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Barley Endosperm Bioassay for Gibberellins. II. Application of the Method

B. G. Coombe, D. Cohen[1] and L. G. Paleg

Department of Plant Physiology, Waite Agricultural Research Institute, The University of Adelaide, South Australia

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Summary. The use of the modified endosperm bioassay in conjunction with techniques for extracting and paper chromatographing extracts, and the application of statistical analyses to the results, is reported. The modified procedure has not altered the relative response to different gibberellins, though an analysis of many standard curves indicates that at least 1 feature of the bioassay system is still uncontrolled. A comparison of this bioassay with 15 others indicates that the endosperm response may have wide applicability in explorations of the physiological effects of gibberellins.

In the previous paper of this series (5) many conditions affecting the gibberellin-induced reducing sugar release of barley endosperm were examined, and a basic bioassay procedure was outlined. Since, however, the usefulness of a bioassay is determined in large part by its adaptability, the applicability of the procedure when used in conjunction with various methodological and statistical techniques has been investigated.

Methods

All methods, except as stated, were those indicated previously (5). All water was distilled, autoclaved and dispensed with automatic syringe pipettes.

Adaptions and Results

Paper Chromatography. The results obtained by direct bioassay of paper chromatograms containing either 10^{-8} g GA_3 or an extract of grape pericarp are illustrated in figure 1.

Ovaries of the cultivar Doradillo, taken at anthesis, were lyophilized, separated from pollen and anthers, and the seeds dissected out to leave only pericarp tissue. Of this tissue, 36 mg, representing 52 berries, was extracted with 10 ml ethyl acetate for 8 hours, filtered, and re-extracted with 5 ml ethyl acetate for 16 hours. The combined extracts were divided into 6 equal parts, evaporated under reduced pressure, and spotted on 25 mm wide strips of Whatman No. 1 paper (spot = 7 × 14 mm).

The chromatograms were equilibrated over the solvent (80 % isopropyl alcohol) for 12 hours and developed (ascending) for 19 cm. The developed and dried chromatograms were cut into 15 12.6 mm pieces which were placed directly into vials with 1 ml water and 2 endosperm. Incubation and sugar analysis were carried out in the normal way. The results (fig 1) indicate that the bioassay is capable of detecting gibberellins and gibberellin-like substances separated in this way, without any requirement for elution of the active substances from the paper.

The GA_3 spot on the chromatogram was localised at R_F 0.4 to 0.6, and the other areas of the chromatogram produced a relatively low and uniform response. However, because each value was only a single observation, it was not possible to demonstrate that the amounts of sugar released in the vials assaying R_F segments 0.4 to 0.6 of the GA_3 chromatogram were significantly different from those in vials assaying the other chromatogram areas. On a qualitative basis, the results are clearly indicative and meaningful, and preliminary techniques for overcoming the difficulties of adapting a statistical approach to chromatographic results are explored below.

Statistical Procedures. The ability to handle large numbers of treatments with this bioassay makes it possible to meet the statistical requirements for good bioassay procedure. Two aspects of statistical procedure will be discussed here:

A) *Quantitative Determination of Promotive Activity in an Extract.* Procedures for this are well established and are described by Bliss (2). To determine relative potency (i.e. activity) of

[1] Present address: Biology Department, Carleton University, Ottawa, Canada.

