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PLANT HORMONES—III.[1] IDENTIFICATION OF GIBBERELLIC ACID IN IMMATURE BARLEY AND IMMATURE GRASS

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Abstract—Gibberellic acid has been identified in extracts of immature barley seed and immature grass seed by techniques based on thin layer chromatography, fluorimetry and bioassay.

THE OCCURRENCE of gibberellins in higher plants has been established by the isolation of gibberellins A_1, A_5, A_6 and A_8.[1-4] In addition crude extracts of many plant species have been found to possess biological properties similar to the gibberellins,[5] and attempts were made to identify the compounds responsible by paper chromatographic comparison with known gibberellins.[5-7] A disadvantage of this technique is that biologically inactive derivatives of the gibberellins cannot readily be detected. Thin layer chromatography provides a more convenient and sensitive method of separating and identifying all nine known gibberellins[8-10] and their derivatives[8] and we are currently applying this technique to the identification of some of the plant gibberellin-like substances. This paper presents circumstantial but compelling evidence for the presence of gibberellic acid (gibberellin A_3) in immature barley ears and in immature seed heads of a grass species.

Three groups have presented evidence for the presence of gibberellic acid-like substance in higher plant extracts. Adler *et al.*[6] using paper chromatography in conjunction with bioassay and sulphuric acid spray, detected the presence of a gibberellin, having R_f values in two solvent systems similar to gibberellic acid, in extracts of the following plants: Kudzu vine, pinto bean, alfalfa, sweet corn, dandelion and lima bean. Using similar techniques Radley[7] has demonstrated the presence in malt of a gibberellin also similar to gibberellic acid. Lazar *et al.*[11] using an isotope dilution technique and chromatography have demonstrated the presence in malted barley of a substance having properties similar to gibberellic acid which gives a methyl ester similar to methyl gibberellate. These authors however did not demonstrate that the initial extract had plant growth promoting activity, or that their purification procedures are unique to gibberellic acid. In the absence of confirmation of identity by

[1] J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **18**, 349 (1962).
[2] J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **11**, 60 (1960).
[3] A. KARAWADA and Y. SUMIKI, *Bull. Agr. Chem. Soc. Japan* **23**, 343 (1959)
[4] C. A WEST and B. O. PHINNEY, *J. Am. Chem. Soc.* **81**, 2424 (1959).
[5] B. O. Phinney and C. A. West, *Ann. Rev. Plant Physiol.* **11**, 411 (1960).
[6] N. ADLER, T. MEDWICK, R. G. JOHL and M. A. MANZELLI, *Advances in Chem. Ser.* **28**, 26 (1961).
[7] M. RADLEY, *Chem. & Ind. (London)* 877 (1959).
[8] J. MACMILLAN and P. J. SUTER, *Nature* **197**, 790 (1963).
[9] M. KUTÁČEK, J. ROSMUS and Z. DEYL, *Biol. Plant.* **4**, 226 (1962).
[10] G. SEMBDNER, R. GROSS and K. SCHREIBER, *Experientia* **18**, 584 (1962).
[11] L. LAZER, W. E. BAUMGARTNER and R. R. DAHLSTROM, *J. Agr. Food Chem.* **9**, 24 (1961).

characterization of several derivatives the evidence available, although highly suggestive of the presence of gibberellic acid in plants, is not unequivocal.

In our initial studies, whole ears of barley, *Hordeum vulgare* L. var Proctor, harvested 1 week and 4 weeks after anthesis, were processed by ethanol extraction and chromatography on charcoal to give an extract which was applied in ethanol to dwarf pea seedlings. An estimate of the amount of gibberellin present was made by comparison of the growth of these plants with that of similar plants treated with known amounts of gibberellic acid.[12] Chromatography of the extract on paper, identification of the active zones by bioassay and rechromatography of the active materials on paper in benzene–acetic acid–water as described by Bird and Pugh[13] revealed two active substances having R_f values corresponding to gibberellins A_3 and A_7 (and/or A_4) respectively. The latter substance showed significant activity on the cucumber bioassay system,[14] but little on lettuce seedling bioassay.[15]

There appeared to be more activity in the younger sample and in subsequent work a batch of approximately 50 kg of barley ears harvested about 10 days after anthesis was processed to give a crude acidic extract which was partially purified by chromatography on a column of charcoal–celite followed by chromatography on a column of celite–silicic acid. This procedure gave 14 mg of a fraction, bioassay of which indicated the presence of approximately 150 μg of gibberellin. Paper chromatography and bioassay on peas revealed that activity was concentrated in a narrow zone having an R_f value close to that of gibberellic acid. No zone of activity corresponding to the R_f value of gibberellin A_7 was observed.

