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# Barley Endosperm Bioassay for Gibberellins. I. Parameters of the Response System

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*Summary.* Parameters of the bioassay based on the gibberellin-induced reducing sugar release of barley endosperm were investigated. Procedures for the rapid handling and processing of up to several hundred treatments without loss in sensitivity of the test are described, and the effects of variations in many aspects of the bioassay were assessed.

In general, the variations in varieties, techniques, additives, conditions, and even gibberellins, all illustrate the stability, sensitivity, and adaptability of the hormone-induced response and emphasize its utility as a gibberellin bioassay.

Among the recent bioassay procedures suggested for gibberellins, the barley endosperm test exhibits several characteristics which are most desirable: it is relatively quick in that a response to gibberellin can be measured within 24 hours; the response seems to be specific for gibberellins since other substances which have been tested by various authors have not elicited similar reactions; the range of active gibberellins is broad, and the sensitivity of the test, to $GA_1$ and $GA_3$ in particular, is 10 to 100 times greater than that found in most other bioassays.

Experiments designed to elaborate on the basic requirements of the tissue and the techniques, as well as increase the usefulness of the test, are described in the following papers.

## Materials and Methods

Initially, the procedures of Nicholls and Paleg (1)[2] were used. Barley seed, after calcium hypochlorite sterilization, were cut transversely 3 mm from the distal end; the endosperm pieces were weighed in groups of 4 and incubated for 48 hours at 30° in 24 × 50 mm lidded vials containing 1 ml of test solution and 500 μg streptomycin sulfate. Three replicates of each treatment were used. The content of reducing sugar was assayed by the method of Paleg (2).

Least significant differences were calculated from an analysis of variance for the concentration × treatment relationship and are shown as single vertical bars in each figure. The response due to increasing $GA_3$ concentrations was always highly significant (with variance ratio in the hundreds) and is not mentioned when other statistical results are referred to. The significance of treatment or concentration × treatment interaction, as shown by F-tests, is indicated in each legend.

## Results

In an assay involving the production and measurement of relatively large amounts of sugars, contamination by micro-organisms becomes a major concern. To overcome this possibility, Nicholls and Paleg treated a naked barley cultivar, Triple Awned Lemma, with 4 % (w/v) calcium hypochlorite for 24 hours. This is a severe treatment and a shorter period was tested. Seed of the naked cultivar, Pearl (which was used in the preliminary experiments), was shaken in 5 % hypochlorite (120 ml/100 seed) for either 3 or 24 hours at 25°. Those shaken for 3 hours were rinsed in running tap water for a further 20 hours. At the end of 24 hours both lots of seed were washed liberally with doubly-distilled water, cut in half with a razor blade, weighed and incubated (4 endosperm/vial) at 30° for 48 hours in 1 ml of test solution. After incubation, aliquots were taken for reducing sugar measurements. The results (fig 1) indicated that a 3 hour exposure to 5 % hypochlorite followed by a 20 hour rinse is sufficient to provide sensitive and relatively uniform test material.

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[2] Mention should be made here of the error in ordinate units published in this reference; values presented should have been μg glucose equivalents/mg dry weight.

Using the modified sterilization procedure, the useful length of exposure to $GA_3$ was investigated. The results of a 24 and 48 hour exposure to 1 ml solution in either slowly rotating (1 rpm, 45° angle) 16 × 150 mm test tubes or stationary 24 × 50 mm vials, are presented in table I. The values indicated that tubes had no virtue when compared with vials which were, in fact, less cumbersome. A 48 hour exposure to either water or $GA_3$ greatly increased the level of sugar release but did not materially alter the sensitivity. This experiment provided an opportunity to express the data in 2 ways; as weight of reducing sugar per mg initial dry weight of endosperm or as weight of reducing sugar per vessel. The coefficients of variability, calculated from an analysis of variance of the data after transformation to its logarithmic equivalents, were identical (0.320 and 0.312 respectively); thus it is not necessary to weigh the endosperm pieces. The table also shows that log transformation of reducing sugar data is necessary because of the hundred-fold range of values; all subsequent results are presented as curves of $\log_{10}$ dose against $\log_{10}$ response.

