GIBBERELLIC ACID, PART VI. THE BIOLOGICAL ACTIVITY OF ALLOGIBBERIC ACID AND ITS IDENTITY WITH GIBBERELLIN B

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Mild acid hydrolysis of gibberelic acid (I) yields allogibberic acid (II) (5, 6) with aromatization of ring A and evolution of carbon dioxide. Cross (5) has presented evidence that allogibberic acid is identical with gibberellin B, a substance obtained from culture filtrates of Gibberella fujikuroi by Yabuta et al (10) along with gibberellin A, and also obtained (11) by treatment of gibberelin A with mineral acid at low temperature.

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\begin{align*}
\text{OH} & \quad \text{CO} \quad \text{CH}_3 \\
\text{CHOH} & \quad \text{CH}_3 \\
\text{I} & \\
\text{II} \\
\end{align*}
\]

Gibberellin B was reported by Yabuta et al (10) to produce elongation of rice seedlings (i.e., the "bakanane" effect) in the same way as gibberellin A, but that it was markedly less active. Cross (5), on the basis of data on growth of dwarf peas supplied by one of us, reported that allogibberic acid was biologically inactive. Stowe and Yamaki (8) have drawn attention to this apparent contradiction, and have suggested that the presumed identity of allogibberic acid and gibberellin B should be reconsidered. As they rightly point out, it is of some importance to know whether or not ring A of gibberelic acid is necessary for biological activity.

In this paper we present in greater detail the evidence for the identity of allogibberic acid and gibberellic B and report further evidence that allogibberic acid is inactive in inducing shoot extension. In addition, since Stowe and Yamaki (8) have also stated that gibberellin B may stimulate root growth, whereas gibberelic acid and other gibberellins usually do not, we have examined the effects of allogibberic acid on root growth.

METHODS AND MATERIALS

DWARF PEA TEST: The materials to be examined were applied to 14-day-old dwarf pea seedlings, var. Meteor, in doses of 0.01 to 10.0 µg in 2 µl ethanol solution. The height of the plants was measured at the beginning of the experiment and 3, 7 and 10 days afterwards. Sixteen replicates of each treatment were used. The method has been described in greater detail by Cross (5).

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LITERATURE CITED

detail previously. A dose of 0.01 µg gibberellic acid produces a detectable growth response (2).

Cress Root Growth Test: The method used was that of Audus (1) modified as previously described (4). Six replicates of 20 seedlings were used for each treatment.

Materials: An improved preparation of allogibberic acid was used. Gibberellic acid (100 mg) was dissolved in water (67 ml) at 50°C, the solution was cooled to room temperature, treated with dilute hydrochloric acid (33 ml, 3 N) and allowed to stand at room temperature for 4 days. Allogibberic acid hydrate separated in needles (58 mg, 66%), melting point 125 to 130°C (loss of water) resetting ca. 140°C and remelting 195 to 197°C. Found: C, 71.4; H, 7.5. Calc. for C₁₈H₂₀O₃·H₂O requires C, 71.5; H, 7.3%.

Further crystallization of some specimens from benzene-methanol (plates) and toluene raised the melting point to 200 to 203°C [α]₅°D = 84° ± 3 (c, 0.94 in ethanol) (Found: C, 76.2; H, 7.1%).

The infra-red spectra of allogibberic acid and its monohydrate were identical (CO band, 1735 cm⁻¹, monomeric carboxyl) in solution in dioxan but the spectra of the crystalline compounds in Nujol mulls showed characteristic differences (figs 1 and 2) owing to different modes of hydrogen bonding in the solid state. Anhydrous allogibberic acid showed a double peak at 1715 and 1679 cm⁻¹ in the double bond stretching region and hydroxyl absorption at 3460 and 3160 cm⁻¹ whereas the hydrate showed a single carbonyl band (1700 cm⁻¹), a band at 1636 cm⁻¹ (? water) and bands at 3350 and 3480 cm⁻¹ (hydroxyl groups).

Three samples of allogibberic acid were tested in the work reported in detail below: (a) Needles, melting point 198°C [α]₅°D = 73° ± 3, (c, 1.115 in ethanol) obtained from the standard preparative

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Figs. 1 and 2. Infra-red spectra in Nujol: 1 (top) allogibberic acid hydrate and 2 (bottom) allogibberic acid.
grade (above) of allogibberic acid hydrate by crystallisation from toluene. (b) Plates, melting point 200 to 202°C [$\alpha$]$_{D}^{19}$ = 84° ± 3, (c, 1.10 in ethanol) obtained by recrystallization of (a) from benzene-methanol (Found: C, 76.3; H, 7.2). (c) Needles, melting point 198-201°C [$\alpha$]$_{D}^{20}$ = 84° ± 3, (c, 1.12 in ethanol) obtained as follows: (b) was heated for one hour under reflux with N sodium hydroxide solution and the product obtained by acidification and filtration crystallized from toluene. allogibberic acid is stable under these conditions of alkaline hydrolysis (5) which decompose gibberellic acid.