Isolation of the gibberellin in the active concentrate and preliminary characterization was effected by two-dimensional thin layer chromatography on silica gel; development was carried out first with ethyl acetate and secondly with water. Development in ethyl acetate caused no significant displacement of the known gibberellins but slightly displaced some of the contaminants thereby improving the resolution obtained with water. The chromatographic behaviour of the known gibberellins in this system is presented in Table 1. The R_f values vary slightly from plate to plate but the mobilities relative to gibberellic acid are practically constant. The system separates gibberellins A_6, A_8 and A_9 from each other and from the unresolved pair of gibberellins A_1 and A_3 and the unresolved group of gibberellins A_2, A_4, A_5 and A_7. Detection was effected by spraying with ethanolic sulphuric acid, heating at 120° and viewing under ultraviolet light as described by MacMillan *et al.*[8] These authors noted that the colour of the fluorescence induced by heating for 10 min provided a useful adjunct to R_f value in characterizing the gibberellins. We have now found that the period of heating required to induce fluorescence is a further distinguishing feature of the gibberellins. Thus heating for 1 min reveals only gibberellins A_3 and A_7; the former as a green–blue spot and the latter as a bright yellow spot. Further heating is required to reveal the other gibberellins (see Table 1).

Chromatography of the active concentrate in the above system and detection by sulphuric acid spray and heating for 1 min, revealed a green–blue fluorescent spot having an R_f value identical to that of a comparison spot of gibberellic acid. Co-chromatography of the active concentrate with gibberellic acid gave a single spot, and with gibberellin A_7 two spots. Purified gibberellin was obtained from untreated chromatograms by acetone elution of adsorbent scraped from the zone corresponding in R_f value to the green–blue fluorescent zone of a

[12] P. W. BRIAN and H. G. HEMMING, *Physiol. Plantarum* **8**, 669 (1955).
[13] H. L. BIRD and C. T. PUGH, *Plant Physiol.* **33**, 45 (1958).
[14] P. W. BRIAN and H. G. HEMMING, *Nature* **189**, 74 (1961).
[15] B. FRANKLAND and P. F. WAREING, *Nature* **185**, 255 (1960).

marker spot of active concentrate. The eluate showed growth-promoting activity typical of gibberellic acid on both peas and lettuce. These results indicated identity of the gibberellin with gibberellic acid.

Further characterization of the gibberellin was effected using the solvent systems described by MacMillan and Suter.[8] These authors have shown that gibberellins A_5, A_6 and A_9 are separated from each other and from the unresolved pairs of gibberellins A_2 and A_8, A_1 and A_3, and A_4 and A_7 by adsorption chromatography on a thin layer of silica with di-isopropyl ether–acetic acid, and that gibberellins A_1 and A_3 are resolved by partition chromatography on a thin layer of kieselguhr with benzene–acetic acid–water. The purified gibberellin, on chromatography in these systems and detection by both bioassay and sulphuric acid spray,

TABLE 1. R_f VALUES OF THE GIBBERELLINS

Gibberellin	R_{GA}* ±0·01	Fluorescence induction period (min)	Colour of spot
Gibberellic acid (A_3)	1·0	1–3	Green–blue; blue on prolonged heating
Gibberellenic acid[16]	1·07	1–3	As gibberellic acid
A_1	1·0	30–40	Blue
A_5	0·83	10–20	Blue
A_6	0·93	10–20	Blue
A_8	1·07	10–20	Blue
A_7	0·89	1–2	Bright yellow; pale yellow on prolonged heating
A_4	0·89	4–8	Purple
A_2	0·89	4–8	Purple
A_9	0·78	4–8	Purple

* Mobilities in water relative to gibberellic acid which has an $R_f \sim 0{\cdot}90$.
System: Two-dimensional adsorption chromatography on silica in (a) ethyl acetate and (b) water.
Detection: Ethanolic sulphuric acid spray, heated at 120° and viewed under ultraviolet light.

exhibited R_f values and colour reactions identical to those of a comparison spot of gibberellic acid.