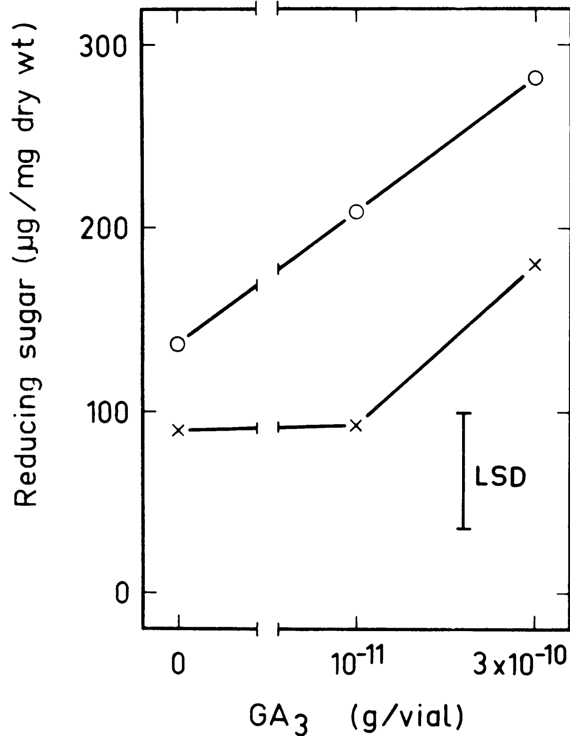



FIG. 1. Release of reducing sugar by barley endosperm incubated in $GA_3$ solutions after sterilizing with 5 % hypochlorite for 24 hours (X) or only 3 hours, plus 20 hours in running tap water (○). Values are averages of triplicates of 3 incubation conditions; tubes, vials, vials with seed cut longitudinally. F tests : hypochlorite treatment significant ($p < 0.001$); interaction of hypochlorite × $GA_3$ concentration not significant.

In addition to the necessity for securing uniform endosperm pieces, cutting and the attendant handling of seed are important parts of the technique since these procedures occur after the sterilization step. Contact with fingers, or a succession of implements and/or surfaces increases the risk of micro-organism contamination. To reduce this risk, and, at the same time, speed the cutting process, brass cutting blocks were made (fig 2). These blocks, together with forceps and razor blades, can be stored in alcohol, or flamed, as desired.

Following the sterilization and water soaking steps, the seed are aligned on the brass cutting block with forceps with the distal or endosperm end of the grain resting against a raised ledge. Alignment is facilitated by transverse grooves, which approximate the diameter of the grain. A holding bar about 3 mm wide, is laid over the grain to hold them in place while a razor blade is moved down the length of the block in a groove situated

Table I. *Effect of Time and Incubation Vessel on Release of Reducing Sugar by Barley Endosperm*

| Treatment | $GA_3$ concn (g/ml) | Reducing sugar release (means of triplicates) µg/mg* | µg/vessel | log µg/mg* | log µg/vessel |
|---|---|---|---|---|---|
| 24 hr in tubes | 0 | 5.1 | 210 | 0.680 | 2.306 |
| | $10^{-11}$ | 5.6 | 248 | 0.745 | 2.382 |
| | $3 \times 10^{-10}$ | 31.4 | 1493 | 1.491 | 3.161 |
| 24 hr in vials | 0 | 7.0 | 283 | 0.840 | 2.452 |
| | $10^{-11}$ | 15.7 | 670 | 1.192 | 2.823 |
| | $3 \times 10^{-10}$ | 48.9 | 2213 | 1.689 | 3.345 |
| 48 hr in tubes | 0 | 99.3 | 4197 | 1.982 | 3 603 |
| | $10^{-11}$ | 173.0 | 8027 | 2.182 | 3.844 |
| | $3 \times 10^{-10}$ | 306.7 | 13000 | 2.474 | 4.099 |
| 48 hr in vials | 0 | 95.3 | 4473 | 1.976 | 3.637 |
| | $10^{-11}$ | 198.0 | 7990 | 2.264 | 3.878 |
| | $3 \times 10^{-10}$ | 243.7 | 10410 | 2.381 | 4 009 |
| L.S.D. $p < 0.05$ | | 27.7 | 1177 | 0 228 | 0.223 |
| $p < 0.01$ | | 37.6 | 1600 | 0.304 | 0 297 |

* µg Reducing sugar per mg initial dry weight of endosperm.

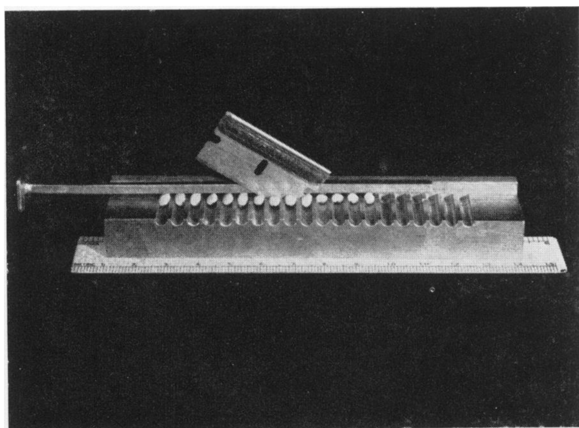


FIG. 2. Seed cutting implement.

4 mm from the raised ledge. In this way, the embryo halves can be speedily discarded leaving a uniformly cut group of endosperm halves 4 mm in length. The endosperm, after cutting, are collected in a petri dish with moistened filter paper until somewhat more than the required number have been cut. This procedure was adopted to facilitate randomization and decrease time lapses between the beginning and end of apportionment of endosperm particularly in large experiments.

