GIBBERELLINS

A collection of papers comprising the Symposium on Gibberellins, presented before the Division of Agricultural and Food Chemistry at the 138th National Meeting of the American Chemical Society, New York, N.Y., September 1960.

Number 28

ADVANCES IN CHEMISTRY SERIES

American Chemical Society

Washington, D. C.

1961

Library of Congress Catalog Card No. 61-11135

PRINTED IN THE UNITED STATES OF AMERICA

ADVANCES IN CHEMISTRY SERIES

Robert F. Gould, Editor

AMERICAN CHEMICAL SOCIETY APPLIED PUBLICATIONS

The Structures of the Fungal Gibberellins

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Investigations in Japan in the 1920's of the bakanae disease of rice, caused by the fungus *Gibberella fujikuroi*, led in 1939 to the isolation of crude gibberellin, a crystalline material with plant growth-promoting properties. As a result of intensive research in the past five years, the fungal gibberellins now comprise a group of six chemically closely related metabolites of *Gibberella fujikuroi*. The structure of gibberellic acid, $C_{19}H_{22}O_6$ the most readily available of the gibberellins, has been elucidated and studies of its biosynthesis have shown that it can be classed as a diterpenoid. The other fungal gibberellins—A_1, A_2, A_4, A_7, and A_9—have been chemically related to gibberellic acid. Evidence for the structures of the gibberellins is reviewed and the stereochemistry of gibberellic acid is discussed. The study of the fungal gibberellins has led to the discovery that gibberellins occur in higher plants.

In September 1957, the American Chemical Society held its first Gibberellin Symposium. At that time only four gibberellins were known, all metabolites of the fungus *Gibberella fujikuroi*.

Today *nine* gibberellins have been isolated and fully characterized. Five have only been isolated from the fungus, three from a higher plant, and one from both fungus and higher plants.

The five new gibberellins which have been isolated recently in our laboratories have been named gibberellins A_5 to A_9 as they were isolated and shown to be structurally related to the known gibberellins. No distinction has been made between fungal gibberellins and those of higher plants.

This paper presents a brief review [for a more comprehensive review see (*3*)] of the chemistry of the fungal gibberellins, gibberellic acid $C_{19}H_{22}O_6$ (*14*), gibberellin A_1 $C_{19}H_{24}O_6$ (*31*), gibberellin A_2 $C_{19}H_{26}O_6$ (*34*), gibberellin A_4 $C_{19}H_{24}O_5$ (*38*), gibberellin A_7 $C_{19}H_{22}O_5$ (*6*), and gibberellin A_9 $C_{19}H_{24}O_4$ (*7*).

Gibberellic acid is obtained most readily pure and in quantity and knowledge of the chemistry of the gibberellins comes largely from study of the chemistry of

gibberellic acid. I would like therefore to summarize the evidence for our structure of gibberellic acid and deal, at the same time, with gibberellin A_1. We can then consider the other fungal gibberellins and their relationship to gibberellic acid. Finally, I would like to discuss the stereochemistry of gibberellic acid.

Structure of Gibberellic Acid

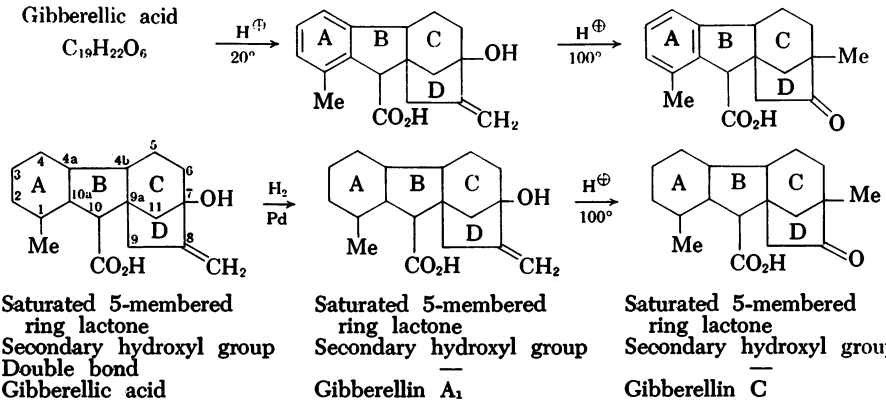


Figure 1. Gibberellic acid and derivatives

Gibberellic acid, $C_{19}H_{22}O_6$ (I) is a monobasic acid of p*K* 4.0. With dilute mineral acid at 20° C. (*4*) it gives allogibberic acid (II) which with acid at 100° C. yields gibberic acid (III). Structures of these two important degradation products have been conclusively established by us. The evidence has been published in full (*11, 26*) and it is not dealt with here.

Gibberellic acid is tetracarbocyclic and the ready transformation to allogibberic acid suggests that it possesses the same carbocyclic structure as allogibberic acid and differs only in ring A which can be readily aromatized. This view is supported by many facts (*5, 10*), only one of which is mentioned here.

Controlled reduction (*17*) of gibberellic acid (as its methyl ester) with palladium on charcoal reduces one double bond—not the terminal methylene double bond, for in the product, gibberellin A_1 methyl ester can be ozonized (*27*) to formaldehyde and an α-ketol (Figure 1). Reduction of this nonterminal methylene double bond blocks the ready aromatization to ring A of allogibberic acid, yet the ring C/D chemistry of gibberellin A_1 parallels (*5*) that of allogibberic acid (*26*). To take only one example, Takahashi and his colleagues (*34, 35*) have shown that gibberellin A_1 undergoes acid-catalyzed rearrangement exactly analogous to the rearrangement of allogibberic acid. The product, a saturated 5-membered ring ketol (Figure 1), was named gibberellin C by the Japanese workers. This ring C/D rearrangement is important diagnostically as it does not occur when the tertiary hydroxyl group is absent and it has been used for diagnosing the C/D ring structure in gibberellins of higher plants.

The carboxyl group is in the same position in both gibberic acid and allogibberic acid, since the methyl esters of each give the methyl ester of gibberic acid on boiling with mineral acid (*5, 21*).

Ring A of Gibberellic Acid. Gibberellic acid possesses a C-methyl group so that ring A must be 6-membered. The methyl group is placed at position 1 (see Figure 1 for numbering) because it appears there in the acid hydrolysis

products and also in dehydrogenation products. Ring A must also accommodate a secondary hydroxyl, one olefinic double bond, and a 5-membered ring lactone group. Each of these groupings is considered in turn.

Secondary Hydroxyl Group. It has been shown to be at position 2 both by Seta and coworkers (*28*) and by Cross (*5*). Although, the Japanese workers were the first to demonstrate this, the method of Cross is shown in Figure 2, since it is the simpler.

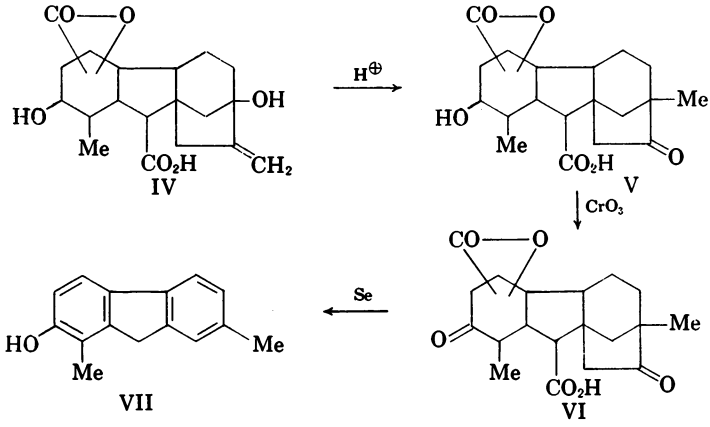


Figure 2. Determination of the position of the hydroxyl group

Rearrangement of gibberellin A_1 (IV) to the ketone (V) and oxidation of the latter gave a diketone (VI) which, on dehydrogenation gave 1,7-dimethyl-fluoren-2-ol (VII) whose structure was established by synthesis (*13*).

Olefinic Double Bond. The position of the double bond in ring A is uniquely defined as follows (Figure 3).

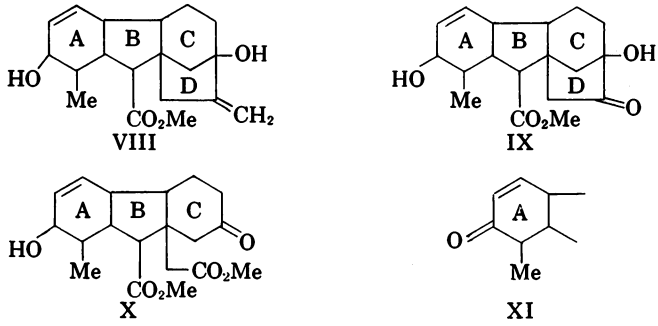


Figure 3. Determination of the position of the double bond; all structures less ring A lactone

Methyl gibberellate (VIII), the corresponding ketol (IX), and the keto acid methyl ester (X) are oxidized to α,β-unsaturated ketones (XI) (*5*). The hydroxyl group is therefore allylic to the ring A double bond. Moreover the λ_{max} (228 to 229 mμ) of these α,β-unsaturated ketones is such that the double bond must be disubstituted. A disubstituted double bond, allylic to C-2, can only be placed as shown in structure (VIII).

γ-Lactone. It only remains to locate the position of the γ-lactone.

One end of the lactone ring must be at position 1. This is apparent from the nuclear magnetic spectra of gibberellic acid and its derivatives (*30*).

In methyl gibberellate, methyl acetyl gibberellate, gibberellin A_1 methyl ester, and methyl benzoyl gibberellate there is a peak at $\sigma = 4.2$. This peak is sharp, has the same intensity as the methyl of the ester grouping in methyl benzoyl gibberellate and is typical of a methyl attached to a carbon atom which does not carry a proton. Therefore, one end of the lactone bridge must be attached to the carbon carrying the methyl group.

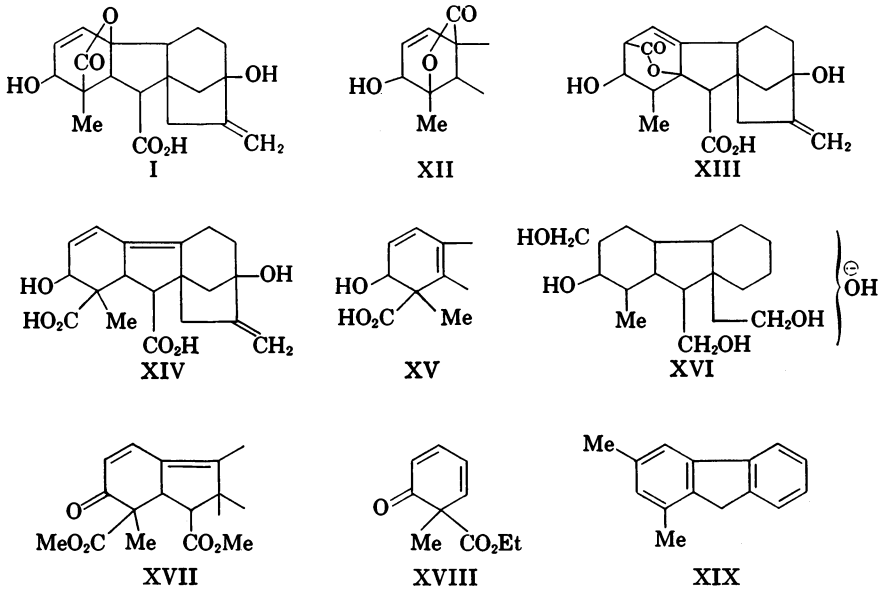


Figure 4. Determination of the position of the γ-lactone

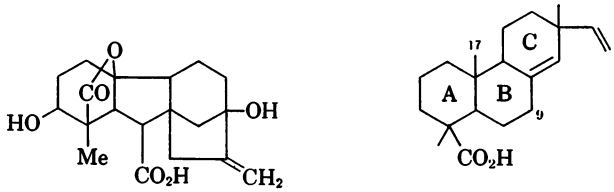
With regard to the other end of the lactone bridge, hydrogenation of gibberellic acid and methyl gibberellate is accompanied by hydrogenolysis. This suggests that the hydroxyl function of the lactone is allylic to the double bond, defining the structure of gibberellic acid as (I) (Figure 4).