MacMillan and Suter[8] have also shown that the methyl esters of the known gibberellins are resolved by a combination of thin layer chromatography on silica in di-isopropyl ether–acetic acid, and on silica in benzene–acetic acid–water. In these systems the methyl ester of the gibberellin, prepared by reaction with diazomethane, behaved exactly as a comparison spot of methyl gibberellate. Acetylation of the gibberellin methyl ester by heating in acetic anhydride gave a product chromatographically identical to di-O-acetyl methyl gibberellate on silica gel in di-isopropyl ether–acetic acid.

Acid hydrolysis of a few microgrammes of the gibberellin and thin layer chromatography of the products on silica in di-isopropyl ether–acetic acid, with detection by aqueous permanganate spray, gave two spots corresponding to the known acid hydrolysis products of gibberellic acid; allogibberic acid[17] (R_f 0·48) and gibberic acid[18]† (R_f 0·70). The acid hy-

† Although it lacks olefinic unsaturation, gibberic acid has a benzylic methylene group readily susceptible to oxidation.

[16] J. S. MOFFATT, *J. Chem. Soc.* 3045 (1960). [17] T. P. C. MULHOLLAND, *J. Chem. Soc.* 2693 (1958).
[18] B. E. CROSS, J. F. GROVE, J. MACMILLAN and T. P. C. MULHOLLAND, *J. Chem. Soc.* 2520 (1958).

drolysis products of the other known gibberellins have mobilities relative to gibberic acid in this system as follows: Gibberellin A_5, 0·5; A_6, 1·0, 1·27; A_8, 0·05; A_7, 0·57. The hydrolysis products of the remaining gibberellins are not revealed by aqueous permanganate.

Final confirmation of the identity of the gibberellin as gibberellic acid was given by the activation–emission spectrum determined after heating in ethanolic sulphuric acid at 50° for 25 min. The spectrum reproduced in Fig. 1 is identical to that of gibberellic acid, showing an activation peak at 418 mμ and an emission peak at 463 mμ. Gibberellins A_1, A_5, A_6 and A_8 do not fluorescence under these conditions; gibberellins A_2, A_4 and A_9 all show activation peaks at 390 mμ and 440 mμ and emission peaks at 420 mμ and 468 mμ,and gibberellin A_7 shows activation peaks at 390 mμ and 455 mμ and emission peaks at 420 mμ and 473 mμ.[19]

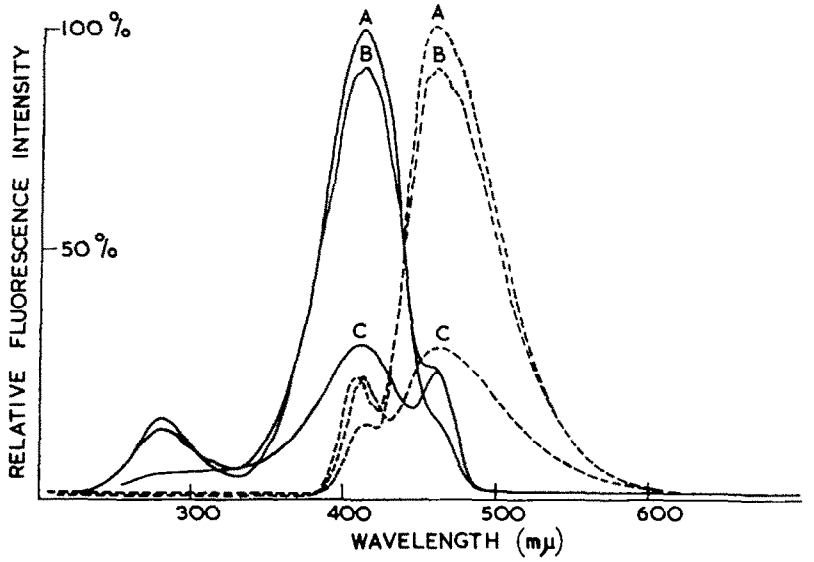

FIG. 1. FLUORESCENCE SPECTRA IN 50% ETHANOLIC SULPHURIC ACID (UNCORRECTED).

Full line, fluorescence activation; broken line, fluorescence emission. A, gibberellic acid (1 mg); B, barley gibberellin; C, blank eluate from chromatogram.