Maintaining a 24 hour exposure to $GA_3$ and varying the rinsing time in running tap water from 4 to 36 hours after a 3 hour hypochlorite sterilization period, gave definite differences though no evidence of an interaction. Four hours rinsing did not either remove sufficient hypochlorite from the endosperm, or enable the endosperm to imbibe sufficiently to respond adequately within the subsequent 24 hours; sensitivity at $10^{-10}$ g/ml was very low. A 36 hour rinse, on the other hand, raised both the control level and the $GA_3$ response at $10^{-11}$ g/ml and extended the period of the test almost another day. A consideration of these points led to the adoption of the 3 + 20 hour pretreatment and a 24 hour treatment period.

Time relationships and manual procedures impose some of the severest restrictions on the applicability of a bioassay, and accordingly, attempts were made to shorten some of the steps. The technique followed in the previous experiments involved immediate dilution of all or a portion of the ambient solution from the vials to 10 ml in a second vessel, addition of Amberlite IR-120($H^+$) resin (1 g), filtration of the diluent into a third vessel and analysis of reducing sugar content of an aliquot of the filtered diluent. These techniques were modified in 2 ways. Instead of sampling all of the vials immediately after the 24 hour incubation period, some were frozen for 20 hours prior to sampling. This variation permits much greater flexibility in time schedules and enables the collection of larger numbers of treatments before sugar assays are run. The second modification involves a shortening of the steps used in the sugar test. To half of the unfrozen and frozen vials, 9 ml of water and a scoop (ca. 1 g) of resin were added. The vials were recapped, placed on their side and shaken for 5 minutes. A filter paper cup (Whatman No. 1, made by shaping a 9 cm disc over a 19 mm test tube) was then inserted into the vial slowly enough to ensure that the contents were not forced over the lip of the cup, and an aliquot of the filtered liquid in the center of the cup was removed and analysed. All liquids were dispensed with automatic syringe pipettes. The results of many tests indicated that both modifications may be used without affecting the values, but with substantial savings in time and glassware. Frozen vials have, in fact, been stored for up to 3 days without any detectable change in the results. The shortened method of assaying reducing sugars was adopted for all subsequent tests.

Using a 3 + 20 hour sterilization and rinse treatment, 24 hours incubation in $GA_3$, freeze storage of vials after incubation and the shortened sugar method, a comparison of varying numbers of endosperm per vial was carried out. The results (fig 3) showed that 4 pieces per vial were not, in fact, necessary and that 2 endosperm were sufficient to produce a sensitive and linear response.

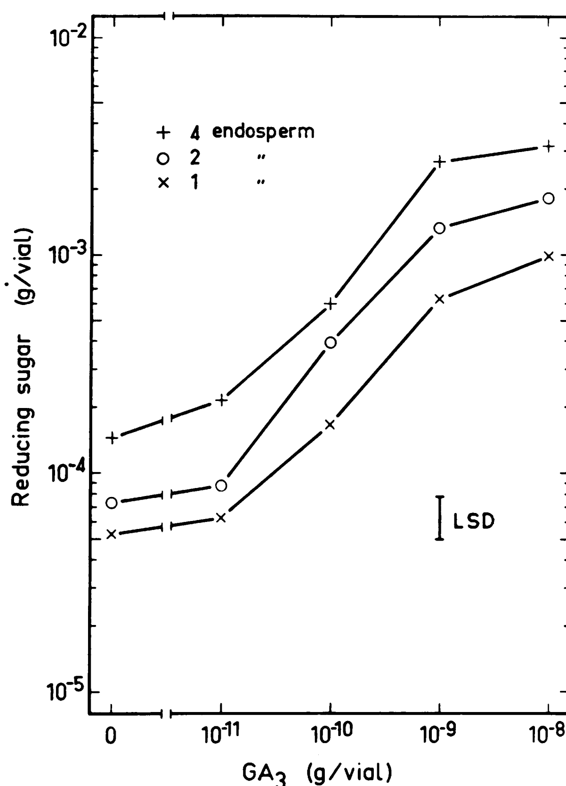



FIG. 3. Dose/response curves with 1, 2 and 4 endosperm per vial. F tests : endosperm number significant ($p < 0.001$) ; interaction not significant.

Table II. *Coefficients of Variation (CV)**

CV's calculated from triplicate determinations of reducing sugar ($\log_{10}$ $\mu$g/ml) classified according to number of endosperm per vial and $GA_3$ concentration.

| $GA_3$ concn (g/ml) | Coefficients of Variation (2 df**) 1 | 2 (endosperm per vial) | 4 | *CV's for $GA_3$ concn (6 df**) |
|---|---|---|---|---|
| 0 | 0.161 | 0.116 | 0.052 | 0.116 |
| $10^{-11}$ | 0.241 | 0.058 | 0.433 | 0.314 |
| $10^{-10}$ | 0.944 | 0.261 | 0.258 | 0.585 |
| $10^{-9}$ | 0.201 | 0.169 | 0.065 | 0.156 |
| $10^{-8}$ | 0.054 | 0.125 | 0.127 | 0.108 |
| CV's for No. of endosperm (10 df**) | 0.408 | 0.161 | 0.236 | |

* CV's calculated from root of average mean squares × 2.303.