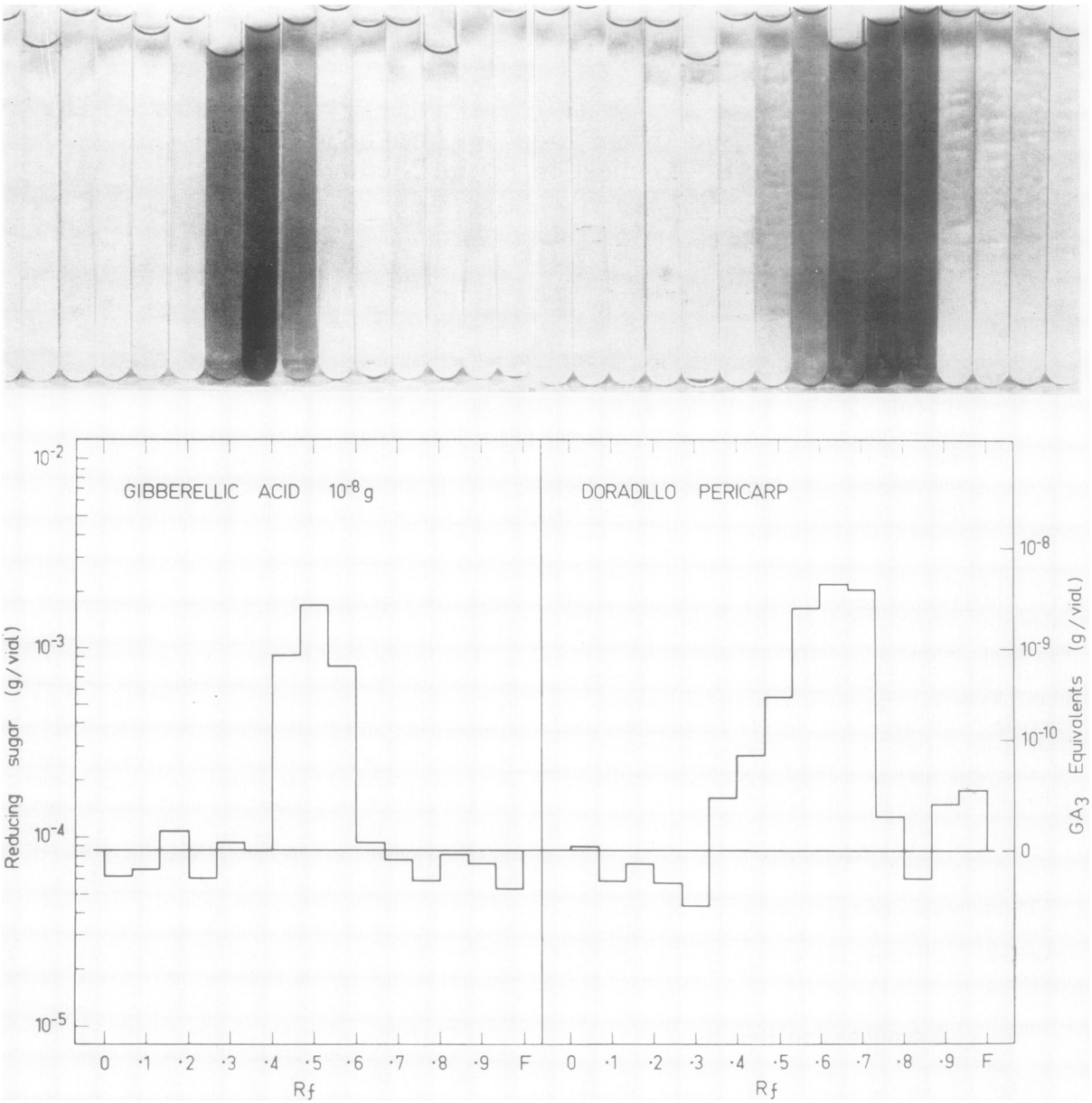

FIG. 1. Assay tubes and histograms of sugar release values obtained from segments of paper chromatograms of 10^{-8} GA_3 and grape pericarp extract. (No significant amount of reducing sugar was found on control chromatogram of extract tested in the bioassay without endosperm.)

an unknown in comparison with a standard, replicates of each should be tested at 2 or more dosage levels within the linear part of the dosage-response curve. From this, slopes of the responses to known amounts of standards and unknowns, mean responses, and experimental errors, can be calculated. If 3 or more dosages are compared, the significance of curvature of the dilution curves, if any, can be tested. Usually preliminary tests are required to ensure that the dosage levels are within the linear part of the dosage-response curve. No estimate of extract activity can validly be made if the response falls outside the fitted range of the regression line calculated for the standard curve. The Biometry Section of the Waite Institute has prepared a computer programme which incorporates equations 23a and 32 of Bliss and provides for the following: mean dose, mean response, slope, standard error of slope and residual mean square of both the standard and unknown, the log ratio of potency and its standard error and confidence limits, and the combined slope and its variance ratio.