Schander,[20] Kirsop and Pollock,[21] and Yomo[22] have reported evidence indicating that during germination of a barley grain a substance is secreted from the embryo to the endosperm where it is implicated in the release of hydrolytic enzymes. Recently, Paleg[23] and MacLeod and Millar[24] have shown that modification of barley endosperm, essentially similar to that in normal germination, can be induced in the absence of the embryo by exogenous gibberellic acid and these authors have independently suggested that the substance secreted from embryo to endosperm in normal germination is a gibberellin. The demonstration of the presence of gibberellic acid in immature barley lends credence to this view and supports the

[19] G. W. Elson, personal communication.
[20] H. Scharder, *Z. Botan.* **27**, 433 (1934).
[21] B. H. KIRSOP and J. R. A. POLLOCK, *J. Inst. Brewing* **64**, 227 (1958).
[22] H. YOMO, *Hakkô Kyôkaishi* **18**, 603 (1960) and preceding papers.
[23] L. PALEG, *Plant Physiol.* **37**, 798 (1961) and preceding papers.

suggestion[24] that gibberellic acid added to malting barley merely enhances the effects of the endogenous component.

Concurrent with the studies on barley an investigation of the endogenous gibberellins in the leaves and immature seed of grass was carried out. Five species (*Lolium perenne, Dactylis glomerata, Phleum pratense, Festuca pratensis, Poa pratensis*) were harvested at a number of stages of development between the seedling stage and the formation of seedheads. They were extracted with ethanol, chromatographed on charcoal and then on paper. Bioassay on peas indicated the presence in all cases of a single gibberellin with an R_f value similar to that of gibberellic acid. Extracts of vegetative tissue contained 0·2–2·9 μg gibberellic acid equivalent per kg fresh weight of tissue. The least activity was found in *P. pratense*. Seedheads of *L. perenne*, *D. glomerata* and *F. pratensis* harvested a few days after anthesis contained considerably more active material, 15–90 μg gibberellic acid equivalent per kg. An extract from *F. pratensis* seeds, containing 24 μg of gibberellic acid equivalent, was examined by thin layer chromatography. Repetition of all the experiments described above positively identified the gibberellin as gibberellic acid. These results suggest that gibberellic acid may be of widespread occurrence in the Gramineae.

EXPERIMENTAL

Bioassay

Three systems were used (a) dwarf pea test,[12] (b) lettuce seedling test,[15] (c) cucumber seedling test.[14]

Thin layer chromatography was carried out on layers (0·275 μ thick) of Silica gel G and Kieselguhr gel G (E. Merck, A. G., Darmstadt) prepared by the method of Stahl.[25] Detection of gibberellins was effected by spraying with ethanolic sulphuric acid (95:5), heating at 120° and viewing under ultraviolet light.[8] (Hanovia Model 11.)

Paper Chromatography of Extracts of Grass and Barley

Batches of leaves of grass and of immature ears of grass (*Phleum pratense* L., *Lolium perenne* L., *Dactylis glomerata* L. and *Festuca pratensis* Huds. (approx. 1 kg) and immature ears of barley (*Hordeum vulgare* L. var. Proctor) (approx. 1 kg) were macerated in ethanol and the extract processed by the published procedure[26] in which the acidic fraction was chromatographed on a celite–charcoal column. Eluates from the column were chromatographed on paper (Whatman No. 1) using benzene–acetic acid–water (4:2:1). Eluates of strips of the paper were bioassayed on test (a) and parts of the active fractions were rechromatographed on paper in the same solvent system using the overflow elution technique described by Bird and Pugh.[13] The chromatograms were again bioassayed. In all the materials examined the activity was found in a region having an R_f similar to that of gibberellic acid.

An extract of *Festuca pratensis* purified by the above procedure and containing 24 μg gibberellic acid equivalent is referred to below as *Festuca concentrate.*

Preparation and Partial Purification of Acidic Extract of Barley

Ears of barley (var. Proctor) (50 kg fresh wt), harvested 10 days after anthesis, were processed by the procedure described by MacMillan *et al.*[1] except that 50% aqueous $NaHCO_3$

[24] A. M. MacLeod and A. S. Millar, *J. Inst. Brewing* **68**, 322 (1962).
[25] F. Stahl, *Chemiker Z.* **82**, 323 (1958).
[26] M. Radley, *Ann. Bot.* **27**, 373 (1963).

was substituted for the phosphate buffer. The acidic portion of the extract was triturated with basic lead acetate, filtered and the soluble acids recovered by acidification and extraction with ethyl acetate which yielded a gummy mixture of acids (3·4 g). Bioassay of this crude extract on test (a) indicated 150 μg gibberellic acid equivalent. The crude extract was further purified by chromatography on celite–charcoal as described by MacMillan *et al.*[1]

The fractions obtained from the charcoal–celite column were bioassayed on test (a). The active fraction (250 mg), eluted with 40–45% acetone in water, was further fractionated by chromatography on celite–silicic acid as described previously.[2] Bioassay on test (a) revealed that the activity (approx. 150 μg gibberellic acid equivalent) was concentrated in the fraction (14 mg) eluted with pure ethyl acetate. This fraction is referred to below as the "barley concentrate".