** df = degrees of freedom.

This experiment was analysed to determine statistically the effect and value of 1, 2 or 4 endosperm per vial, in terms of variability. The coefficient of variation (CV) is 40 % when only 1 endosperm is present in each vial (table II). This falls to about 20 % when 2 or more are present, with no apparent virtue of more than 2 endosperm per vial. Although there is careful preliminary selection of both seed and, later, endosperm, it is not possible to predetermine whether all endosperm are, in fact, responsive. The decrease in CV when 2 or more endosperm are present appears to be larger than one might expect from the increase in number and suggests that there may be an interaction between endosperm within a vial. This might be due to the diffusion of enzymes from a reacting endosperm into the ambient solution where they are free to cause sugar release from an endosperm which has not reacted to the exogenous gibberellin. By continuing with 2 endosperm per vial, the work involved in 1 step of the preparation of test tissue was thus halved.

The CV for data derived from different treatments can be reduced by increasing the number of replicates. The CV for any individual value (derived from vials containing 2 or more endosperm) is about 20 %. This figure falls to 14.2, 11.6, and 10 % when 2, 3 or 4 replicates are included. By contrast, 11 replicates are required of values derived from vials with only 1 endosperm, to reduce the CV from 40 % to 12 %. On the basis of a comparison between effort expended and increased statistical accuracy, it was decided to continue using 3 replicates, each containing 2 endosperm.

To counter periodic variability and excessively erratic results produced by our distilled water source, routine procedures were altered to include autoclaving of all distilled water and sterilization of all glassware. Running tap water was replaced by autoclaved distilled water at 3° for the rinse period. With these precautions streptomycin sulfate can be omitted from the incubation solution.

Huskless barley, such as the cultivar Pearl, which was used in all of the preceding experiments, is not widely grown and supplies of seed are often difficult to obtain. To provide alternative test cultivars, and to broaden the usefulness of the bioassay, 4 husked cultivars were tried; these were treated for 3 hours with 50 % (v/v) $H_2SO_4$, as recommended by Pollock et al. (3). The acid treatment serves both to remove husks and to sterilize the seed. These seed were compared with seed of 4 huskless cultivars treated with hypochlorite for 3 hours. After the respective sterilization treatments the seed was shaken 10 times with distilled water (to remove glumes from the husked seed), soaked at 3 to 5° for a further 20 hours, cut and incubated (2 endosperm/vial) at 30° for 24 hours.

The results of the comparisons can be seen in figure 4. Husked and huskless cultivars responded equally well, demonstrated similar sensitivities, and indicated that either a 3 hour hypochlorite (huskless) or acid treatment (husked) was satisfactory. The time (2, 3 or 4 hr) in acid is not critical, and temperature during acid treatment can also be varied from 20° to 30°, neither variation significantly influencing the results. The variations in acid treatment caused large differences in degree of husk removal (the longer period also caused carbonizing of the tips of the seed) but did not increase variability in the responsiveness of the endosperm to gibberellin.

Subsequent experiments indicated that the bioassay is only slightly sensitive to the temperature (3°, 13° or 23°) and length (9, 15 or 21 hr) of the soak in water after a 3 hour acid treatment at 25°. Since 3° retards the growth of micro-organisms, provides a linear response over the $GA_3$ concentration range, and is readily obtainable, it was decided to continue with the combination of 3° for 20 to 21 hours.

The influence of variation in seed size and positioning of endosperm during incubation were