Structure (XII) with the attachment of the lactone bridge reversed can be ruled out for the following reason. Gibberellic acid decomposes slowly in aqueous solution to give gibberellenic acid first described by Gerzon, Bird, and Woolf (*16*) who suggested the homoannular diene structure (XV). We consider that the ultraviolet absorption (λ_{max} 253 mμ; ε 22,400) is more in accord with the heteroannular structure (XIV) and this is confirmed (*25*) by ultraviolet absorption (λ_{max} 309 mμ; ε 16,500) of the derived dienone (XVII) which is decisive [cf. the ultraviolet absorption (λ_{max} 310 mμ; ε 3900) of model cyclohexadienone (XVIII)].

Takahashi and coworkers (*37*) have suggested structure (XIII) for gibberellic acid. This structure is based mainly on results (*29*) of dehydrogenation of a pentol (XVI)—the Japanese formulation—derived from gibberellin A_1. This gave a fluorene m.p. 94° to 97° C., said to be identical with a synthetic sample of 1,3-dimethylfluorene (XIX) m.p. 99° to 100° C. Now we and others have found that 1,3-dimethylfluorene has a melting point of 87° C. In an exchange of letters, Professor Sumiki (*33a*) says that the published melting points for their fluorene are wrong and should be the generally accepted figure for 1,3-dimethylfluo-

rene. Nevertheless, structure (XIII) proposed by the Japanese workers does not explain the ring A chemistry of gibberellic acid. It does not fit the following:

1. The infrared spectra of gibberellic acid and derivatives which all show absorption at ca. 1780 cm.$^{-1}$ in solution, too high a frequency for the δ-lactone of the Japanese structure.
2. The nuclear magnetic resonance spectra.
3. Manganese dioxide oxidation to an α,β-unsaturated ketone.
4. The formation of gibberellenic acid.



XX XXI

Figure 5. Gibberellin A_1 and its diterpene precursor

In our view, structure (I) (Figure 4) represents gibberellic acid. Gibberellin A_1 is then the ring A dihydro derivative (XX) (*17*, *36*). These structures, unlike the Japanese structure, can be regarded as diterpenoid, and derived from a tricyclic diterpene of type (XXI), from which rings C/D arise by the rearrangement proposed by Wenkert (*39*) and ring B by contraction and expulsion of the sixth carbon atom as a carboxyl group (Figure 5). The correctness of these views was established by Birch and coworkers (*1*, *2*) by degradation of gibberellic acid derived from 2-C^{14}-acetate and 2-C^{14}-mevalonic lactone. Loss of the 17-methyl groups of the diterpenoid precursor may occur in situ or after migration. Such loss may or may not be synchronous with lactone formation.

The remaining fungal gibberellins, A_2, A_4, A_7, and A_9 have all been interrelated as shown in Figure 6.

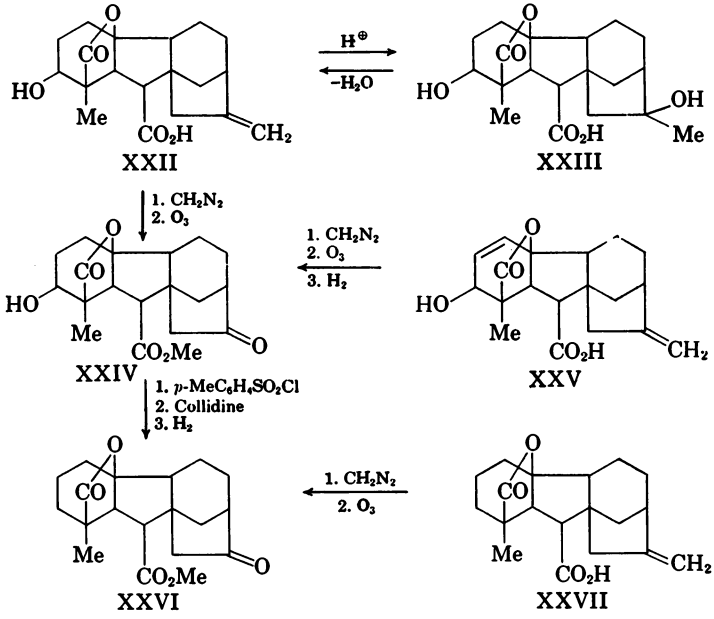


Figure 6. Structural relations among fungal gibberellins A_2, A_4, A_7, and A_9

Gibberellin A_4 (XXII) has one hydroxyl group less than gibberellin A_1; the methyl ester is ozonized (*19*, *38*) to formaldehyde and a norketone (XXIV) which is not an α-ketol, so that gibberellin A_4 does not have the C/D bridgehead hydroxyl group. Gibberellin A_2 (XXIII) is gibberellin A_4 with the terminal methylene group hydrated. We have shown that gibberellin A_4, treated with acid, gives gibberellin A_2 and Kitamura and coworkers (*23*) have shown that dehydration of gibberellin A_2 with phosphorus oxychloride after protection of the secondary hydroxyl group by acetylation, gives acetyl gibberellin A_4.

Gibberellin A_7 (XXV) has been shown by Cross, Galt, and Hanson (*6*) to have the same ring A chemistry as gibberellic acid; for example, ring A is readily aromatized with acid. The methyl ester is ozonized to a norketone which is not an α-ketol. This norketone is reduced to the A_4 norketone (XXIV), the reduction being accompanied by much hydrogenolysis. Gibberellin A_9 (XXVII) is the simplest of the known gibberellins. It has no hydroxyl groups, but has a terminal methylene group. Methylation followed by ozonolysis gives a norketone (XXVI) which is also obtained from the gibberellin A_4 norketone methyl ester (XXIV) by formation of the tosyl derivative, elimination with collidine, and finally reduction (*7*).

This group of gibberellins which do not have the C/D hydroxyl group has been related to gibberellin A_1. Kitamura and others (*22*) report that treatment of the diacetate (XXVIII; R = OAc) of the norketone, derived from gibberellin A_1 methyl ester with zinc and acetic anhydride gives the acetate (XXVIII; R = H) of the methyl ester, of gibberellin A_4 norketone. We have not been able to effect this hydrogenolysis, but Cross, Galt, and Hanson (*7*) have converted the methyl ester (XXVIII; R = H) derived from gibberellin A_4 methyl ester (Figure 7) into the lactone (XXIX) by Baeyer-Villiger oxidation. This lactone has also been prepared from gibberellin A_1 methyl ester (XXX) via the diester (XXXI).

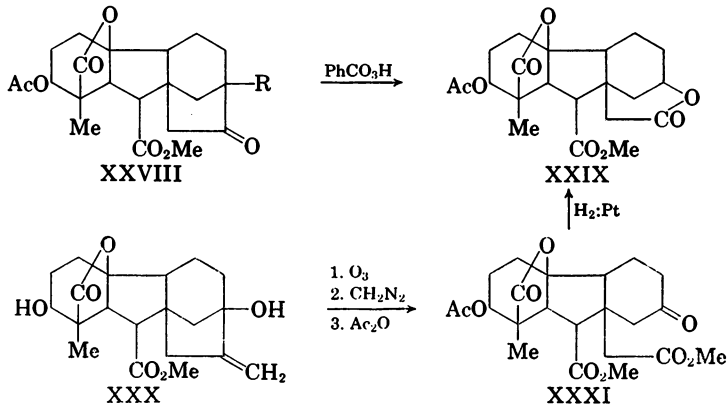


Figure 7. Derivatives from gibberellins A_1 and A_4

Before discussing the stereochemistry of the gibberellins there are two rearrangements (*12*) I would like to mention.

First is the epimerization of the ring A hydroxyl group.

Gibberellins which have a saturated ring A undergo epimerization at C-2 with dilute alkali at room temperature, presumably by a reversed aldol mechanism via intermediate (XXXIII) as first suggested by J. W. Cornforth (*3a*). This epimerization has been shown to occur with gibberellin A_1 and its methyl ester (XXXIV), with both C-8 epimeric tetrahydro derivatives of gibberellic acid and

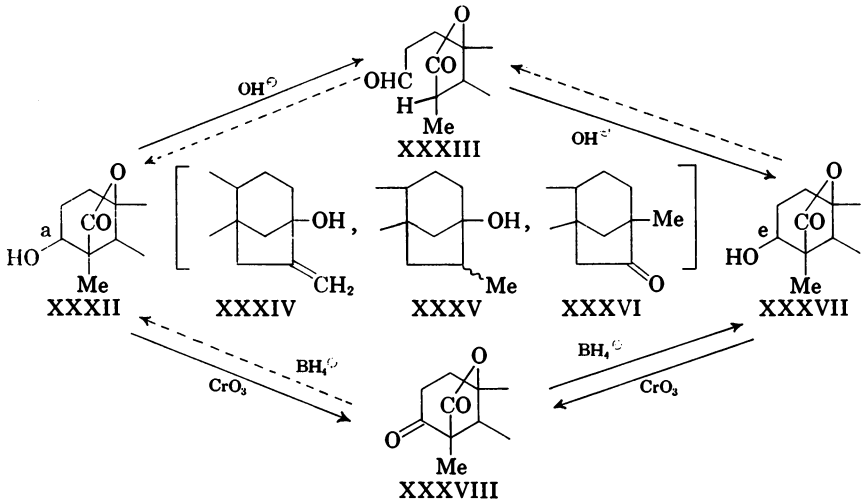


Figure 8. Epimerization of the ring A hydroxyl group

methyl esters (XXXV), and with ring C/D rearrangement products (XXXVI). There is an equilibrium well on the side of the epimer (XXXVII) which presumably has the equatorial 2-hydroxyl group. Each pair of epimers is oxidized to the same ketone (XXXVIII) which upon hydride reduction gives the equatorial epimer (XXXVII) in over 60% yield with less than 10% of the axial epimer (XXXII) (Figure 8).

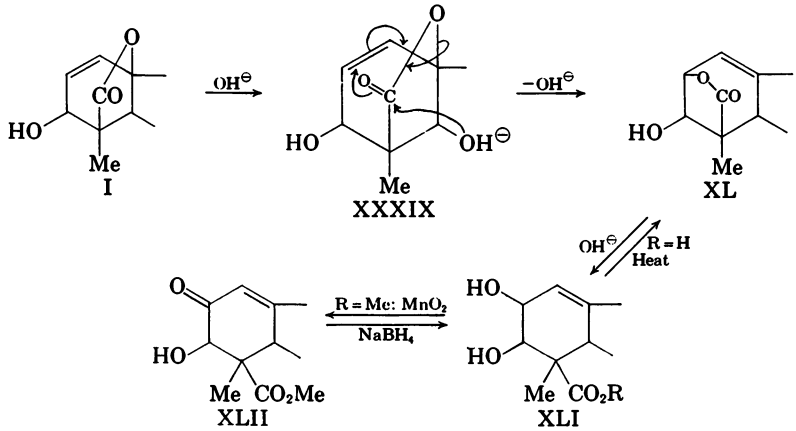


Figure 9. Alkali-induced changes in gibberellic acid

Secondly, this epimerization does not occur in gibberellic acid (I). Instead (Figure 9) allylic rearrangement of the lactone occurs giving the 1 → 3 lactone system (XL) possibly by the mechanism indicated (XXXIX). This occurs with both gibberellic acid and the methyl ester. Prolonged treatment with alkali or use of stronger alkali opens the 1,3-lactone to give (XLI; R = H) which can be relactonized to (XL). In view of our experience with saturated ring A derivatives, we interpreted at first this alkali-induced change simply as a C-2 epimerization, and thought that methyl gibberellate and the ester m.p. 174° C. (XL) obtained from methyl gibberellate with dilute alkali were simply C-2 epimers. We therefore proposed structure (XL, with 2-axial–OH) for ring A of gibberellic acid.

This view was abandoned (9) when it was discovered that methyl gibberellate was oxidized to an α,β-unsaturated ketone with manganese dioxide.

The structure of the methyl ester m.p. 174° C. (XL) was proven (5) mainly by the fact that the corresponding dibasic acid (XLI; R = H) and its dimethyl ester (XLI; R = Me) are oxidized with periodate and that the dimethyl ester is oxidized by manganese dioxide to a trisubstituted α,β-unsaturated ketone (XLII) which regenerates (XLII; R = Me) with sodium borohydride.

Epimerization of the 2-hydroxyl does not occur here, since methyl gibberellate and the methyl ester m.p. 174° C. give the same hydrogenolysis acid (see below).