Chromatography of a portion of the barley concentrate on Whatman No. 1 paper in isopropanol–water (4:1) by the ascending technique and detection by bioassay on test (a) revealed activity in a zone having R_f 0·58 to 0·70. Under identical conditions gibberellins A_3, A_6, A_1, A_7 and A_9 had R_f values of 0·70, 0·70, 0·75, 0·90 and 0·95 respectively.

Thin Layer Purification and Preliminary Identification of the Gibberellins

The following materials, in acetone solution, were spotted from left to right at 1·5 cm intervals in a line 2 cm from one edge of a plate (20 × 20 cm) of silica G: Four portions, each of 100 μg, of barley concentrate; barley concentrate (100 μg) admixed with gibberellic acid (2 μg); barley concentrate (100 μg) admixed with gibberellin A_7 (2 μg); gibberellic acid (2 μg) and gibberellin A_7 (2 μg). The plate was developed in ethyl acetate for 18 cm with the solvent front running from left to right perpendicular to the line of spots. After drying in air (15°, ½ hr) the plate was developed in water for 14 cm in a direction perpendicular to the previous run. After drying (15°, 3 hr) narrow zones (3 mm) were scraped from the plate in the region corresponding to three of the four barley spots of barley concentrate and the scrapings eluted with acetone. Bioassay on tests (a) and (b) of these eluates revealed that activity was concentrated in the zone having R_f 0·88 to 0·90. The behaviour of the active material in the two assay systems was consistent with that of gibberellic acid rather than the other known gibberellins.[27] The active material eluted from this zone of the chromatogram is referred to below as the "purified gibberellin".

The remaining portion of the chromatogram was sprayed with ethanolic sulphuric acid and heated at 120°. After 1 min the following fluorescent spots were visible in ultraviolet light: a green–blue spot, due to barley concentrate, at R_f 0·89; a similar spot due to gibberellic acid at the same R_f value; a single green–blue spot, due to barley concentrate admixed with gibberellic acid, at R_f 0·89; and a green–blue spot, R_f 0·89, and a yellow spot R_f 0·79, due to barley concentrate admixed with gibberellin A_7. In addition, the barley concentrate gave a broad band (R_f 0·5 to 0·73) having a grey–pink fluorescence. On further heating (1 hr.) no additional fluorescence appeared but the spots due to barley concentrate and gibberellic acid became blue. The behaviour of the other known gibberellins in the system is presented in the Table 1.

A similar experiment performed with *Festuca* concentrate gave the following result: Activity on tests (a) and (b) in the region R 0·88 to 0·86 and green-blue fluorescent spots due to *Festuca* concentrate, *Festuca* concentrate admixed with gibberellic acid, and gibberellic acid all at R_f 0·87 and all revealed by heating for 1 min.

[27] P. W. BRIAN, H. G. HEMMING and D. LOWE, *Nature* **193**, 946 (1962).

Further Characterization of the Purified Gibberellins

(a) *Adsorption Chromatography*

Two spots, each of 1 μg, of the purified gibberellin of barley and a comparison spot of gibberellic acid (2 μg) were chromatographed on silica G in di-isopropyl ether–acetic acid (95:5)[8] and, after drying at room temperature (3 hr), narrow zones of the chromatogram corresponding to one spot of the gibberellin were removed, eluted with acetone and the eluates bioassayed on test (a). Activity was concentrated at R_f 0·093–0·095. Detection of the remaining portion of the chromatogram in the usual way revealed a green–blue spot due to the gibberellin at R_f 0·093 and a similar spot due to gibberellic acid at the same R_f value.

A similar experiment performed with the purified gibberellin of *Festuca* gave a chromatogram showing activity on test (a) in the region R_f 0·092–0·095, a green–blue spot due to the gibberellin at R_f 0·093 and a similar spot due to gibberellic acid at the same R_f value.