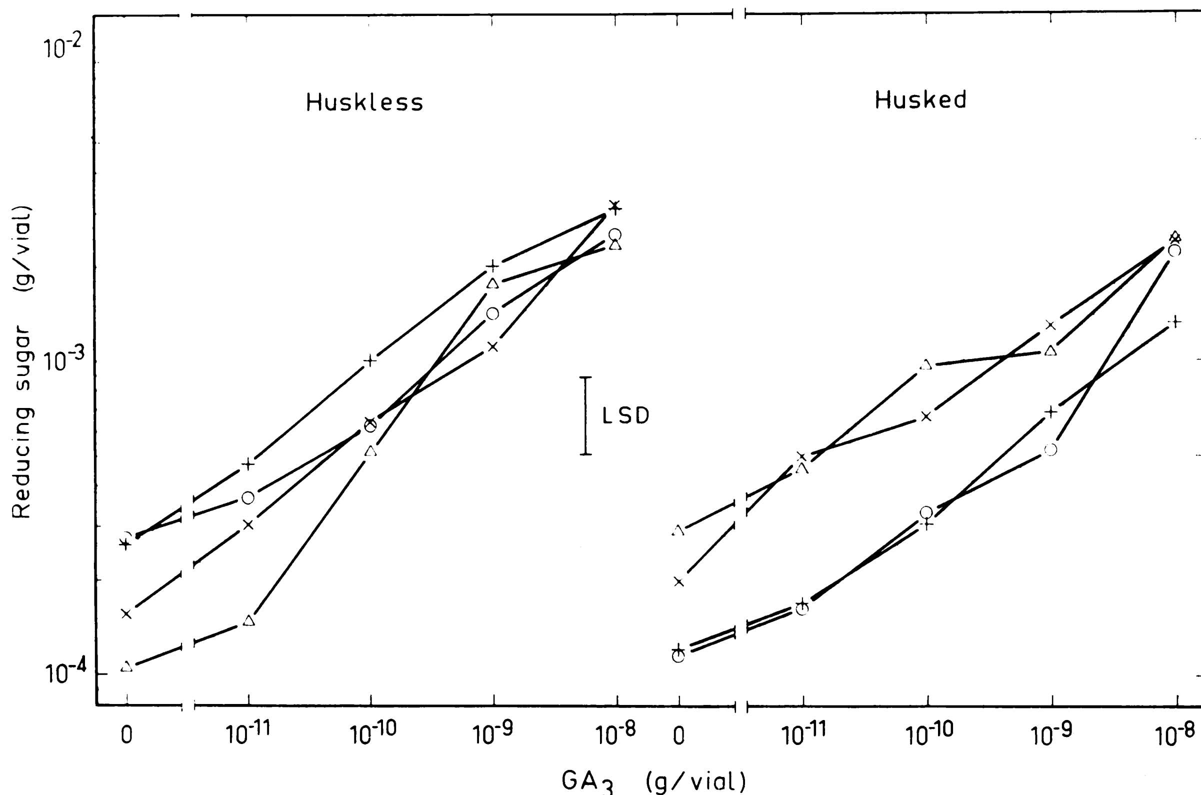



FIG. 4. Comparison of the dose/response curves of 4 huskless [Arabian Blue, (+); Pearl, (△); Triple Awned Lemma, (○); Red Rachis, (X)] and 4 husked [Prior, (+); Research 1960 harvest, (△); Research 1962 harvest, (○); Resibee, (X)] cultivars of barley. F tests : cultivars significant ($p < 0.001$); interaction not significant.

briefly investigated. Endosperm from seed whose transverse axis measured 3.4 to 3.8 mm released significantly more reducing sugar than seed of 3.0 to 3.4 mm. However, the response curves were parallel indicating no greater sensitivity of large than small seeds. When the response of endosperm lying on their side was compared with that of endosperm lying on their cut face, no significant differences were obtained. This was surprising since diffusion of chemicals and oxygen was thought to be aided by the former position. It is possible that the pronounced bulging which occurs as the endosperm imbibes and expands renders this aspect less important, but constancy of conditions should be a major concern for users of the test.

A variable feature of the bioassay is the amount of liquid in the incubation vial. Standard procedures of 2 endosperm and 1 ml of solution in a 24 × 50 mm flat-bottomed vial, produce a depth of about 2 mm, and under these conditions a portion of each endosperm protrudes above the surface of the liquid. Effects of different volumes of solution (as indicated in fig 5) were determined. Since all previous tests were run with 1 ml solution the results can be interpreted on either a per ml (concentration) or per vial (total amt) basis. In this and the next experiment, however, varying amounts of solution were used. The results have been presented on a per ml (concentration) basis although total amounts have also been examined.

The results (fig 5) show that as the depth is increased sugar release is diminished. Response curves to the different treatments were not parallel: the slopes decreased with increasing depth, indicating that some factor other than dilution is also involved. The most likely explanation is that aeration, necessary for the manifestation of the $GA_3$ response (4), becomes progressively more limiting as depth is increased.

By altering the diameter of the incubation vessel and maintaining constant depth, the effect of aeration can be separated from other influences. In vessels of 12, 24 and 42 mm diameter, depths of 1.5 and 4.5 mm were obtained with volumes of 0.2, 0.75, 2.25 ml and 0.5, 2.25, 6.25 ml respectively. The area of the vessels, and volumes at each depth, were in the ratio of approximately 1 : 4 : 12. In the shallower series the endosperm projected above the surface of the solution, but in the deeper series they were completely submersed. The results (fig 6) are presented in the same way as in figure 5.