Stereochemistry

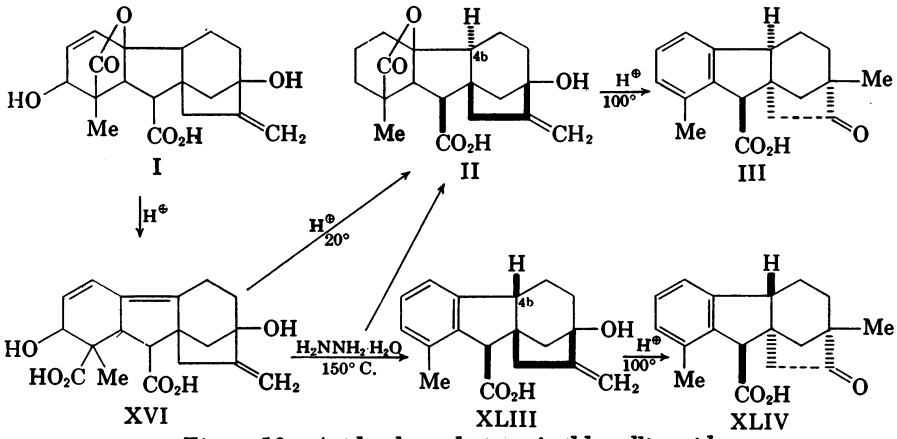


Figure 10. Acidic degradation of gibberellic acid

The acidic degradation of gibberellic acid (I) is shown in detail (Figure 10). First formed is gibberellenic acid (XVI) which with acid at 20° C. gives allogibberic acid (II) which in turn gives gibberic acid (III). With hydrazine hydrate at 150° C., gibberellenic acid gives, in addition to allogibberic acid, an epimer, epiallogibberic acid (XLIII) which gives epigibberic acid (XLIV) upon treatment with acid at 100° C.

I would now like to present evidence that each of these epimeric pairs (II and XLIII) and (III and XLIV) are C-4*b* epimers, with the absolute configuration as shown in Figure 10.

Allo- and Epiallogibberic Acids (*20*). Keto acid (XLV), derived by ozonolysis of methyl allogibberate, when treated with aqueous alkali gives a little of the corresponding acid (XLVI) (Figure 11). The main product is dibasic acid (XLVII) where the 9-carboxyl group is epimeric. Both dibasic acids give the same anhydride (XLVIII), but hydrolysis gives only dibasic acid (XLVI) derived directly from allogibberic acid. Allogibberic acid must therefore have the carboxyl group and ring D *cis*. So must epiallogibberic acid, since it undergoes an analogous series of reactions.

The methyl esters of keto acids (XLVI) and (XLVII) from both allo and epiallogibberic acids show positive Cotton effects so that the acetic acid side chain at C-8*a* must be β-oriented. This means that the carboxyl group and ring D must be β-oriented in allo- and epiallogibberic acids.

Allo- and epiallogibberic acids are epimeric at position 4*b*. This is shown in the following way.

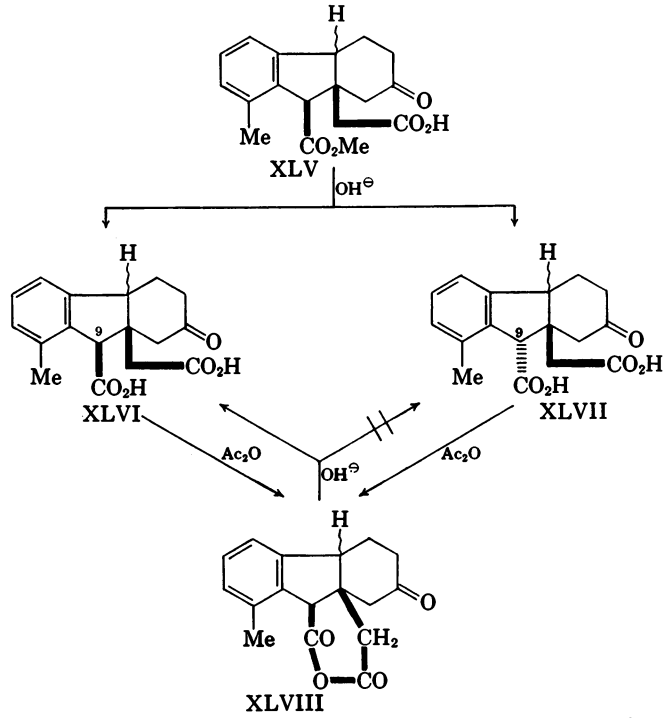


Figure 11. Derivatives of allo- and epiallogibberic acids

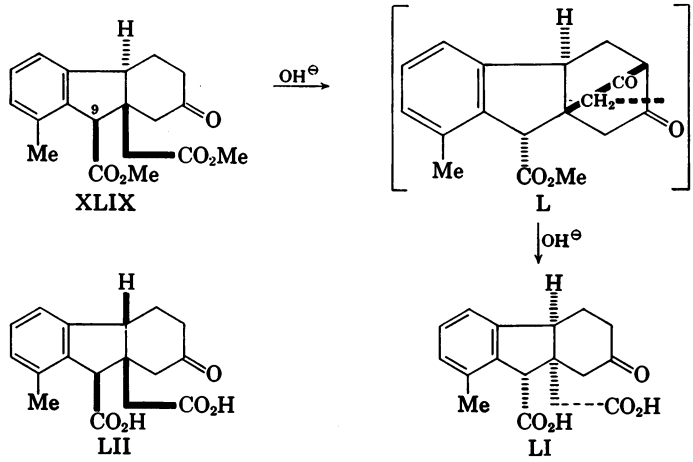


Figure 12. Evidence to show that allogibberic and epiallogibberic acids are epimers at C-4b

When the dimethyl ester (XLIX) (Figure 12) of the dibasic acid, obtained by opening ring D of allogibberic acid, is treated with alkali not only does the C-9 center epimerize, but the C-8*a* center is also inverted. This is shown by the fact that the dimethyl ester of the product (LI) shows a negative Cotton effect in contrast to the starting keto ester (XLIX). Epimerization at C-8*a* presumably takes place via the 1,3-diketone (L) followed by fission as shown by the dotted line. After having epimerized at C-8*a* and C-9, the dibasic acid (LI) and its

dimethyl ester and anhydride are mirror images of the dibasic acid (LII), its dimethyl ester and anhydride which are derived directly by oxidation of epiallogibberic acid without epimerization. This clearly shows that allogibberic and epiallogibberic acids are epimers at C-4*b*.

That allogibberic acid has rings B/C *trans* fused can be deduced as follows.

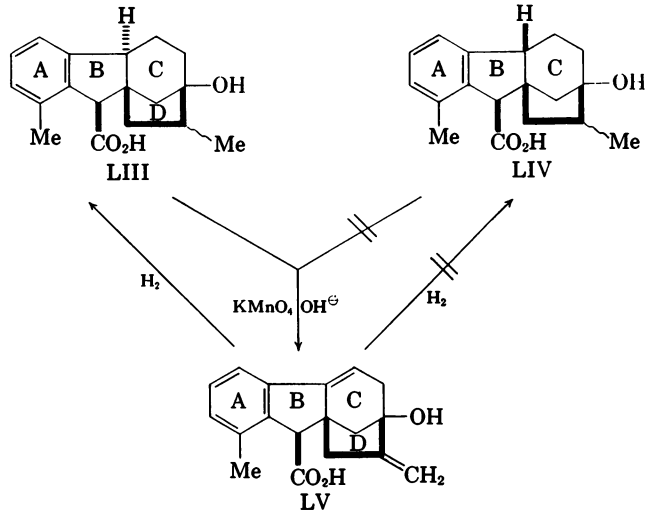


Figure 13. B/C ring fusion in allogibberic acid

Dihydroallogibberic acid (LIII) (Figure 13) is oxidized to the dehydro derivative (LV). Dihydroepiallogibberic acid (LIV) is not. Reduction of the dehydro compound (LV) under a variety of catalytic conditions gives only dihydroallogibberic acid (LIII) and no dihydroepiallogibberic acid (LIV). Since reduction would be expected to occur from the less hindered α-side, allogibberic acid has rings B/C *trans* fused and epiallogibberic acid is the *cis*-B/C compound (20).

Similar arguments for this absolute configuration (II; Figure 10) of allogibberic acid have been adduced by Stork and Newman (32).

It follows that epiallogibberic acid has the absolute configuration (XLIII, Figure 10).

Gibberic Acid (19)

Gibberic acid is obtained from allogibberic acid with mineral acid at 100° C. We first suggested (*10*, *26*) a Wagner-Meerwein mechanism for this change which would mean a change in configuration (LVII) of ring D in gibberic acid. Birch and coworkers (*1*, *2*) suggested a pinacol-pinacolone mechanism which means in effect migration of the methyl produced by hydration of the terminal methylene group (Figure 14). This mechanism would involve no change in configuration (LVI) of ring D; Birch produced labelling evidence to support his view. It has now been established (*19*) that the Wagner-Meerwein mechanism is the operative one. This conclusion is supported by O.R.D. evidence. Gibberic acid and epigibberic acid show a negative Cotton effect, antipodal to those of the norketones (LVIII, R = OH and R = H) derived from allogibberic acid and gibberellin A_4 respectively.

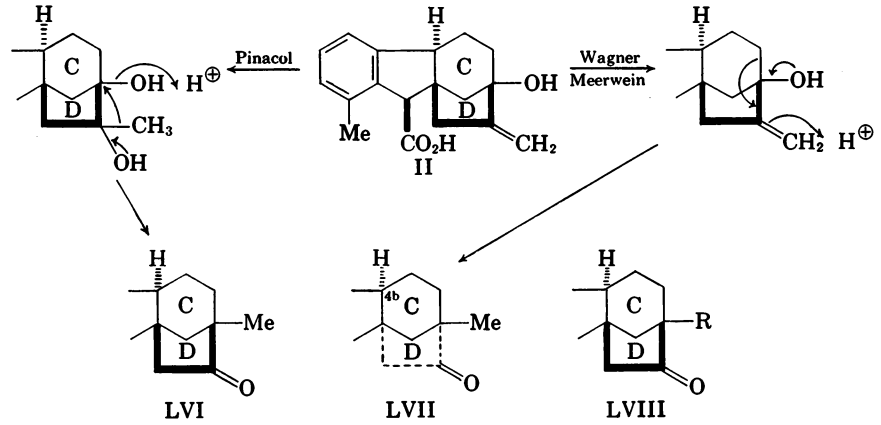


Figure 14. Reaction mechanisms for the conversion of allogibberic acid into gibberic acid

Gibberic acid must therefore have the stereochemistry shown in (LVII) [Stork and Newman (32) have reached the same conclusion by similar arguments], and epigibberic acid must be the 4*b*-epimer of (LVII).

Stereochemistry of Gibberellic Acid

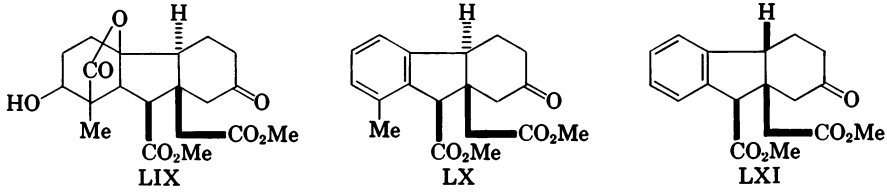


Figure 15. Ring C ketones obtained from gibberellin A_1, allogibberic acid, and epiallogibberic acid

The ring B/C fusion appears to be the same in both gibberellic acid and allogibberic acid. The O.R.D. curves, after correction for background dispersion, of the ring C ketones (LIX) and (LX) (Figure 15), derived from the methyl esters of gibberellin A_1 and allogibberic acid respectively are very similar and unlike that of the corresponding epiallogibberic acid derivative (LXI).

Ring A and A/B Fusion. The secondary hydroxyl is axial. This is shown by:

a. The base catalysed epimerization of the hydroxyl at C-2, described above, must imply an axial to equatorial transformation.

b. Similarly reduction of ring A ketones with sodium borohydride gives in every case the C-2 epimer in predominant amount over the gibberellin derivative.

c. The relative ease of dehydration of the hydroxyl group in the pairs of C-2 epimers leads to the same conclusion.

Hydrogen at C-10*a*. Hydrogenolysis of methyl gibberellate (LXII) and the isomeric ester, m.p. 174° C. (LXIII) with Adams catalyst yields the same product (LXIV) (Figure 16). The derived ketone (LXV) with boiling water, is decarboxylated to a mixture of two ketones, one of which (LXVI) is converted into the other (LXVII) with alkali. The simplest interpretation of the O.R.D. curves shown for these two ketones suggests the absolute conformations depicted (Figure 16) with the C-10*a* hydrogen β-oriented. Ketonization of the enol which is pre-

sumably the intermediate in decarboxylation must be subject to some steric control giving the axial methyl compound as well as the equatorial one.