(b) *Partition Chromatography*

Two spots, each of 1 μg of the purified gibberellin of barley and a comparison mixture of gibberellic acid (2 μg) and gibberellin A_1 (2 μg) were chromatographed on Kieselguhr G in benzene–acetic acid–water (8:3:5) as described by MacMillan and Suter[8] and the chromatogram dried at room temperature (3 hr) and examined by bioassay and sulphuric acid spray as described above. The gibberellin gave a zone active on test (a) at R_f 0·086 to 0·088 and a green–blue fluorescent spot at R_f 0·087 revealed during heating for 1 min; gibberellic acid gave a similar spot at the same R_f value. Gibberellin A_1 gave a blue spot at R_f 0·13 after heating for 10 min.

A similar experiment performed with the gibberellin from *Festuca* gave a chromatogram showing activity on test (a) due to the gibberellin at R_f 0·072–0·075, a green–blue spot due to the gibberellin at R_f 0·073, a similar spot due to gibberellic acid at the same R_f value and a blue spot due to gibberellin A_1 at R_f 0·11.

(c) *Chromatography of Derivatives*

(i) *Methyl ester.* The purified gibberellin from barley (2 μg) was treated in methanol solution with excess ethereal diazomethane and the product and a comparison spot of methyl gibberellate (2 μg) were chromatographed on silica G in di-isopropyl ether–acetic acid (95:5).[8] Detection in the usual way revealed a green–blue spot due to the methyl gibberellin at R_f 0·33 and a similar spot due to methyl gibberellate at the same R_f value.

Chromatography of the methyl gibberellin and a comparison mixture of methyl gibberellate (2 μg) and gibberellin A_1 methyl ester (2 μg) on silica G in benzene–acetic acid–water (8:3:5)[8] and detection gave a green–blue spot due to the methyl gibberellin at R_f 0·25 and a similar spot due to methyl gibberellate at the same R_f value. Both spots were revealed on heating for 1 min. Further heating (10 min) revealed gibberellin A_1 methyl ester as a blue spot having R_f 0·32.

Similar experiments performed on the methyl ester of the gibberellin from *Festuca* yielded the following results: (a) in di-isopropyl ether–acetic acid; a green–blue spot due to the methyl gibberellin at R_f 0·3 and a similar spot due to methyl gibberellate at the same R_f value, (b) in benzene–acetic acid–water; green–blue spots due to the methyl gibberellin and methyl gibberellate both at R_f 0·26 and a blue spot due to gibberellin A_1 methyl ester at R_f 0·35.

(ii) *Acetyl derivatives of ester.* The methyl ester (2 μg) of the gibberellin from barley was heated in acetic anhydride (0·2 ml) during $\frac{1}{2}$ hr, the acetic anhydride removed by distillation

under reduced pressure and the product and a comparison mixture of 2-*o*-acetyl methyl gibberellate and 2,7-di-*o*-acetyl gibberellate chromatographed on silica in di-isopropyl ether–acetic acid (95:5). Detection yielded a green–blue spot due to the acetyl derivative of the methyl gibberellin at R_f 0·57 and similar spots due to mono-acetyl methyl gibberellate and di-acetyl methyl gibberellate at R_f values 0·30 and 0·57 respectively.

A similar experiment performed with methyl ester of the gibberellin of *Festuca* gave green–blue spots at R_f 0·30 and 0·57.

Acid Hydrolysis

The purified gibberellin of barley (5 μg) was heated in 0·2 ml 0·1 N-hydrochloric acid during 0·5 hr. The products were isolated in ethyl acetate (5 × 0·5 ml) and chromatographed together with a comparison mixture of gibberellic acid, *allo*gibberic acid and gibberic acid, on silica G in di-isopropyl ether-acetic acid (95:5). Detection with 0·5% aqueous potassium permanganate spray revealed brown spots due to the hydrolysis products at R_f 0·09, 0·48 and 0·70 and brown spots due to gibberellic acid, *allo*gibberic acid and gibberic acid at R_f 0·09, 0·48 and 0·70 respectively.

A similar experiment performed with the gibberellin of *Festuca* gave an identical result.

Activation—Emission Spectra

Determined by G. W. Elson. A solution of the purified gibberellin (1 μg) in 50% ethanolic sulphuric acid was heated at 50° during 25 min and the activation-emission spectrum determined on an Aminco–Bowman spectrophotofluorometer. The spectra obtained from the gibberellin of barley and the gibberellin of *Festuca* were both identical to that of gibberellic acid showing activation peaks at 418 mμ and emission peaks at 463 mμ (see Fig. 1).

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