Collectively, the slopes of the response curves in the deeper series (4.5 mm) were less than those in the shallow solutions (1.5 mm), confirming the

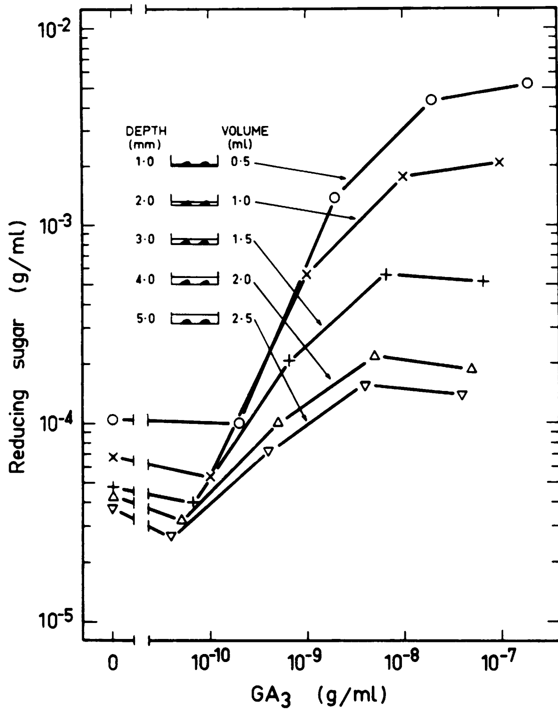
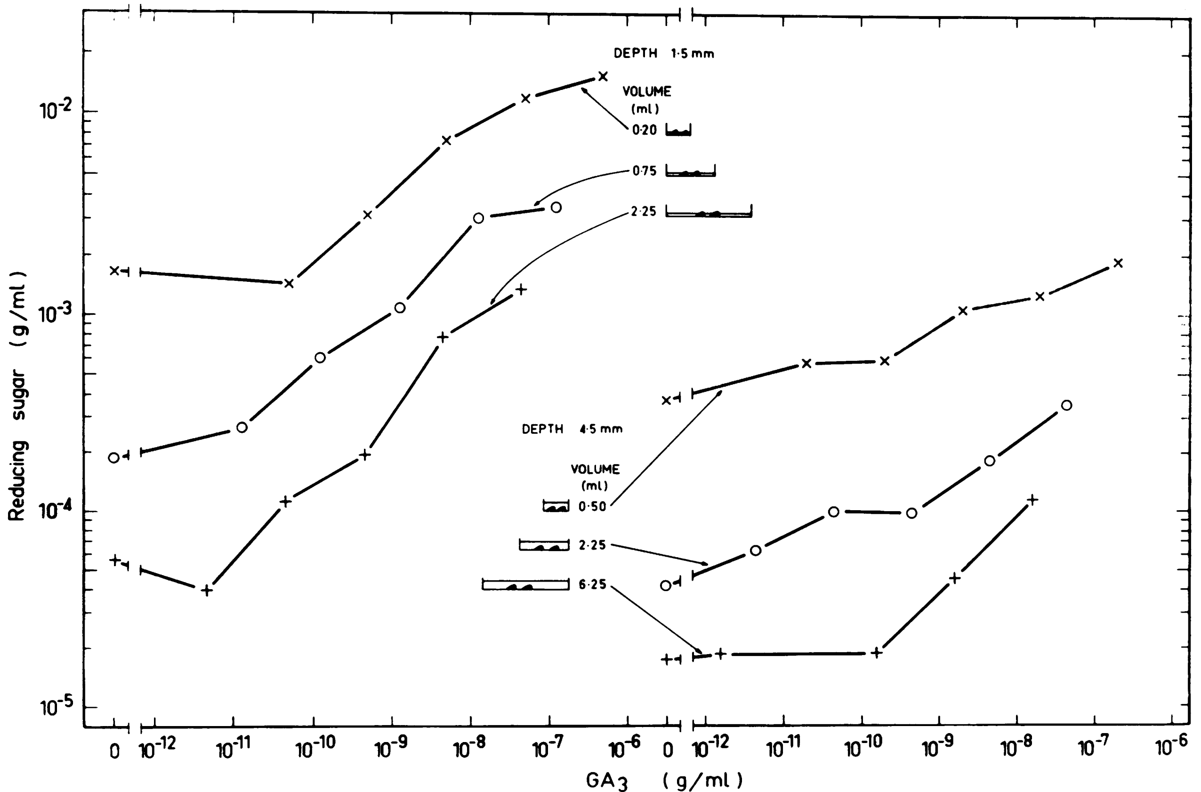



FIG. 5. Effect on the gibberellin-induced response of varying the depth of solution from 1 to 5 mm in a 24 × 50 mm vial. The lessening of the slope in the steep part of the curves, as measured by regression analysis, is highly significant.

results in figure 5 and reflecting the inhibited response to $GA_3$ under conditions of limited aeration. Within each depth condition the sugar release curves are parallel indicating that the endosperm are reacting to $GA_3$ similarly, despite differences in amounts of solution. That diffusion is not exerting an influence on the results is shown by the fact that the curves coincide when sugar release is plotted on a per vial (total amt) basis. The induced response depends completely on the concentration of gibberellin and aeration rather than other factors, so that if these 2 variables are held constant comparable results may be expected regardless of amount of solution.

A comparison of 12, 24, 36 and 48 hour incubation periods at 20° and 30° (fig 7) showed a steady increase in the amount of reducing sugar released with time at all levels of $GA_3$ indicating that the test becomes more sensitive with longer incubation periods. Nicholls and Paleg (1) used a 48 hour incubation period but, for the sake of speed, we decided to persist with 24 hour.