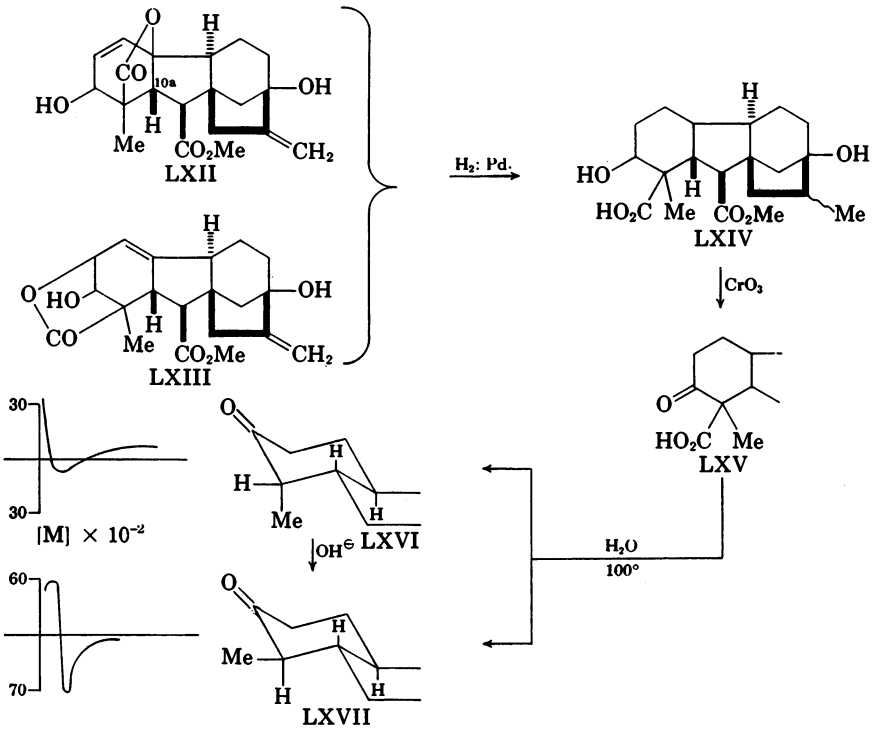
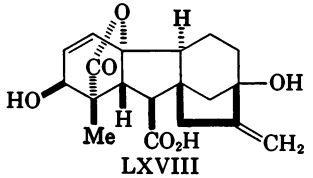
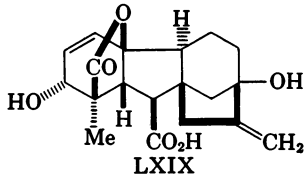
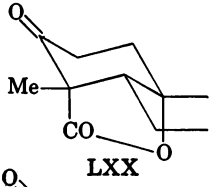


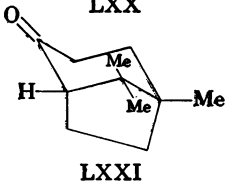
Figure 16. Reactions to show the conformation of the C-10a hydrogen

Assuming β-orientation of the C-10*a* hydrogen atom, this result leaves two possible absolute configurations (LXVIII) and (LXIX) for gibberellic acid (Figure 17).









[α]D Lactone-Acid	
α	
Dihydropimaric-	−130°
Isopimaric-	−50°
β	
Isorosenono-	+36°
Dihydroisorosenono-	+52°
2-Epitetrahydrogibberellic-	+75°

Figure 17. Configurations of gibberellic acid

The α-oriented lactone configuration (LXVIII) was originally favored by us (*8*) and by Stork and Newman (*33*). The latter authors arrived at this conclusion from their observed molecular rotation difference between the C-2-epi-tetrahydrogibberellic acid and the corresponding dibasic acid obtained by opening the lactone ring. They interpreted this value (+75°) in a rather doubtful manner in terms of Klyne's application (*24*) to polycyclic compounds of Hudson's lactone rule.

Our reasons for preferring (LXVIII) to (LXIX) were:

1. The ketones (LXX) derived from each of the C-8 epimers of methyl tetrahydrogibberellate show a strong positive Cotton effect similar to an unpublished curve for (+)—homo*epi*camphor (LXXI), kindly supplied by Professor Ourrison.

2. The A/B *trans* fusion is less strained, since the two fused 5-membered rings are *cis*. This was thought to account more satisfactorily for the stability of the lactone ring and its ease of reclosure, than did the alternative with the two 5-membered rings *trans* fused and more rigidly locked.

Edwards and coworkers (*15*) have advanced a rotational difference argument in favor of the β-lactone configuration (LXIX) (Figure 17). With dihydropimaric and isopimaric acid lactones which are known to possess α-oriented lactone rings, the rotation difference (lactone-acid) is negative and large. In the case of isorosenonolactone and dihydrorosenonolactone, with β-oriented lactones, the rotation differences are positive. They point out that the rotation difference for the C-2 epimer of tetrahydrogibberellic acid, published by Stork and Newman, is large and positive. Edwards and others (*15*) concluded therefore that the lactone ring in gibberellic acid was β-oriented. If so, gibberellic acid can be represented by the absolute configuration (LXIX).

If the interpretation of the results, shown in Figure 16, is correct and the hydrogenolysis product of methyl gibberellate has a *trans* ring A/B fusion with the C-10*a* hydrogen atom β-oriented then some recent results of ours (*18*) provide proof that the lactone is, in fact, β-oriented. The argument is as follows (Figure 18):

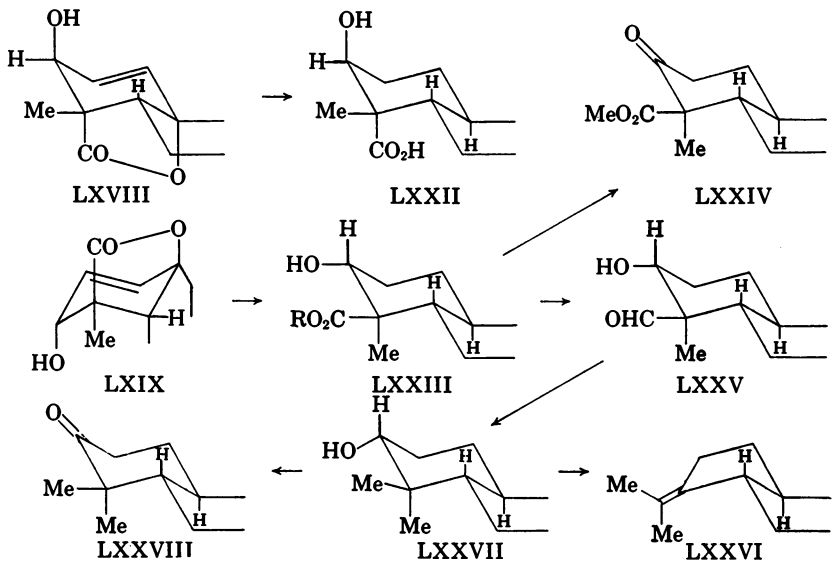


Figure 18. Evidence for orientation of the γ-lactone

To get a hydrogenolysis product of methyl gibberellate possessing *trans* fused rings A/B with the 10*a*-hydrogen β-oriented, hydrogenolysis of the α-lactone configuration (LXVIII, Figure 18) must occur with retention of configuration and the product (LXXII) must possess the original axial-2-hydroxyl group. In the case of the β-lactone configuration (LXIX), hydrogenolysis must occur with inversion, so that the hydrogenolysis product (LXXIII, R = H) would possess an equatorial 2-hydroxyl group.

In fact, the hydrogenolysis product of methyl gibberellate has been shown to possess an equatorial 2-hydroxyl group (LXXIII). This has been established in three ways.

a) The methyl ester (LXXIII; R = Me) is oxidized to the ketone (LXXIV) which regenerates (LXXIII; R = Me) with sodium borohydride in over 60% yield, giving less than 10% of the axial 2-hydroxyl epimer (vide supra the borohydride reduction of the ring A ketones of gibberellin A_1 derivatives which gives less than 10% of the gibberellin derivative and over 60% of the epimer with an equatorial 2-hydroxyl group).

b) The carboxyl group in the hydrogenolysis product has been converted to methyl via the aldehyde (LXXV). The *gem*-dimethyl derivative (LXXVII) undergoes a retropinacol rearrangement with phosphorus pentachloride, analogous to that undergone by triterpenes with a 3-equatorial hydroxyl group. The product (LXXVI) yields a diol derivative which was oxidized to a ketone and acetone (the latter was isolated as the dinitrophenyl hydrazone in 43% yield).

c) The *gem*-dimethyl derivative (LXXVII) is regenerated from the corresponding ketone (LXXVIII) in over 50% yield by reduction under strict thermodynamic control with sodium in alcohol.

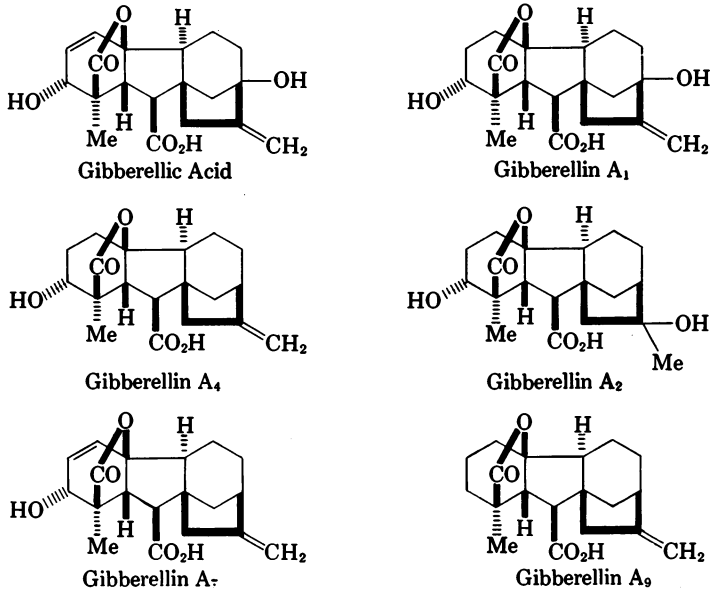


Figure 19. Configurations of gibberellic acid and gibberellins A_1, A_2, A_4, A_7, and A_9

These results leave little doubt that the hydrogenolysis product of methyl gibberellate possesses an equatorial hydroxyl group (LXXIII). If the interpretation of the O.R.D. data for the ketones (LXVI and LXVII; Figure 16) is correct,

gibberellic acid must therefore have the β-lactone bridge configuration (LXIX), and, consequently, the absolute configuration shown in Figure 19. It would then follow that gibberellins A_1, A_2, A_4, A_7, and A_9 have the absolute configurations shown in Figure 19.

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RECEIVED September 23, 1960.

Isolation and Structures of Gibberellins from Higher Plants

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The wide variety of normal growth responses, induced in plants by gibberellic acid, suggested that gibberellins, or biologically similar compounds, are present in higher plants. The isolation from many sources of crude plant extracts having gibberellin-like properties supported this idea. Chromatography of acidic material, extracted from immature runner bean seed (*Phaseolus multiflorus*) has enabled us to isolate the four pure gibberellins, A_1, A_5, A_6, and A_8. The structures of the new gibberellins, A_5, A_6, and A_8, have been determined by relating them to gibberellic acid. Paper chromatographic data on the known plant and fungal gibberellins are presented and compared with published data for some unidentified gibberellin-like substances, present in crude plant extracts. The isolation of four pure gibberellins from a higher plant establishes the gibberellins as a new class of plant growth hormones.

The study of the fungal gibberellins has had a very important consequence—the discovery that the gibberellins are native plant growth hormones.

The presence of gibberellins, or biologically similar compounds, in higher plants was first indicated by the wide range of essentially normal growth responses of plants to gibberellic acid. The isolation of crude plant extracts with biological properties indistinguishable from the fungal gibberellins supported this idea. The presence of gibberellins in plants has now been established by the isolation of the pure hormones; to date, we have isolated four gibberellins, all from the immature seed of scarlet runner bean (*Phaseolus multiflorus*). These plant gibberellins are listed in Table I. Gibberellin A_1, is a known metabolite of *Gibberella fujikuroi*. Gibberellins A_5, A_6, and A_8 are new gibberellins and are not produced by the fungus, so far as is known.

Table I. Gibberellins from *Phaseolus multiflorus*

Gibberellin		*Acid, Decomp. Pt., °C.*	*Me Ester, M.P., °C.*
A_1	$C_{19}H_{24}O_6$	255–58	234–35
A_5	$C_{19}H_{22}O_5$	260–61	191–93
A_6	$C_{19}H_{24}O_6$	205–09 222–25	136–37
A_8	$C_{19}H_{24}O_7$	210–15	221–24 231–36

Isolation Procedures

Immature seed was examined because Phinney, West, Ritzel, and Neely (*16*) and Radley (*17*) had shown it to be the richest source. Runner bean was chosen because it was grown locally.