Frequently, in plant hormone bioassays, the

activity of an extract is tested at only 1 concentration level in comparison with dilutions of a standard. The comparison may even be made mathematically, by regression analysis (e.g. equations 23 and 32 of Bliss) or analysis of variance, or by interpolation on a graph drawn with a straight line between points or with a smoothed (or fitted) curve. This practice has serious shortcomings in spite of the use of statistics since, with only a single concentration of the unknown, there is no way to determine whether the activity observed is in the ascending or descending part of the response curve, or, in fact, is due to a compound in any way similar to the standard. The problem is still more acute when a bioassay of unknown or low specificity is used. Even if several levels of the unknown are compared it is not meaningful to measure potency if the slope differs significantly from that of the standard. (As an expedient under these circumstances, some arbitrary measure, such as the dose giving mid-response, can be used for

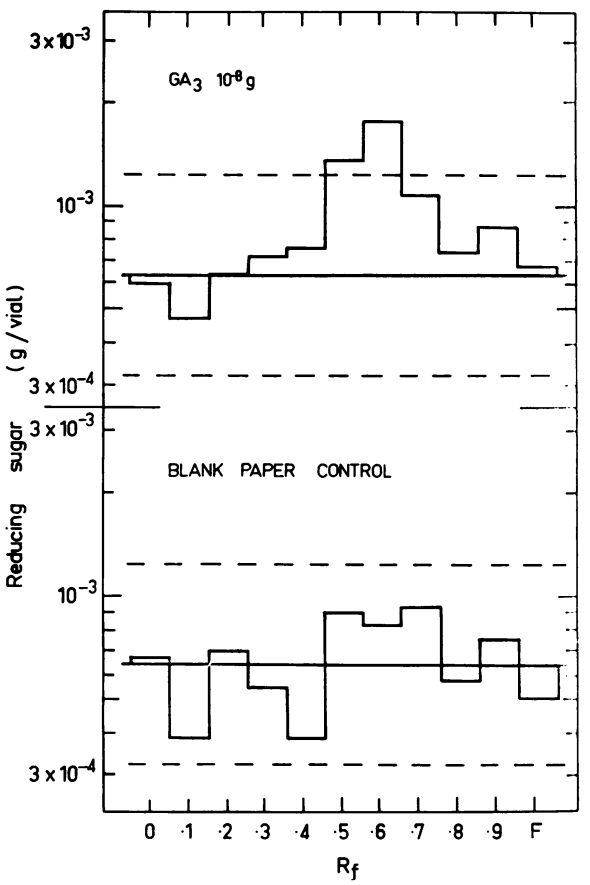

FIG. 2. Histograms of sugar release values obtained from segments of paper chromatograms of control and 10^{-8}g GA_3 run (ascending) after 1 hr equilibration over solvent (isopropanol:water – 4:1). Solid horizontal line is mean endosperm response to segments of control chromatogram. Dotted horizontal lines are 5 % confidence limits calculated from the control chromatogram.