Using a 24 hour period, the effect of 4 temperatures of incubation (20°, 25°, 30°, 35°) was tested. The results (fig 8) show an increase in the response to $GA_3$ concentrations with an increase in temperature from 20° to 30°. The further increase to 35° lowered the response significantly. Hence 30° appears to be the optimum temperature under these conditions.



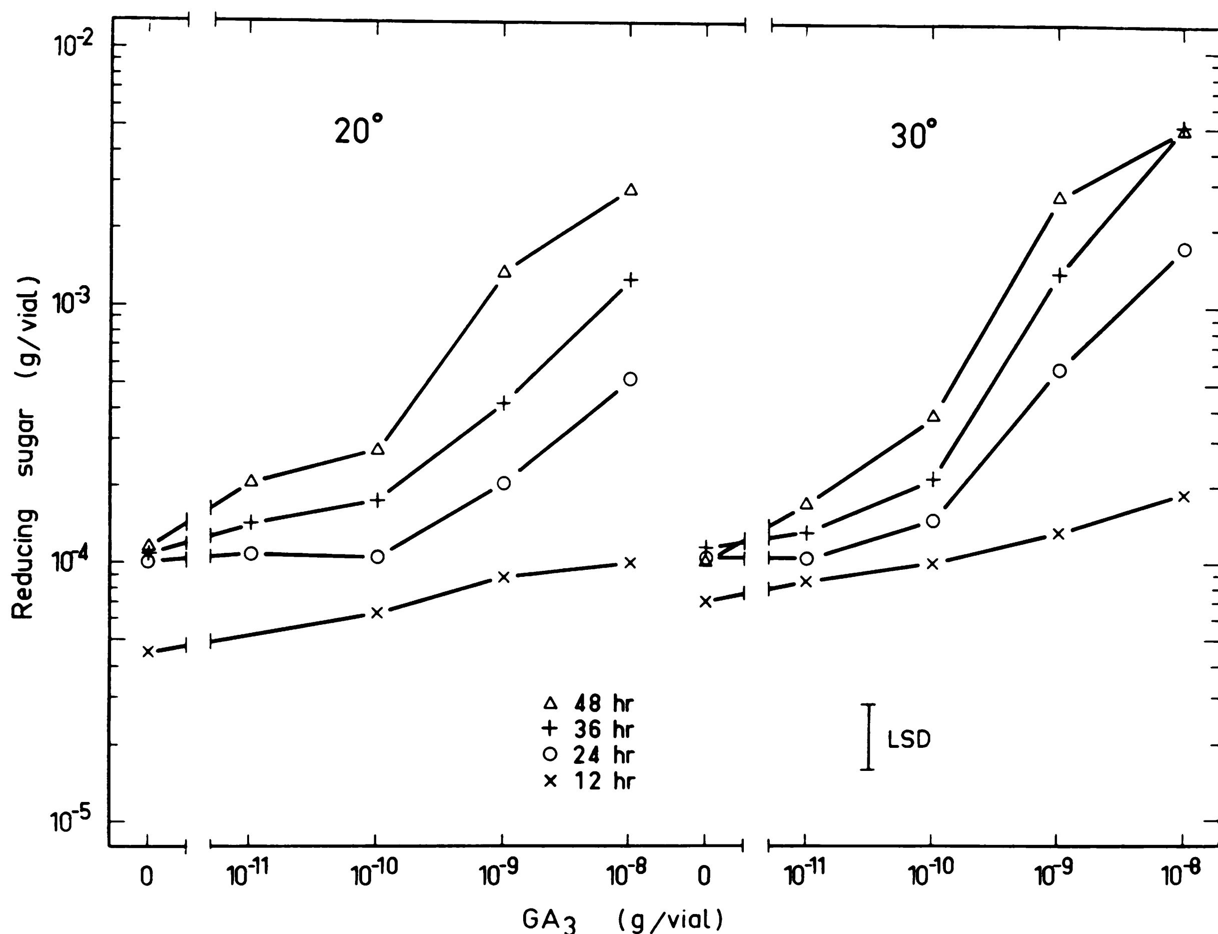



FIG. 7. Development of the gibberellin response with time (12, 24, 36 and 48 hrs) at 20° and 30°. F tests : temperature, time, concentration × temperature, and concentration × time are all significant ($p < 0.001$) ; temperature × time, and concentration × temperature × time are not significant.

Endosperm have been incubated with varying concentrations of indoleacetic acid (IAA) and 6-furfuryl-amino-purine (kinetin) up to $10^{-6}$ g/ml (about $5 \times 10^{-5}$M) without any significant promotive effect on reducing sugar release.

Since this assay system is suitable for direct elution of pieces of chromatogram paper, it was of interest to determine whether filter paper had any effect on the response. No significant effect was noted with Whatman No. 1 paper either unwashed (2 sizes), or washed with water or isopropyl alcohol (80 %).