The seed is deep-frozen at −30° for at least one month and then extracted twice with 70% aqueous ethyl alcohol. The ethyl alcohol is then removed at low temperature and the residue is separated by buffer into an acid and a nonacid fraction. The crude acid fraction is further fractionated by column chromatography, by procedures similar to those described by West and Phinney (*18*). First the crude acidic extract is chromatographed on a column of charcoal-Celite, which effectively separates the gibberellins from each other but not from other acids.

The column is eluted by gradient elution; the concentration of acetone in water increases almost linearly from 0 to 95%. Gibberellins A_8, A_1, A_6, and A_5 were eluted with the following percentages of acetone in water: 27 to 38, 38 to 41, 43 to 49, and 51 to 56%, respectively. The gibberellins are obtained pure after further chromatography on a column of silicic acid and Celite, followed by crystallization.

Structures

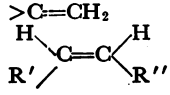
Gibberellin A_1 was the first gibberellin to be isolated from a higher plant (*10, 12*). It was readily identified by infrared spectrum, elementary analyses, optical rotation, and conversion to the methyl ester. Our highest yields have been 2 mg. per kg. fresh weight of seed.

Gibberellin A_1 has since been isolated from the seed of French bean by West and Phinney (*18*) and from water sprouts of citrus by Karawada and Sumiki (*5*).

Gibberellin A_5 was isolated in a yield of about 1 mg. per kg. of seed. For structure determination (*9, 10*) we had a total of 37 mg.

Gibberellin A_5 ($C_{19}H_{22}O_5$) has a melting point of 260–61° and forms a methyl ester (m.p. 190–91°). The infrared spectra of Nujol mulls of the acid and ester (see Table II) show the presence of alcoholic hydroxyl, hydroxyl of carboxylic acid, unconjugated five-ring lactone, carboxyl (or ester) carbonyl, exocyclic methylene group, and a cis-disubstituted double bond. Catalytic hydrogenation of the methyl ester confirmed the presence of two double bonds.

Table II. Infrared Spectra of Nujol Mulls

	$\nu_{max.}^{Nujol}$, *Cm.*$^{-1}$	
Grouping	*Gibberellin A_5*	*Gibberellin A_5 methyl ester*
—OH	3420	3475
—OH of CO_2H	2700	...
5-ring lactone	1759	1754
>CO of CO_2R	1732	1730
$>C{=}CH_2$	1659, 893	1656, 843
	1624, 694	1624, 700

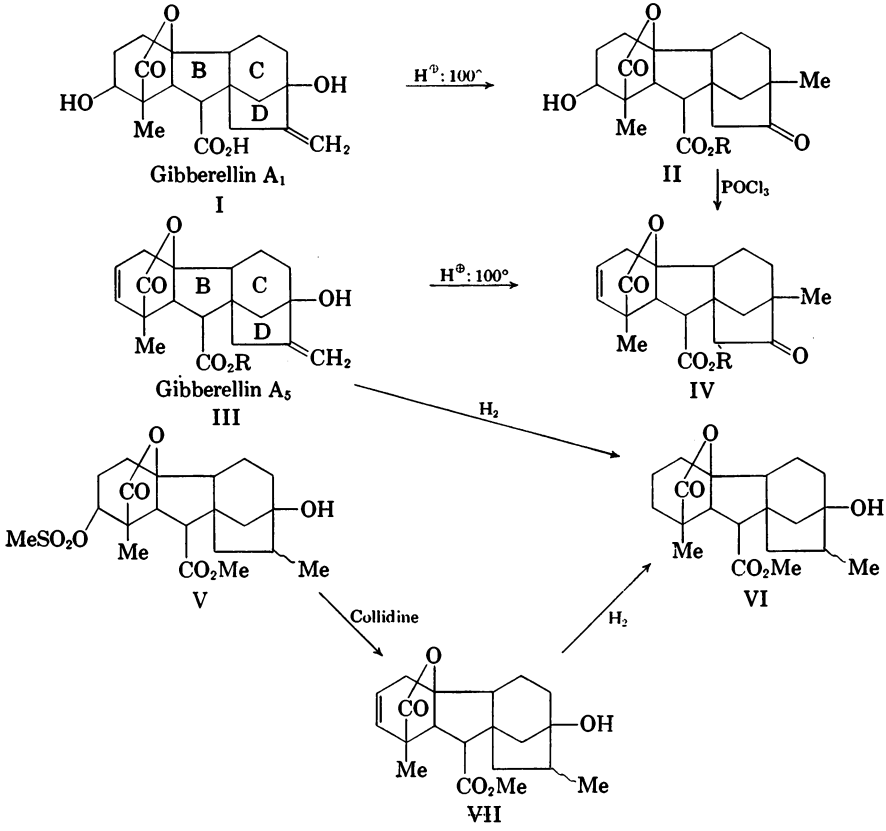


Figure 1

Gibberellin A_1 (I) with boiling mineral acid undergoes ring C/D rearrangement to a ring D saturated ketone (II; R = H). Gibberellin A_5 (III; R = H) undergoes analogous rearrangement to IV (R = H), showing that it has the same ring C/D structure as gibberellin A_1.

Hydrogenation of gibberellin A_5 methyl ester (III; R = Me) gives a tetrahydro derivative (VIII), a mixture of C-8 epimers, which was also obtained from the monomesylate (V) of the C-8 epimeric mixture of methyl tetrahydrogibberellates by treatment with collidine followed by hydrogenation of VI. This result showed that gibberellin A_5 was an anhydrogibberellin A_1. Since catalytic hydrogenation of gibberellin A_5 methyl ester causes no hydrogenolysis of the lactone, the cis-disubstituted double bond in ring A of gibberellin A_5 was unlikely to be allylic to the hydroxyl end of the lactone bridge. Thus there is only one possibility—that shown in III—and this structure was confirmed by direct dehydration of the methyl ester (II; R = Me) of the gibberellin A_1 rearrangement product to the methyl ester (IV; R = Me) of the rearrangement product from gibberellin A_5.

Gibberellin A_5 (III; R = H) can be prepared from gibberellin A_1 (I) (Figure 2). Monotosylation of the methyl ester (VIII) of gibberellin A_1 gave IX, which with boiling collidine yielded gibberellin A_5 methyl ester (X) which was hydrolyzed by aqueous alkali to gibberellin A_5 (III).

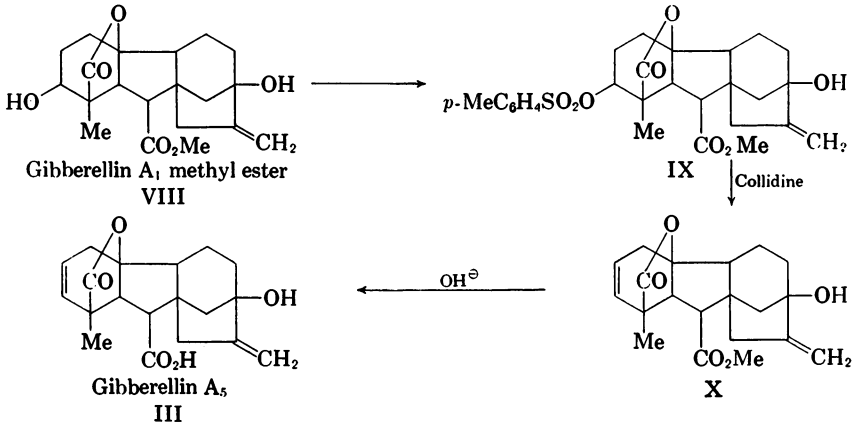


Figure 2

Gibberellin A_5 and bean factor II, isolated by West and Phinney (*18*) from the seed of French bean, are identical. West has kindly made a direct comparison of the infrared spectra, which are identical.

Gibberellin A_8. Of the four gibberellins isolated from scarlet runner seed, gibberellin A_8 is present in the largest amount; 400 mg. were isolated from 24 kg. of seed—i.e., 16 mg. per kg.

Gibberellin A_8 (XI) (m.p. 210–15°) analyzes as $C_{19}H_{24}O_7$—i.e., gibberellin A_1 with an additional hydroxyl. It gives a methyl ester (XIII) which exists in dimorphic forms (m.p. 221–24° and 231–36°) (Figure 3).

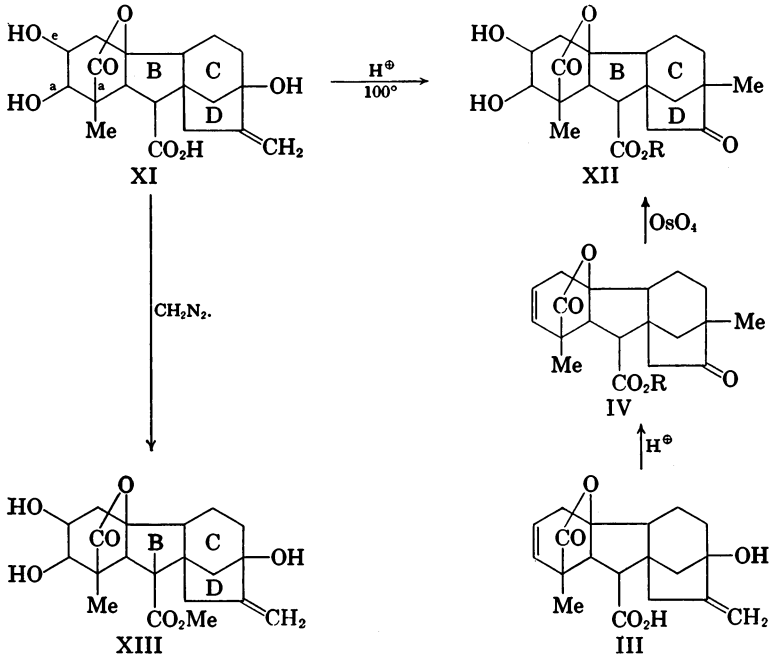


Figure 3

With boiling mineral acid, gibberellin A_8 gave a five-ring ketone rearrangement product (XII; R = H), confirming the ring C/D structure shown. Gibberellin A_8 and derivatives are oxidized by periodate. This fact suggested the 1,2-diol structure (XI), which was proved by hydroxylation of the methyl ester (IV; R = Me) of the rearrangement product of gibberellin A_5 (III), which gave the methyl ester (XII; R = Me) of the gibberellin A_8 rearrangement product. The hydroxylation, performed with osmium tetroxide, gave virtually a single product, showing that the ring A hydroxyl groups are trans to the lactone bridge (as indicated in XI).

Gibberellin A_6. Thirty milligrams were isolated from 24 kg. of seed— i.e., about 1 mg. per kg. Gibberellin A_6 has also been isolated in trace amounts from the sap of runner bean pod.

Gibberellin A_6 exists in two dimorphic forms: form A, m.p. 206–09°; and form B, m.p. 222–25°. When mixtures of both forms are obtained, the melting point is intermediate. Infrared absorption (Table III) shows the presence of alcoholic hydroxyl, unconjugated five-ring lactone, carboxylic acid, and terminal methylene group. Elementary analyses of gibberellin A_6 and its methyl ester (m.p. 136–37°) indicated the molecular formula $C_{19}H_{24}O_6$ for the acid. Microhydrogenation of the methyl ester confirmed the presence of one double bond.

Table III. Infrared Absorption of Gibberellin A_6

	ν_{max}, $Cm.^{-1}$		
	Nujol		
	Form A	*Form B*	*$CHCl_2$ solution*
Alcoholic —OH	3430	3375	...
5-ring lactone	1769	1770	1770
Monomeric carboxyl	...	1773	1738
Dimeric carboxyl	1715	...	...
>C=CH_2	1653, 900	1666, 885	...

Treatment of gibberellin A_6 (XIV) with boiling mineral acid gave a keto acid (XV; R = H) (Figure 4). The keto acid (XV; R = H) and its methyl ester showed absorption in the infrared typical of a five-ring ketone, and still showed hydroxyl absorption, so that gibberellin A_6 has a second hydroxyl in addition to that at the C/D ring junction. Gibberellin A_6 has been related to gibberellin A_5 and thence to the other gibberellins as follows: In an attempt to dehydrate the rearranged keto ester (XV; R = Me) the compound was treated with phosphorus oxychloride, affording a phosphate ester and a chloro compound (XVI). This chloro compound was dechlorinated by hydrogenolysis with a Raney nickel catalyst affording a chlorine-free product which was identical with the dihydro derivative (XVII) of the methyl ester (XVIII) of the keto acid obtained by rearrangement of gibberellin A_5 (III).

The position of the second hydroxyl group in gibberellin A_6 is not yet known and we are actively engaged in locating it.

