysis was over 90% (Fig. 4). After extraction of the GAs into ethyl acetate followed by TLC, recovery, as checked by an internal standard of [^{3}H]GA_1 and [^{3}H]GA_4 was consistently higher than 80%. All results were corrected for recovery.

Dilution series of crude methanolic extracts were run for each plant material to be analyzed. The parallelism of such extract dilution curves with the corresponding standard curves (see Fig. 4) was taken as evidence for the absence of factors in the samples interfering unspecifically in the antigen-antibody reaction. Extracts were then separated by TLC and the distribution of immunoreactive material was recorded (Figs. 5, 6). This demonstrated the absence of interfering material in the crude fractions, or, for the combined assay of GA_1, GA_3, GA_4 and GA_7, the relative proportions of these GAs present.

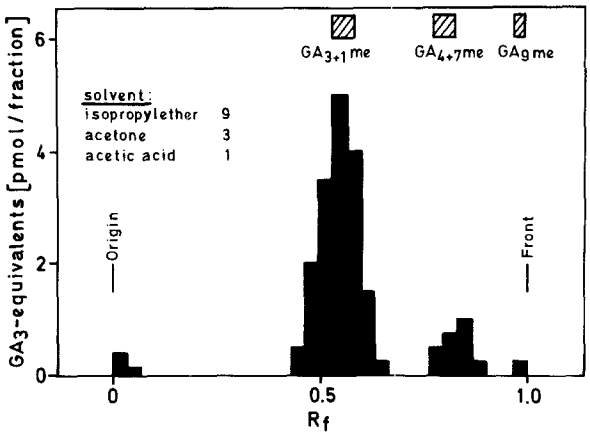

Fig. 5. Distribution of immunoreactive material on a thin-layer chromatogram (silgel) of a crude methanolic extract of *Hyoscyamus niger* L. apices (see Fig. 4). A methylated extract was applied to the plate. The antiserum detects, besides the major immunoreactive band at the R_f of GA_3, a minor fraction co-chromatographing with GA_4 and GA_7. From the chromatographic data, the total level of immunoreactive material was determined as 24.2 pmol g^{-1} FW. The recovery of an internal standard of GA_3 added to a duplicate aliquot of the extract which was processed in parallel was 82% (compare Fig. 4)

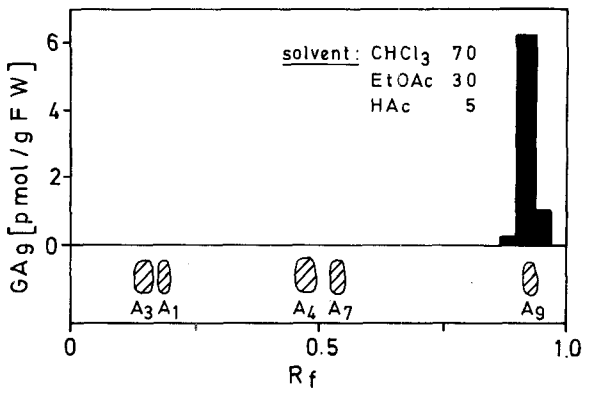

Fig. 6. Extract as in Fig. 5, but chromatographed unmethylated. Individual fractions were eluted, treated with diazomethane and then assayed. The antiserum against GA_9 detects immunoreactive material only at the R_f of GA_9. The level of GA_9 in *Hyoscyamus niger* apices, as calculated from the chromatograms, is 8.9 pmol g^{-1} FW (recovery: 85%, compare Fig. 4)

A further check for accuracy was the comparison of results obtained in TLC-purified fractions with that obtained for crude extracts. Both results had to be identical within the limits of experimental error.

Furthermore, correlation of results obtained using two immunoassays differing in the tracer molecule and in the cross-reactivity distribution of the antisera for the same sets of samples was taken as further evidence for the absence of cross-reacting material of unknown structure in the respective fractions (e.g. Fig. 4; Atzorn and Weiler 1983).

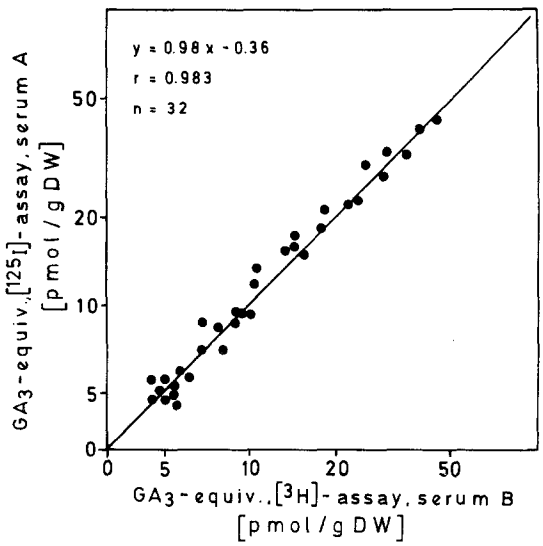

Fig. 7. Correlation of GA_3-radioimmunoassays (^3H-method, this paper; ^{125}I-method, Weiler and Wieczorek 1981). The samples represent a variety of extracts from shoot tissue of different legumes

Discussion

This paper describes techniques for the quantitative determination of several gibberellins. The C_{19}-GAs used here were selected because they are physiologically highly active and/or exhibit very different substitution patterns. Thus it was possible to compare the immunological behaviour of apolar GAs (GA_9) with that of more polar ones (e.g. GA_3). It is demonstrated that highly specific antisera can be raised for all the GAs selected. In combination with tritiated GAs or reduced GA-17-norketones of high specific radioactivity (more than 10^{14} Bq mol^{-1}), the assays allow the detection of as little as 20–40 pg of compound, i.e. are sufficiently sensitive to cope with as little as 1–2 mg of tissue for a single analysis. They are only surpassed in sensitivity by the enzyme immunoassay (Atzorn and Weiler 1983). The assays reported here overcome the restrictions still inherent in the first published method for GA_3 (Weiler and Wieczorek 1981) in that they allow assay development with only a few milligrams of GA and employ highly stable tracer molecules instead of the ^{125}I-tracers of much shorter half-life.

From Table 2 (and data not given herein), a few generalizations can be established which may be useful in the set-up of assays for other GAs. The choice of the carboxyl group for attachment of the GA to the carrier protein results in immunogens eliciting antibodies able to bind to a large part of the GA molecule. The binding area extends

from ring A to ring D and substitutions in either part of the molecule usually reduce cross-reactions considerably. In the vicinity of hydrophobic areas, the addition of polar groups, or alterations of polar substituents result in a sharp loss of the compound's cross-reactivity. Thus, the antiserum against the highly lipophilic GA_9 is extremely specific. On the other hand, changes in hydrophobic parts of a GA carrying polar substituents are usually not discriminated sharply by the antibodies. Thus, the anitserum raised against GA_1 also recognized GA_3.

The techniques reported here should allow similar assays to be carried out for most other GAs as well. As became evident from our work, a range of structurally similar GAs may be quantitated with a single assay (compare Fig. 2, Table 2). Thus it is hoped that with a few group-selective assays in combination with simple separation techniques, a large number of GAs will become accessible to immuno-analysis in the near future.

So far only the carbonyl function of the GAs has been used for immunogen synthesis (Fig. 1). However, other positions of the molecule can be used as well, including hydroxyl groups and the exocyclic methylene carbon, thereby increasing the range of available antisera with desired characteristics.

The techniques described here apply to the immunoassay of GAs with as little as milligram amounts of tissue to yield quantitative results. This analytical technique will be of much help in elucidating functions of gibberellins in various physiological processes.

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