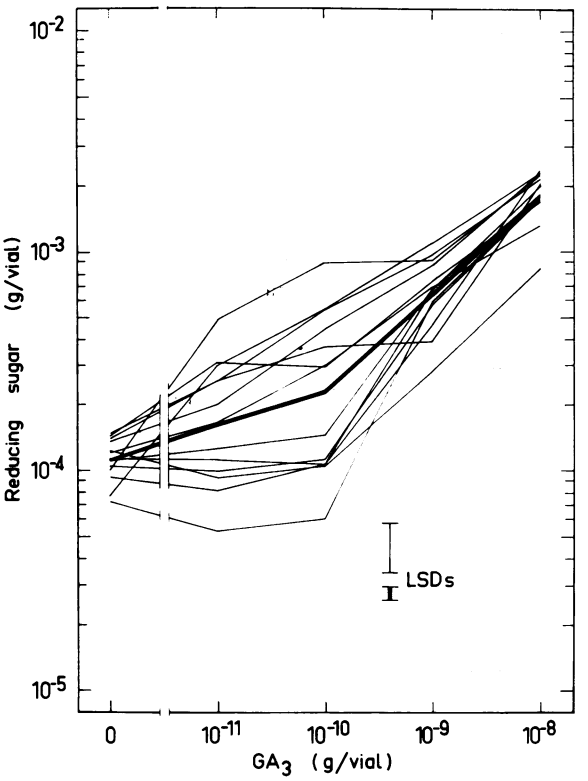

FIG. 3. Reducing sugar release induced by standard solutions of GA_3 in 13 consecutive tests. Mean values represented by heavy line.

analysis.) In this bioassay the slopes obtained with known gibberellins appear to be comparable (15; fig 4), but non-parallelism can result if inhibitors and other interfering substances are present. Purification procedures should be used to overcome this problem.

B) *Statistics of Chromatograms.* Chromatography is a convenient method for separating substances, but it is difficult to assess the results quantitatively and statistically. Usually some subjective assessment has to be made to supplement statistical methods. For instance, the histogram in figure 1 of a paper chromatogram of gibberellic acid shows a clear and obvious peak, yet it was not possible to establish statistically significant activity with only this 1 chromatogram.

If replicate chromatograms are run an analysis of a variance can be made with R_F as a variable.

Another approach is to bioassay 1 or more blank chromatograms spotted with the extraction solvent and developed at the same time as the extract chromatograms. The mean and confidence limits of the blank can be calculated and used to estimate which parts of the extract chromatogram have significant activity. The results in figure 2 illustrate this approach; the values for the blank chromatogram, though variable, have given a mean and confidence limits which, when superimposed

on the histogram of the GA_3 chromatogram, show that the latter has a significant peak of activity at R_F 0.6. Confidence in the determination of a peak is greater if, as in this figure, 2 or more adjoining pieces of chromatogram have significant activity.

An estimation of the quantity present in a peak can be made by comparing the peak response with that of standards assayed at the same time (the difficulties in doing this have been stressed above). To make quantitative determinations, replicate chromatograms at 2 or more dosages are desirable, and can be obtained by separately spotting 2 dilutions of the extract or by developing 1 chromatogram, eluting pieces, and measuring 2 dilutions of the eluate. It is considerably easier to interpret the unknowns if the standard compound is assayed similarly.

Standard Curve. In spite of all the precautions adopted to ensure uniformity, when the standard curves reported in many of the experiments in this series of papers are compared, a degree of heterogeneity is apparent. Thirteen such standard curves, obtained in thirteen consecutive tests with the same procedure are shown in figure 3. The most variable feature of the curves, and, indeed, of the bioassay, is the sensitivity of the endosperm to the 2 lowest concentrations of GA_3. When the curves were analyzed statistically, evidence for 2 populations was obtained. One group of curves showed higher average control values and greater sensitivity to GA_3 than the second group. Though the conditions (including atmospheric) were thoroughly examined, it has not been possible, as yet, to relate the 2 types of responses to any particular treatment either prior to or during the bioassay.

Activity of Other Gibberellins. In a previous paper (15) the activity of 8 numbered gibberellins and allogibberic acid, in the barley endosperm test, was compared. It was of interest to determine whether the modifications adopted in the development of the bioassay influenced the responses induced by at least some of these gibberellins and figure 4 illustrates the results obtained with $GA_{1, 3, 5}$ and $_6$. Sugar release is qualitatively almost identical with that reported earlier (15) with the possibility that the endosperm were slightly more sensitive to this sample of GA_6. It can be concluded that the bioassay procedure has not altered, to any appreciable degree, the relative response of the endosperm to the different gibberellins previously tested.