Figure 5 shows the four gibberellins we have isolated from runner bean seed.

The biological activity of these gibberellins has not yet been studied in detail. They are all active in promoting the growth of dwarf Meteor pea plants, A_1, A_5, and A_6 having about the same order of activity. Gibberellin A_8 is much less active. They are all active in the lettuce seedling test described by Frankland and Wareing (3), except A_8, which is inactive. We do not yet know their activity on Phinney's single-gene mutants of maize, except for gibberellin A_5, tested by Phinney and West, who found that it was more active on d-3 and d-5 than d-1, as they had previously found for their bean factor II.

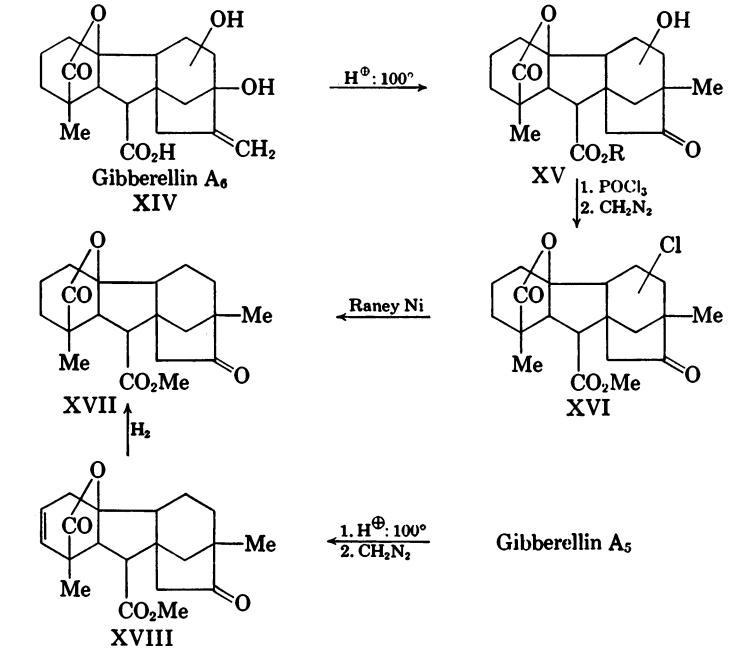


Figure 4

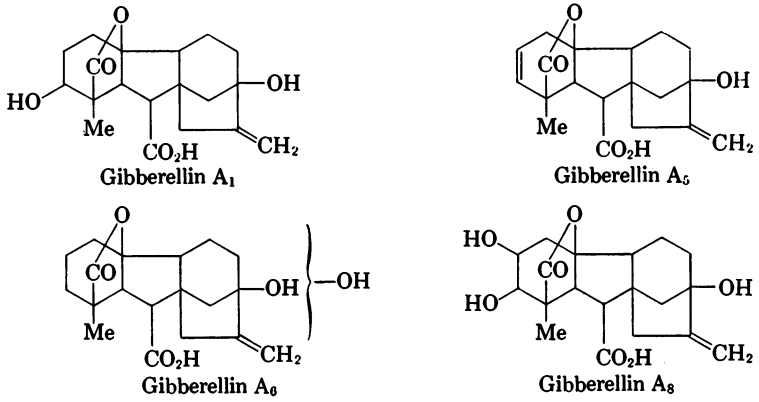


Figure 5

Gibberellin-like Substances of Higher Plants

In addition to the isolation of these four pure gibberellins, a vast body of evidence indicates the widespread occurrence in higher plants of extractable substances with "gibberellin-like" biological properties. It is instructive to compare published R_f values for unidentified gibberellin-like substances of crude extracts with the R_f values of the known gibberellins.

Table IV shows the R_f values for all the gibberellins except A_2, on paper and in four different solvent systems. The gibberellins are arranged in order of increasing mobility (in fact, in increasing number of hydroxyl groups). The relative mobilities (R.M.) in brackets are given to compare with relative mobilities derived from published R_f values for unidentified gibberellin-like materials.

Table IV. R_f Values of Gibberellins

Gibberellin[a]	Solvent System[b] 1	2	3	4
	R_f Values[c,d]			
A_8	0.15 (50)	0.40 (80)	0.25 (69)	0.35 (70)
A_3	0.29 (100)	0.50 (100)	0.40 (100)	0.48 (100)
A_1	0.31 (105)	0.52 (105)	0.40 (100)	0.49 (100)
A_6	0.33 (115)	0.51 (105)	0.42 (105)	0.51 (105)
A_5	0.45 (155)	0.59 (120)	0.49 (120)	0.56 (120)
A_4	0.60 (205)	0.72 (145)	0.57 (140)	0.72 (150)
A_7	0.61 (210)	0.71 (145)	0.57 (140)	0.71 (150)
A_2	0.725 (250)	0.77 (155)	0.65 (160)	0.78 (160)

[a] Detected by 0.5% aqueous potassium permanganate spray.
[b] Solvent system. 1. *n*-Butyl alcohol–1.5*N* ammonium hydroxide (3:1), descending.
2. Isopropyl alcohol-water (4:1), ascending.
3. *n*-Butyl alcohol-tert-amyl alcohol-acetone-ammonia-water (5:5:5:2:3), descending.
4. Isopropyl alcohol–7*N*-ammonium hydroxide (5:1), ascending.
[c] Values in brackets give relative mobilities (R.M.) in terms of gibberellic acid (As) = 100 to compare with values derived from *Rf* values of other investigators.
[d] Values for Whatman's paper No. 1 and at 20°C.

The relative mobilities of zones with gibberellin-like activity obtained by Phinney *et al.* (*16*) in solvent system 1 from lupin seed (R.M. = 44) and seed of buckeye and pea (R.M. = 170) correspond to A_8 and A_5, respectively. The zones (R.M. = 100) obtained by them from seed of many plants correspond to gibberellic acid, A_1, and A_6. The zone (R.M. = 200) which they obtained from *Echinocystis* seed in the same solvent system corresponds to A_4 and A_7 and it is tempting to put forward the following argument: *Echinocystis* is a member of the Cucurbitaceae; Lockhart and Deal (*7*) and Bukovac and Wittwer (*2*) have shown A_4 to be more active for cucumber than gibberellic acid and A_1. Recently Brian (*1*) has shown that both A_4 and A_7 are more active than gibberellic acid, A_1, and A_8 in promoting hypocotyl growth of two varieties of cucumber. This fact and the coincidence of relative mobilities suggest that the gibberellin of *Echinocystis* is A_4 and/or A_7.

The active zone (R.M. = 280 to 370), obtained in solvent system 1 from pea plants by McComb and Carr (*8*), may represent an unknown gibberellin, since its R.M. value is higher than that of the unhydroxylated A_9.

Nitsch (*15*) and Harada and Nitsch (*4*) have reported R_f values for gibberellin-like substances in solvent system 2. The R.M. values of 122 and 175 from immature apple seed (*15*) correspond to A_5 and A_9, respectively; substance E (R.M. = 130–195) from photo-induced *Rudbeckia* (*4*) corresponds to A_9.

Of the active zones obtained by Lang (*6*) in solvent system 3 from extracts of vegetative and photo-induced *Hyocyamus*, the one (R.M. = 70 to 90), obtained from both sources, corresponds to A_8 while the other (R.M. = 135), obtained from photo-induced *Hyocyamus* only, corresponds to A_4, A_5, and A_7.

Finally, the very low R.M. values of 20 to 40, reported by Murakami (*13*, *14*) for active zones of extracts of seed of several legumes, suggest a heavily hydroxylated gibberellin.

Summary

Four gibberellins–A_1, A_5, A_6, and A_8–have been isolated from *Phaseolus multiflorus* and their structures established by relating them to gibberellic acid. Paper chromatographic evidence suggests there are others to be isolated. Thus the gibberellins, which were initially studied as metabolites of a single species of fungus, can now be regarded as a new class of plant hormones.

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RECEIVED September 23, 1960.

Evidence for the Widespread Occurrence of a Gibberellic Acid-Like Substance in Higher Plants

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A gibberellic acid-like substance occurs widely in higher plants. Positive detection was affirmed only after the following criteria were met: chromatographic behavior similar to a known sample of gibberellic acid, yellow fluorescence following reaction with a sulfuric acid spray only when viewed under ultraviolet light, and appreciable biogrowth response only for the eluates of the properly located chromatographic bands. As a preliminary step, plant extracts were purified by combinations of column chromatography, dialysis, extraction, and repetitive paper chromatography. Semiquantitative estimates, made by visual comparison of the fluorescence on the chromatograms to standard gibberellic acid, indicated the following approximate levels (parts per billion): kudzu vine 80 to 500, pinto bean 12, sweet corn kernels 15 to 45, Lima bean 15, and alfalfa 15 to 75.

The belief has become widespread in recent years that the gibberellins, although initially isolated from fungal sources (*1*, *3*, *23*, *27*), actually form part of a natural growth-regulatory hormone system in higher plants (*22*, *26*). Extensive biogrowth evidence for gibberellin-like activity in numerous plants (*15*, *18–22*, *26*) has recently been supplemented by the chemical isolation of gibberellin A-1 (GA1) in bean seeds (*13*) and in the sprouts of the Mandarin orange (*25*). (For convenience, the five known gibberellin acids are designated as GA1, GA2, ... GA5. Gibberellic acid is therefore GA3 in this system.) In addition, a new gibberellin, GA5 (bean factor II), has been discovered and isolated from strictly plant, rather than fungal, sources (*12*, *26*). The close similarity in structure of GA1, GA3, and GA5, coupled with their changing relative growth-stimulation activity from plant to plant (*18*, *26*), suggests that GA3 may also be a natural constituent of plants. The demonstration of its presence would provide important support and broaden the above theory.

The possible presence of endogenous GA3 is also of significance from the public health standpoint, particularly in view of the potential widespread commercial use of GA3 on plants ultimately involved in human consumption. Here,

the demonstration of naturally occurring GA3 in a cross section of plants and in amounts commensurate with use levels would be important additional evidence for the absence of a chronic toxicity hazard. Peck and associates (*17*) have shown that rats fed for 8 weeks on a diet containing 5% GA3 show no appreciable effect.

This work was performed in an attempt to gather evidence concerning the possible presence of endogenous GA3 in a wide variety of higher plants. Previous studies using paper chromatographic and biogrowth techniques (*15, 19, 21*) have shown the presence of endogenous gibberellin-like substances, but have not permitted differentiation of these gibberellins. In particular, the active substances isolated on paper in these works have given no positive response to the sulfuric acid–induced fluorescence test, which is believed to be specific to GA3 among the currently known gibberellins (*26*). In this work, after purification procedures had been selected and developed by which it was possible to detect added GA3 in plant extracts, positive fluorescence tests were obtained in correlation with paper chromatography and plant growth stimulation assays. Although short of actual isolation and characterization, this combination gives a high degree of over-all specificity, and is taken as evidence for the presence of an endogenous gibberellic acid-like substance in the plants examined.

Detection Techniques

Positive detection was affirmed only after the following criteria were met:

1. Chromatographic behavior similar to a known sample of GA3.
2. Yellow fluorescence following reaction with a sulfuric acid spray only when viewed under ultraviolet light.
3. Appreciable biogrowth response only for the eluates of the properly located chromatographic bands.

Chromatography. Descending paper chromatography (Whatman No. 1 paper) was used with the following solvent systems.

Mitchell's or Amyl System (*14*). 25 parts of *tert*-amyl alcohol, 25 parts of *n*-butyl alcohol, 25 parts of acetone, 10 parts of concentrated ammonia, and enough water to make 100 parts.

Capryl System (*1*), Reversed Phase. The paper is impregnated with capryl alcohol, and aqueous 0.2*M* phosphate buffer at pH 6.5 is used as developing solvent.

A standard was run as part of every chromatogram. The Mitchell system permits unique detection of GA3 among its early decomposition products. For this system, the R_f of GA3 (based on the leading edge of the band) is usually reproducible within 0.05 in a given chamber on a given day, but has an over-all range (over 100 runs) from 0.3+ to 0.5. This range is not unduly wide for chromatography of a weak acid anion when volatile ammonia is used in the solvent system. Mitchell (*14*) reports several narrower ranges (0.49 to 0.55, 0.51 to 0.58) for this system. For the capryl system, the daily reproducibility was about 0.02, with over-all range about 0.85 to 0.92, appearing to depend to some extent on the geometry of the chromatographic chamber. It is not as useful as the amyl system in distinguishing GA3 from its decomposition products.