Specimens (a), (b) and (c) of allogibberic acid, gibberellic acid, and an aqueous solution of gibberellic acid which had been kept at room temperature for 7 weeks were chromatographed on Whatman's no. 3 MM paper (18½ x 22½ inches) using the solvent system n-butanol : ammonia (d 0.880) : water (4 : 5 : 1; descending technique). After equilibration of the paper for 1 to 16 hours in the presence of the lower phase (see below), development, with the upper phase for 6 to 8 hours, and drying, spots were made to fluoresce in ultra-violet light by spraying with 5% ethanolic sulphuric acid followed by heating for a few minutes at 80°C (7).

allogibberic acid gave very weakly fluorescent brownish spots, not visible unless comparatively large amounts of material were present. Gibberellic acid (violet spot) was detected at a minimum of 0.5 μg per spot. Results are summarized in table I.

When the temperature of development was 18 to 20°C, gibberellic acid gave a spot with $R_f$ ca. 0.40, whereas the aged aqueous solution of gibberellic acid gave, in addition to the above spot, one with $R_f$ 0.02, caused by a degradation product which will be described in a later paper, and an unidentified spot with $R_f$ ca. 0.35.

When development was carried out at 28 to 30°C, gibberellic acid gave results similar to the aged aqueous solution of gibberellic acid; this effect was sometimes obtained with development at 20°C when the time of preliminary equilibration of the paper had been extended to 16 hours. Specimen (c) of allogibberic acid gave a spot with $R_f$ ca. 0.56, and no spot corresponding to gibberellic acid was detected. Specimen (a) gave spots with $R_f$ values corresponding to allogibberic acid, gibberellic acid and the unidentified material with $R_f$ ca. 0.35. The content of gibberellic acid in specimen (a) was roughly estimated as 0.3%. Specimen (b) of allogibberic acid contained only traces of gibberellic acid, not always detectable even at 1200 μg of material per spot.

**RESULTS**

**Effects on Pea Shoot Growth:** The result quoted by Cross (5) was obtained with Meteor peas grown in nutrient solutions, the allogibberic acid being included in the nutrient solution. Two samples were used—anhydrous allogibberic acid, at concentrations of 42.8 and 4.8 μg/ml and allogibberic acid hydrate at 13.75 and 1.5 μg/ml. Neither induced increased growth; indeed the higher concentrations were toxic, though this was attributed to the use of alcohol (0.25% v/v) in making the solutions.

In later tests, where allogibberic acid was applied to the leaves, increases in growth were obtained with some samples. This only happened when large doses (10 to 100 μg/plant) were used and never suggested that allogibberic acid was of an activity exceeding 0.1% that of gibberellic acid. However, in view of the element of uncertainty, the three samples listed under Methods and Materials above were tested at the same time at four dosage levels. The results are presented in table II. Sample (a) of allogibberic acid produced a significant growth increase only at the highest dose (10 μg/plant). The two samples which had been most rigorously purified, (b) and (c), produced no growth increases. It therefore seems most probable that the increase produced by sample (a) was due to the contamination with gibberellic acid demonstrated chromatographically (see Methods and Materials). Pure allogibberic acid had less than 0.1% the activity of gibberellic acid, i.e., to all intents and purposes is inactive. The apparent inhibition of growth caused by the 0.01 μg dose of sample (a) can probably be ignored as it could well occur by chance in an experiment of this size.

**TABLE II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg)</th>
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<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>allogibberic acid:</td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>67 *</td>
</tr>
<tr>
<td>(b)</td>
<td>73</td>
</tr>
<tr>
<td>(c)</td>
<td>82</td>
</tr>
<tr>
<td>Gibberellic acid</td>
<td>104 **</td>
</tr>
<tr>
<td>Untreated control</td>
<td>83</td>
</tr>
</tbody>
</table>

*Significantly different from control at 5% level.
**Significantly different from control at 1% level.

Standard error = 6.1.
TABLE III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Gibberellic acid</td>
<td>29.6</td>
</tr>
<tr>
<td>alloGibberic acid:</td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>16.6*</td>
</tr>
<tr>
<td>(c)</td>
<td>19.8*</td>
</tr>
<tr>
<td>Untreated control</td>
<td>32.1</td>
</tr>
</tbody>
</table>

*Significantly different from control at 1% level. Standard error = 1.65.

Effects on Cress Root Growth: The results of a cress root growth test are given in Table III. Data for all treatments except controls are means of 6 replicates of 20 seedlings; for the untreated controls 24 replicates of 20 seedlings were used. Gibberellic acid had no effect on root growth in this dosage range, as previously reported (4). At no dose did either sample of alloGibberic acid increase root growth; at the highest concentration (10 µg/ml) both samples significantly reduced root growth.