Response to Ethyl Acetate. When using ethyl acetate to extract gibberellin-like substances from plant tissue it was found that controls, without

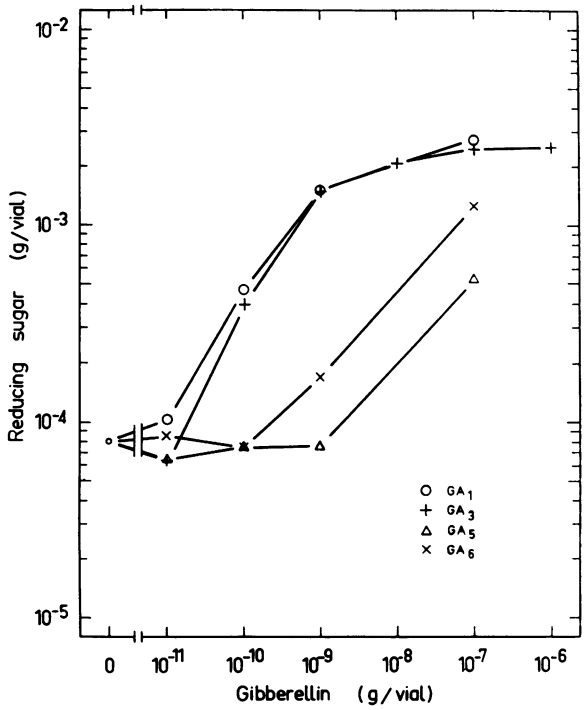

FIG. 4. Reducing sugar release induced by GA_1, GA_3, GA_5 and GA_6.

Table I. *Reducing Sugar Released by Barley Endosperm in the Presence of Residues from Ethyl Acetate*

Treatment	log μg reducing sugar/vial
Ethyl acetate (ml evaporated)*	
0	2.396
15	2.717
30	2.700
60	3.175
L.S.D. at $p < 0.05$	0.212
Water	2.200
Untreated ethyl acetate**	2.241
Untreated ethyl acetate shaken with sodium sulfate	2.812
Redistilled ethyl acetate	2.244
Redistilled ethyl acetate shaken with sodium sulfate	2.259
L.S.D. at $p < 0.05$	0.339

* 2 Samples assayed in triplicate.

** Duplicate 60 ml samples of ethyl acetate were evaporated, dissolved in 4 ml autoclaved distilled water, and triplicate 1 ml aliquots bioassayed.

Table II. *Comparison of the Conditions and Performances of Various Gibberellin Bioassays*

Bioassay	Ref	Conditions needed	Days to complete prep + test	Volume (ml) of solution per test unit	Replication of units used	Useful range of sensitvity to GA_3		Need for pre-elution of chromatograms
						Solution concn μg/ml	Wt. of gibb, μg/test unit	
Intact plants								
Dwarf maize leaf sheath	(17)	30°, White light	7+7	0.1	1–10	0.01–100	0.001–10	Yes
Dwarf rice leaf sheath	(13)	30°, White light	2+7	1.5–3.0	...	0.001–1	0.002–2	No
Dwarf pea internodes	(10)	27°, Red light	5+4	0.005	4	0.1–100	0.005–0.5	Yes
Dwarf pea stem diffusion	(4)	25°, Dark	6+1	0.01	5–15	0.01–1	0.0001–0.01	Yes
Dwarf *Pharbitis* epicotyl	(9)	30°, White light	4+10	0.0025	20–30	0.05–1000	0.0001–2.5	Yes
Cucumber hypocotyl	(8)	24°, White light	0+5	5	3	10–1000	50–5000	No
Lettuce hypocotyl	(7)	28°, White light	2+3	5	...	0.01–100	0.05–500	No
Sections of plants								
Barley endosperm	(5)	30°, Dark	1+1	1	3	0.00001–0.01	0.00001–0.01	No
Avena fatua embryos	(11)	20°, Dark	0+1	0.25	3	0.0001–0.1	0.00003–0.025	No
Oat leaf base	(21)	25°, Dark	4+1	10	...	0.0001–10	0.001–100	No
Oat mesocotyl	(12)	25°, Dark	3+1	1	1	0.0001–0.1	0.0001–0.1	No
Wheat leaf	(18)	20°, Dark	5+1	5	1	0.001–0.1	0.005–0.5	No
Dwarf bean leaf disc	(19)	25°, Dark	6+1	10	...	0.001–1	0.01–10	No
Dwarf pea epicotyl	(16)	25°, Dark then 20°, White light	4+3	4	1	0.00001–1	0.00004–4	No (thin layer) Yes(paper)
Apple leaf disc	(6)	25°, White light	?+4	0.2–4.0	4	0.0001–1	0.0001–1	No
Rumex leaf disc	(20)	25°, Dark	1+4	0.3	4	0.00001–1.0	0.000003–0.3	No