## Discussion and Conclusion

A summary of the experiments performed has been organized into a series of recommended bioassay procedures as follows:

With huskless cultivars, sieved seed is sterilized in 4 % hypochlorite solution for 3 hours at 25°. Husked cultivars respond equally well; instead of hypochlorite sterilization these seed are dehusked and sterilized in 50 % v/v $H_2SO_4$ for 2 to 4 hours at 20 to 30° then washed by vigorous and repeated agitation with sterile water (at least 10 times).

FIG. 6. Effect of 2 depths of solution, 1.5 and 4.5 mm, in vessels of 3 diameters, 12, 24 and 42 mm on the gibberellin-induced response. Within each depth slopes are similar, but the slopes between depths are significantly different ($p < 0.01$).

The sterilized seed is soaked in sterile water for 20 hours at 3°. The seed is then cut transversely 4 mm from the distal end. Two endosperm pieces are placed in 24 × 50 mm lidded vials with 1 ml water containing known or unknown amounts of gibberellin and incubated for 24 to 48 hours at 30°. The variability is increased if only 1 endosperm is used, there is no advantage from the use of 4.

After incubation the vials are frozen (to stop the reaction) and stored in the freezer until ready for sugar measurement. The concentration of reducing sugars is measured by a shortened method as follows: Add about 1 g Amberlite IR120($H^+$) resin and 9 ml water to the frozen incubate, recap and shake 5 minutes. Insert a filter cup and pipette an aliquot of filtered diluent from inside the cup. The aliquot is then heated with copper reagent, color is developed with arseno-molybdate reagent and optical densities are compared at 560 mμ with appropriate glucose standards.

With this procedure, using suitable vial racks, boiling tube racks and automatic syringe pipettes, several hundred vials can be run by 1 or 2 people during a week. Care should be taken to exclude micro-organisms by autoclaving glassware and distilled water. The results also suggest that the length of the incubation period and the volume of solution can be altered to increase sensitivity,

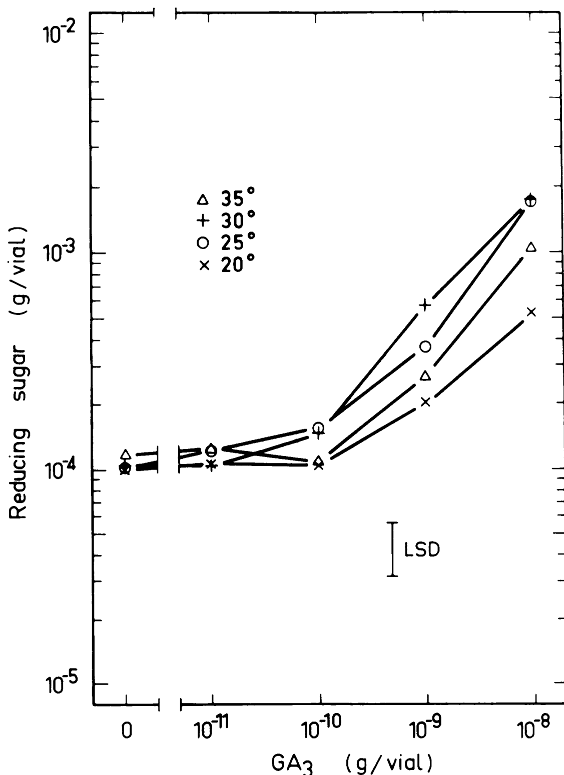



FIG. 8. Effect of incubation for 24 hours at temperatures of 20°, 25°, 30° and 35° on the response to gibberellic acid.

although the introduction of either modification has intrinsic difficulties.

It is interesting to note that from the responses of barley endosperm presented in this paper, 3 main variations are discernible. The first type is illustrated in figures 3 and 6 in which parallel response curves were obtained. In these cases, the $GA_3$-mediated reaction mechanism is unchanged although an outside influence, such as size of diffusing surface or volume of solution, can alter the position of the overall response with respect to the ordinate. The second type is that illustrated by figures 7 and 8 in which the curves diverge from an essentially common starting or initial value. In these cases, it seems likely that some aspect of the $GA_3$-initiated chain of reactions is being at least partially inhibited or promoted. The third variation observed was when the response curves, though having different initial or starting points, converged to a common value as in the left hand side of figure 4. Results similar to these have also been obtained as a result of treating the endosperm with specific compounds. This case can be explained by assuming that the treatment influences the amount of sugar initially available for diffusion into the ambient solution, and that at higher levels of gibberellin the response system approaches saturation thus obscuring the effect of treatment. This type of response is also dependent on a sufficiently high sugar release value in the absence of added gibberellin. Under conditions of very low control sugar release, no such effect would be observed, and the response would take yet a different form. It should be noted that statistical analyses of the 3 types may show differences since interactions may be significant in the last 2 but not in the first type. Classification of the responses in this way may supply valuable information about the nature of the influence exerted.

## Acknowledgments

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