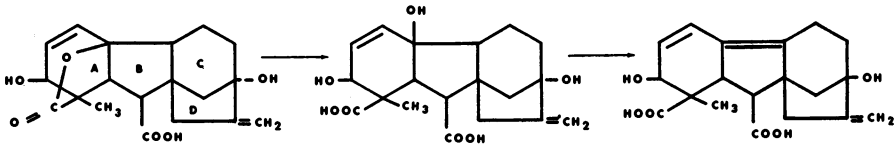
The behavior of GA3 is anomalous in the presence of plant extracts, particularly at low relative concentrations of GA3. Not only are the R_f values altered and the band shapes distorted, but often the GA3 completely fails to respond to the detection test. Although the major causes for this behavior are undoubtably over-

loading and quenching, the possibility of specific interaction with plant components cannot be eliminated.

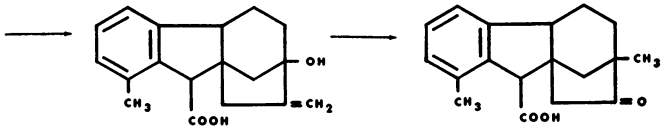
Detection by Fluorescence. The detection test is the ultraviolet light–activated yellow fluorescence which is induced only after spraying with 70% aqueous sulfuric acid (2). A preliminary inspection under ultraviolet light eliminates substances with non-sulfuric acid-induced ultraviolet fluorescence. In practice, the restriction to "yellow" eliminates many predominantly blue components in a plant extract chromatogram. Some yellow fluorescent components have been observed on occasion from plant sources without spraying, and are thus eliminated. Few bands have been observed that give a positive test other than at the R_f of GA3 or its decomposition products.

Specificity of Fluorescence

Differentiation from Decomposition Products. The successive degradation scheme for GA3 in neutral to acid solutions may be crudely portrayed by the sequence I → V. With the exception of II, the existence and structures of these compounds have been amply documented [I(3, 5–7), III (4, 8), IV (7), V (7)]. Although II has not been isolated to date, its hydrogenated counterpart with an alicyclic A ring is known (24).



I, GIBBERELLIC ACID II, HYDROLYSIS PRODUCT III, GIBBERELLENIC ACID



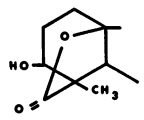
IV, ALLOGIBBERIC ACID V, GIBBERIC ACID

Many other substances may be concurrently and sequentially formed, because of the possible, although kinetically unfavorable, simultaneous occurrence of the C → D ring isomerization, as well as epimerization, esterification in an alcoholic medium, etc.

The kinetics of decomposition of GA3 in 2% methanolic sulfuric acid was qualitatively studied by paper chromatography (Mitchell system) over a period of 300 hours. As the GA3 disappeared (undetectable within one day), three early, very slow-moving components appeared, reached maxima, and subsequently disappeared, whereas two other substances, one of which appeared to be allogibberic acid, continually increased. Of the three transient components, ultraviolet and chromatographic behavior indicated that one was gibberellenic acid. (Circular paper chromatography with the Mitchell solvent system is considerably better than linear chromatography in resolving these components.) Another gave ultraviolet and fluorescent behavior similar to gibberellenic acid, but was chromatographically distinct. It did not appear to occur in a similar kinetic study in an aqueous system; this suggests that it might be the monomethyl ether of gibber-

ellenic acid described by Gerzon, Bird, and Woolf (*8*). The third component gave only fluorescence, but no ultraviolet absorption above 220 mμ; this suggests the absence of conjugated unsaturation. Its low mobility is suggestive of a diacid. (Presumably anionic species are involved in chromatography with the basic Mitchell system. Thus, gibberellenic acid, with two carboxylic groups, is virtually immobile; GA3, with one free carboxy group, moves about halfway; and methyl gibberellate, with no free acid groups, moves slightly behind the solvent front.) It appears to be relatively unstable, as elution with methanol and rechromatography indicate some decomposition. Its early appearance in the degradation, coupled to the above characteristics, suggests that it might be II. GA3 may be readily resolved from the above degradation products with the Mitchell system.

The influence of acid concentration on the absorbance of methanolic GA3 at 253 mμ as a function of time at 23° C. is shown in Figure 1. Because at least two species contribute to this absorbance, the curves give only a crude indication of the initial rate of degradation of GA3. Spectral scans of the solutions show that after the initial rise, the absorbance at 253 mμ begins to contain appreciable contributions from a number of additional components. The number of additional components and their rate of appearance and disappearance increase markedly with increasing acidity. GA3 appears to be relatively stable in neutral methanol.



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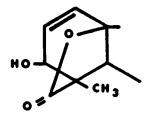

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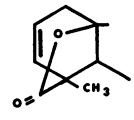

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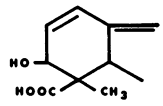

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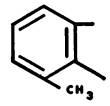

X

Differentiation from Other Gibberellins. Among the currently known gibberellins, solution fluorescence, induced by sulfuric acid, appears to be specific to GA3. West and Phinney (*26*) report no appreciable fluorescence for GA1, GA2, and GA5, and blue-green fluorescence for GA3. This behavior suggests that fluorescence is associated with the A ring (*10, 26*) (VI, GA1, GA2, GA4; VII, GA3; VIII, GA5; IX, gibberellenic acid; X, allogibberic and gibberic acids).

Considering the decomposition products as well, both GA3 and gibberellenic acid have similar activation and fluorescent spectra (*11, 16*), whereas allogibberic and gibberic acids give only weak fluorescence under ultraviolet excitation, none without ultraviolet (*3, 11*). The latter behavior suggests that IX is the precursor moiety of the major fluorogenic species. Extension of the data in Figure 1 suggests that lactone hydrolysis and dehydration occur very rapidly in strong sulfuric acid, and that consequently IX is virtually instantaneously available from GA3.

Several apparently conflicting reports concerning the color of GA3 in sulfuric acid range from a red color with blue fluorescence (*3, 10*) to a bluish yellow fluorescence (*1, 11, 26*). Both phenomena occur. If solid GA3, or its potassium salt, is added directly to the acid, a red color and bluish fluorescence are formed, and persist after dilution. The solutions have absorption peaks in the visible at

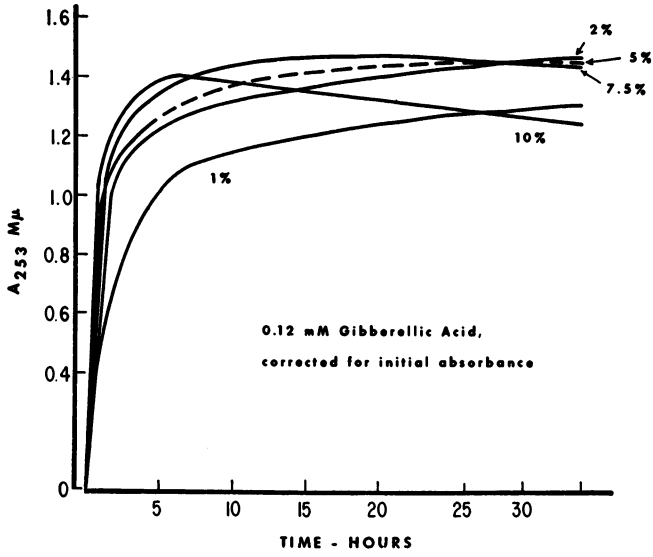

Figure 1. Influence of acid on absorbance at 253 mμ

about 415 and 535 mμ. If, however, the GA3 or its salt is added to the acid as an aqueous solution, a yellow color and bluish fluorescence are formed, and persist after dilution. The 535-mμ band is now absent or greatly diminished. If the GA3 solution and the acid are both cooled prior to mixing, some of the red band occurs. Samples of potassium gibberellate partially decomposed by heating, and shown by infrared to have lost various amounts of the lactone carbonyl, indicate a direct correlation between the loss of red color and the loss of lactone function. This suggests that the local heat generated in the finite time necessary to acidify an aqueous solution of GA3 will permit nearly complete hydrolysis of the lactone ring, and thereby prevent formation of the red product. For the solution reaction, a major yellow fluorogen, a minor blue fluorogen, and many trace components have been reported (*11*).

Plant Growth-Stimulation Activity. As an additional criterion of detection, the plant growth-stimulation activity of methanol eluates of selected chromatographic sections was determined. The principal test used was the pinto bean seedling assay (*9*), in which stem elongation is followed. Kudzu vine was also tested on dwarf maize mutants I and V (second leaf sheath). These plants have been reported to respond only to the gibberellins among the known growth substances (*18, 19, 26*) and to give an approximately equal response to GA1 and GA3, but not to GA2 and GA4 (*18*).

Selection of Plants. Six plants of the Angiospermae class were used: kudzu vine, pinto bean, alfalfa, sweet corn, dandelion, and Lima bean. Of these, kudzu vine (*Pueraria thunbergiana*) is perhaps the least known but the most interesting, in view of its rapid growth (50 to 100 feet per season). It has become established in many parts of the southeastern United States as a soil erosion control agent and minor forage crop. Preliminary biogrowth assays of crude extracts for gibberellin-like activity by pinto bean and dwarf maize I indicated an exceptionally strong positive response. The other plants were selected at random from a list of plants important as foodstuffs for man or animal.

Experimental

Techniques of Purification. The purification techniques have included column and batch adsorption, extraction, dialysis, and column and paper chromatography. Liberal use was made of the fact that the gibberellate anion is not soluble in ethyl acetate, whereas the free acid is soluble, and that charcoal will adsorb GA3 from aqueous solutions but release it with acetone (*26*). As a rough rule, preliminary concentration by a factor of about 10^5 was necessary before significant use of paper chromatography could be made. For kudzu vine and pinto bean, a known amount of GA3 was added to an aliquot of the plant extract and taken through the same procedure as the initial extract. These controls are subsequently referred to as "spiked" extracts, to differentiate them from the initial or "natural" extract.

Kudzu Vine. EXTRACTION. A 1000-gram sample was macerated in a Waring Blendor with 2 liter of water adjusted to pH 3.5 with hydrochloric acid, followed by addition of 2 liters of ethyl acetate. After steeping overnight, the entire filtered solution was concentrated to near dryness by vacuum evaporation below 40° C. and stored under refrigeration until used. Different samples were used for the two experiments.

EXPERIMENT I. The sample was first separated into fractions by a partition column, and the fractions were further fractionated by descending paper chromatography. A schematic representation of these procedures and the results is shown in Figure 2.

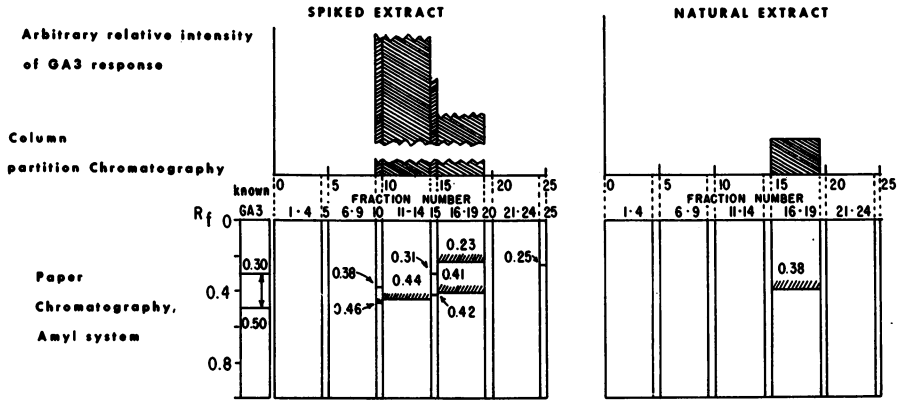

Figure 2. Column partition chromatography of kudzu vine extract followed by paper chromatography of eluent fractions

Partition Column. Two columns packed with coarse powdered cellulose impregnated with capryl alcohol as immobile phase were prepared in an identical manner. The mobile phase consists of 0.2*M* phosphate buffer at pH 6.5. To avoid overloading the column, only a small portion of the extract could be used. The natural extract consisted of 0.20 ml. of the reduced-pressure evaporated solution, corresponding to about 10 grams of kudzu. A similar quantity was added as the spiked extract, except that it contained 500 μg. of added GA3. Ten-milliliter eluent fractions were collected, acidified to pH 2, and extracted with ethyl acetate. The ethyl acetate extracts were concentrated prior to chromatography.

Descending Paper Strip. First, individual fractions spaced five fractions apart were chromatographed. Definite GA3 response was observed in fraction 10 of the spike, weaker response in fraction 15, and no response in the natural extract single fractions. To avoid the possibility of dissipating the fractions in individual runs below the detection limit, the remaining fractions were combined in groups

of four and the resulting total sample was chromatographed as shown in Figure 2.