tissue, sometimes gave a significant response which could be ascribed to neither the presence of reducing power nor to incomplete evaporation of ethyl acetate. This aspect was of obvious interest and was investigated further.

Different volumes of ethyl acetate, which had been stored over sodium sulfate, were evaporated. The residues were dissolved in 4 ml autoclaved, distilled water, and 1 ml aliquots were tested in triplicate in the bioassay. The results (table I) showed that the residue from 60 ml had considerable activity (significant at $p < 0.001$); residues from 30 and 15 ml had smaller, though also significant activity.

The results in table I also demonstrated that the response was associated with storage over sodium sulfate. Following such storage the active agent can be removed by redistillation but in the absence of sodium sulfate, redistillation (of fresh ethyl acetate) is not required.

A similar effect has been reported (1) under conditions in which ethyl acetate was shaken with sodium bicarbonate solution. It was suggested that, like GA_3, such residues induce α-amylase formation in the aleurone layer. Sodium acetate was inferred as the active principle, but in tests where sodium acetate has been included as part of a buffer no such activity has been found (14).

These experiments confirm the report by Briggs (3) that residues from ethyl acetate and other organic solvents cause a reducing sugar release from barley endosperm. The chemical basis for the effect remains obscure but clearly it would be of value to identify the active principle.

Comparison with Other Gibberellin Bioassays. The conditions required for, and the relative performances of, many of the gibberellin bioassays are shown in table II. The column headed "Useful range of sensitivity to GA_3" is somewhat subjective in that the lowest value is that amount which gives a response judged to be greater than control and the highest value is that amount above which no useful increase in response occurs. The range is usually slightly greater than the linear range. All of these responses are on a log-dosage basis; various methods have been used for plotting the response data but log transformation is most common.

In 9 of the tests, sections of plants or organs are used; in the other 7, plants which are intact, or largely so, are treated, and the response of a part of the plant is measured. In general, the intact plant tests are slower and less sensitive than the section tests. Frequently, however, only a small volume of solution (< 0.1 ml) is required to treat the intact plants.

The use of small volumes may be only an apparent advantage since some of the section tests will be able to respond strongly even after the necessary dilution (table II). Larger (1 ml) volumes have the 2 added advantages of enabling larger amounts of compounds to be dissolved before saturation of the solution occurs, and of ensuring reasonably efficient elution of chromatogram sections. (In only 1 test, the dwarf pea epicotyl, has interference due to the presence of chromatogram sections been reported.)

The most sensitive tests are the barley endosperm, the dwarf pea epicotyl, the *Rumex* leaf disc, and the *Avena fatua* embryo tests. These can measure GA_3 at weights less than 10^{-4} μg. Such a level of sensitivity compares favorably with the most sensitive auxin bioassay (the *Avena* curvature and the pea root tests) and, fortunately, the gibberellin bioassays are considerably simpler.

It should be noted that the sensitivity ranges quoted refer only to GA_3, whereas in some bioassays this is not the most active gibberellin. This is particularly true with the cucumber hypocotyl bioassay in which GA_7 is nearly 1000 times more active than GA_3 (8). Specificity in response to different gibberellins is, of course, helpful in qualitative determinations.

Most of these tests are thought to be specific for gibberellins, at least with respect to plant growth substances. Two notable exceptions are the oat mesocotyl which also responds to IAA and the dwarf bean leaf disc which responds to kinetin. In the oat leaf base test, IAA is added to the culture medium.

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