This result raised one further possibility which we have tested by experiment. The surprising lack of effect of gibberellic acid on growth of intact roots could be explained if some natural "gibberellin-like hormone" were always present in optimal quantities. The inhibitory effects of alloGibberic acid could then in turn be attributed to its structural similarity to the gibberellins, only ring A being different. If this were so the inhibition caused by alloGibberic acid should be reduced by gibberellic acid. The data presented in Table IV show that this is not so; gibberellic acid is without effect on cress root growth alone or in the presence of alloGibberic acid.

Identity of AlloGibberic Acid and Gibberellin B: An authentic specimen of gibberelin B received from Professor Sumiki melted at 125° C, reset at 135 to 140° and remelted at 192 to 197°, identical in behaviour with alloGibberic acid hydrate. The identity was confirmed by comparison of the infra-red spectra. After drying gibberelin B at 100° C in vacuo a mixed melting point determination with anhydrous alloGibberic acid showed no depression.

Discussion

Though the identity of alloGibberic acid and gibberellin B is certain there are discrepancies in reported biological activity that require explanation. Stowe and Yamaki (8) have noted two features of the biological activity of gibberellin B: 1) It stimulates shoot growth of rice seedlings, having approximately one tenth the activity of gibberelin A (Gibberellin A, as used by Yabuta et al, is now believed by Takahashi et al (9) to have been a mixture of two or more compounds). 2) Though it might have been suspected that such activity could best be accounted for by contamination with gibberelin A, this explanation must be rejected, because gibberellin B increased root growth, unlike gibberellin A which reduced it. Their conclusions were based on the data of Yabuta et al (10).

Since the alleged stimulation of root growth by gibberelin B is critical for this argument, the results of Yabuta et al (10) merit further examination. They presented data for growth of rice seedlings over periods of 8 and 15 days. These do indicate that root growth was slightly depressed by gibberelin A; this has been our experience with gibberellic acid on wheat seedlings (3) in experiments lasting one or two weeks, but in more critical tests over shorter periods (4) we have not found it to affect root growth in concentrations up to 10 µg/ml. Gibberelin B, on the other hand, had little if any effect on growth as judged by their 8-day data, but the 15-day data indicated marked stimulation of root growth by the two highest concentrations tested (3.5 and 7 µg/ml). It is doubtful whether these results can be accepted at their face value. The mean values presented were each based on measurements of 20 seedlings, growing in a very small container with no nutrients supplied. Such conditions are unsuitable for root growth studies, as is indeed shown by the wide variability within treatments indicated by the maximum and minimum values presented; indeed it is doubtful whether any of the effects on root growth could be considered significant. Moreover, Yabuta et al themselves concluded that gibberelin B had no effect on root growth, in distinction from gibberelin A which was slightly inhibitory.

If this point of view is accepted, it seems reasonable to conclude that our data on shoot growth for alloGibberic acid and those of Yabuta et al for gibberellic acid are more reliable than those of Yabuta et al.
berellin B can best be reconciled by assuming that their material was contaminated with the active gibberellin A. The results we have presented in this paper show the difficulty encountered in obtaining samples of allo gibberic acid uncontaminated with gibberellic acid, using chemical methods for preparation of the former from the latter. The gibberellin B used by Yabuta et al in their physiological studies (10) was extracted from culture filtrates containing more gibberellin A than gibberellin B, so that risks of contamination were even greater than in our case.

Our results indicate that allo gibberic acid does not promote shoot growth. allo Gibberic acid agrees in its properties with the published chemical and physical properties of gibberellin B (10, 11) and the hydrate was identical with a specimen of gibberellin B supplied by Professor Sumiki. Thus if our explanation of the results of Yabuta et al (10) is not accepted, it must be concluded that the gibberellin B used in their physiological experiments differed from that investigated chemically then and later, and differed from the gibberellin B supplied to us. This seems to us unlikely.

We have shown allo gibberic acid to inhibit growth of cress roots slightly at 10 μg/ml. This is not necessarily incompatible with the observations of Yabuta et al. They did not use gibberellin B at so high a concentration as this, and cress roots are in general more sensitive than those of rice or other cereals.

Perhaps the most important conclusion that can be drawn from these results, is that since allo gibberic acid differs from gibberellin acid only in ring A, this part of the molecule must be of importance in determining biological activity of the gibberellins.

**Summary**

Allo Gibberic acid hydrate is shown to be chemically identical with the gibberellin B described by Yabuta et al (10). When rigorously purified it does not induce accelerated shoot growth of dwarf peas. It slightly inhibits the growth of cress roots, and this inhibition is not reversed by gibberellin acid. Earlier claims (10) that gibberellin B stimulates shoot growth are attributed to incomplete removal of gibberellin A. These results indicate that ring A of gibberellic acid is of importance in determining biological activity.

**Literature Cited**