In the spike, GA3 was eluted throughout fractions 10 to 19, but was predominantly located in fractions 10 to 14. Its recovery was confirmed by chromatographing a 1% portion of the 11 to 14 fraction in the capryl system (R_f 0.88 relative to 0.85 for standard). A number of additional bands are seen, presumably due to the concentration and resolution of trace degradation products from the large amount of GA3 used in the spike. Only one band is found for the natural extract, in the range to be expected for GA3. It occurs in a somewhat later fraction compared to the spike, but this is not unusual in the face of a hundredfold GA3 concentration difference between the two extracts.

EXPERIMENT II. The evaporation sample was slurried in benzene and extracted several times with a total of 350 ml. of 0.05*M* phosphate buffer at pH 7.6. A 15% portion of the resulting aqueous phase was spiked with 3.6 mg. of GA3, and carried separately through all subsequent operations. The aqueous phase was batch-treated with 3 grams of chromatographic grade alumina, and then passed through a column of 25% Celite 545 in Darco G-60. After the column had been washed with pH 7.6 buffer, it was eluted with 95% acetone. The eluate was evaporated to dryness and redissolved in methanol. An initial chromatogram was run (amyl system) to effect preliminary purification. For the natural fraction, the sample was spread over two sheets to minimize overloading. Both chromatograms were cut into sections or zones and eluted with methanol. Different zones were taken from each and combined, resulting in somewhat overlapping zones. Each eluate was then rechromatographed by both solvent systems.

The results in Table I show that, in the presence of plant material, the standard GA3 is spread over a considerably wider range than is usual. However, after the sample is further purified by the second run, the mobility of GA3 returns to normal. The same pattern of distribution, with zone III as maximum, is manifest in the natural run, although at considerably lower fluorescent intensities. For both fractions, the presence of GA3 is confirmed by the capryl system. A portion of zone III of the natural extract gave positive response proportional to concentration in the pinto bean seedling assay and in the dwarf maize mutants I and V assay. The relative activity on both mutants was approximately equal, as is required for GA3 (*26*). The correlation of relatively specific biological growth activity with chromatographic and chemical behavior affirms the presence of a GA3-like substance in kudzu vine.

Table I. Chromatography of Purified Kudzu Vine Extract

	R_f[a]				
	Standard	*Zone*			
		I	*II*	*III*	*IV*	*V*
			Spiked Fraction			
First-run zones[b]		0–0.22	0.22–0.44	0.44–0.66	0.66–0.87	0.87–1.09
Second-run bands						
Amyl systems	0.48–0.37	0.35	0.07, 0.45, 0.52	0.45, 0.48	0.43	...
Capryl system	0.85	0.91	0.86	0.86	...	...
			Natural Fraction			
First-run zones[b]		0–0.22	0.18–0.55[c]	0.44–0.73	0.66–0.92	0.87–1.10
Second-run bands						
Amyl system	0.44–0.32	0.17, 0.60	0.53–0.34	0.39–0.28	0.39–0.35	...
Capryl system	0.92		0.09, 0.88	0.89	...	...

[a] Hyphenated values for bands refer to leading and tailing edges of band. Single values refer to leading edge of bands.
[b] Zones eluted with methanol, concentrated, and used for second run.
[c] Aliquot of eluate of this zone gave a positive biogrowth response by dwarf maize hybrid I and V and by pinto bean seedling assay.

Pinto Bean. The extraction and prepurification steps were the same as for kudzu vine (Experiment II). The results are shown symbolically in Figure 3. A lengthwise strip $^1/_3$ the width of the chromatogram was cut off and sprayed; the rest was eluted for the second run. The spiked extract indicated a definite band at an R_f that is somewhat high for GA3, whereas the natural extract barely indicated a faint haze at an even higher R_f. In the second run, however, GA3 response was normal for both extracts. The anomalous behavior in the first run may be due to the high ratio of plant extract residue to GA3. The presence of two distinct bands in adjacent strips of the second run of the natural extract may be due to chromatographic travel of the substance in an irregularly curved path during the first run. Chromatograms that would give similar behavior by this procedure have often been observed for overloaded strips of spiked plant extracts.

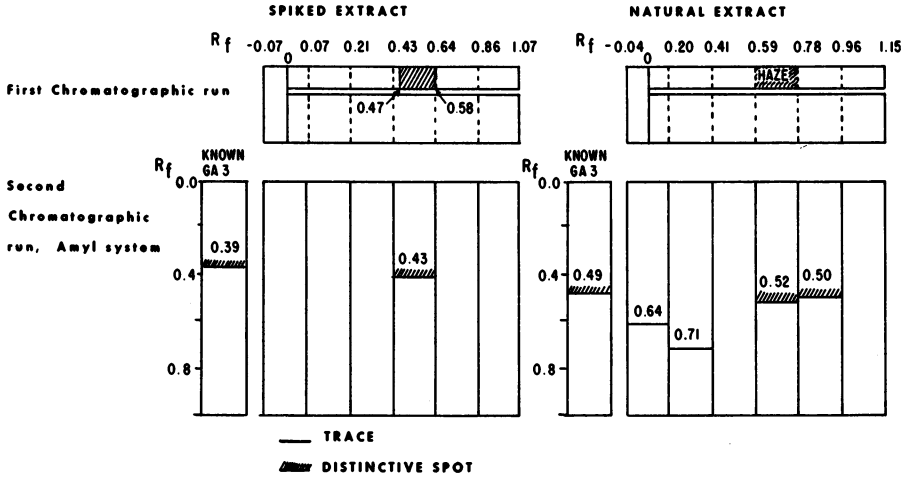

Figure 3. Repetitive paper chromatographic fractionation of purified extract of pinto bean

Alfalfa, Lima Bean, Dandelion, and Sweet Corn Kernels. EXTRACTION. Two hundred-gram samples were treated like kudzu vine, except for an additional ethyl acetate wash after overnight steeping. The combined filtrates were evaporated to about 3 ml., and stored in a refrigerator for several months. The slurries were then vacuum-dried and extracted with aqueous pH 7 phosphate buffer.

PRELIMINARY PURIFICATION OF LIMA BEAN AND ALFALFA. The pH 7 buffer solution was washed once with ethyl acetate, acidified to pH 2 with sulfuric acid, and extracted several times with ethyl acetate. The combined ethyl acetate extracts were evaporated under reduced pressure below 40° C. The residue was dissolved in pH 7 buffer and then added to a charcoal column (10 grams of 1:1 Darco G-60–crushed glass particles). The column was eluted with 95% acetone, the eluate was evaporated to dryness, and the residue was taken up in methanol.

PRELIMINARY PURIFICATION OF DANDELION AND SWEET CORN. The plant extract in pH 7 buffer was sealed in a Visking cellulose dialysis membrane bag, immersed in a volume of aqueous pH 7 buffer equal to twice the volume of its contents, and shaken for at least 2 hours. The outside solution was replaced, and the entire operation repeated twice. The three outside buffer solutions were combined. A dialysis blank was prepared in similar fashion. The dialyzate was washed once with ethyl acetate. The aqueous phase was adjusted to pH 2 with sulfuric acid and extracted several times with ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness, and redissolved in a small volume of methanol.

CHROMATOGRAPHY. Half of each purified extract was chromatographed with the capryl system, the other half with the amyl system. A lengthwise strip $^1/_3$ the width of the chromatogram was cut off and sprayed. The remainder of each strip was cut into sections, eluted with methanol, concentrated, and rechromatographed by the amyl system. A lengthwise strip $^1/_2$ the width of the second chromatogram was again sprayed. The remainder was again sectioned, eluted, and used for the biogrowth assay.

RESULTS. A typical experiment is shown in Figure 4 for alfalfa. The distribution of bands in the second run shows the distortion in position that can be caused by overloading in the first chromatogram. The GA3 standard solution used in the second run shows signs of atypical decomposition, although the major part (leading band) was still present as GA3. The stippled areas represent the material eluted for the biogrowth assays. Portions labeled with the same subscript were run as combined samples in this test. Plus signs indicate strong growth stimulation in the pinto bean seedling test; negative signs indicate no significant growth relative to controls. Correlation is obtained between fluorescent bands located within 0.05 R_f of the standard in the second run and biogrowth activity for both portions of the extract.

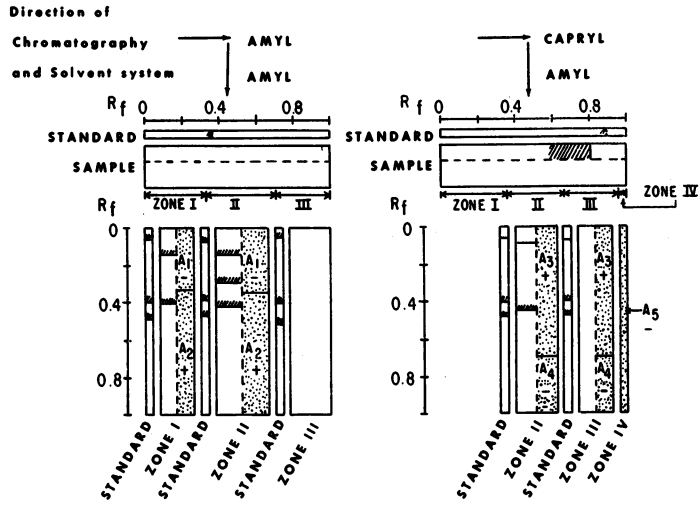

Figure 4. Repetitive paper chromatographic fractionation of purified extract of alfalfa

For dandelion, a trace fluorescent band in the GA3 R_f range in the chromatograms derived from both the amyl and capryl systems was noted, but the biogrowth response was not large enough to be unmistakably positive. For sweet corn kernels, a suitable band with biogrowth confirmation was noted only in the capryl-amyl combination; for Lima bean, only in the amyl-amyl combination.

Other Fluorescent Bands. The principal other fluorescent band observed, with R_f about 0.2 in the Mitchell system, was present in sweet corn and to a far lesser extent in Lima beans. In Figure 5, the four apparently distinct bands in the first chromatogram were found in the second chromatogram to be the same substance. The gradation in intensity in the first chromatogram, greatest in the band closest to the point of application and then dropping off rapidly, was duplicated by the 0.2 R_f band in the second run. The reason for the discontinuous nature of the

appearance of this substance is not clear, but some form of decomposition or dissociation during the chromatography is indicated. The substance does not appear to have any plant growth–stimulating activity. A band of similar R_f was observed in the spiked kudzu run (Figure 2).

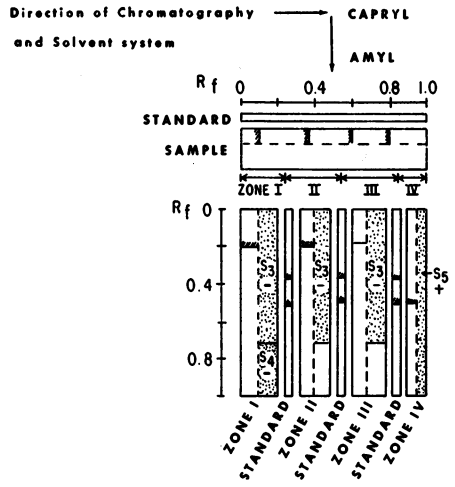

Figure 5. Repetitive paper chromatographic fractionation of purified extract of sweet corn

Concentration Level. The level of GA3-like activity has been estimated by the visual comparison of the fluorescence of the appropriate band to that of standard GA3. This technique gives only crude estimates, but does allow a minimum level to be selected based on the intrinsic sensitivity of the test. No correction has been applied for losses in the extraction and purification procedures, for losses due to degradation of GA3 in the chemical steps, or for extract consumed in testing each first-run chromatograph.

Table II. Apparent Minimum Concentration of Gibberellic Acid-Like Substance in Higher Plants

Plant	*Min. Concn. Level, P.P.M.*
Kudzu vine	0.5, 0.08
Pinto bean	0.01
Alfalfa	0.01–0.08
Sweet corn	0.01–0.05
Dandelion	?
Lima bean	0.01

The distinctly higher level of apparent GA3 indicated for kudzu vine as compared with the other plants is in agreement with earlier observations based only on relative biogrowth response of plant extracts. It is significant, as an implication of a widespread phenomenon, that five of the six plants selected give definite indications of an endogenous GA3-like substance, while the sixth does not give a completely negative response. The results also suggest that kudzu vine may be a prime choice for the extension of this work to the actual isolation and characterization of crystalline material.

Acknowledgment

The authors thank Thomas Poznanski and Samuel Stein for assistance in the early stages of this work and Merck & Co., Inc., for permission to publish this report.

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RECEIVED October 20, 1960.

Properties of Gibberellins from Flowering Plants

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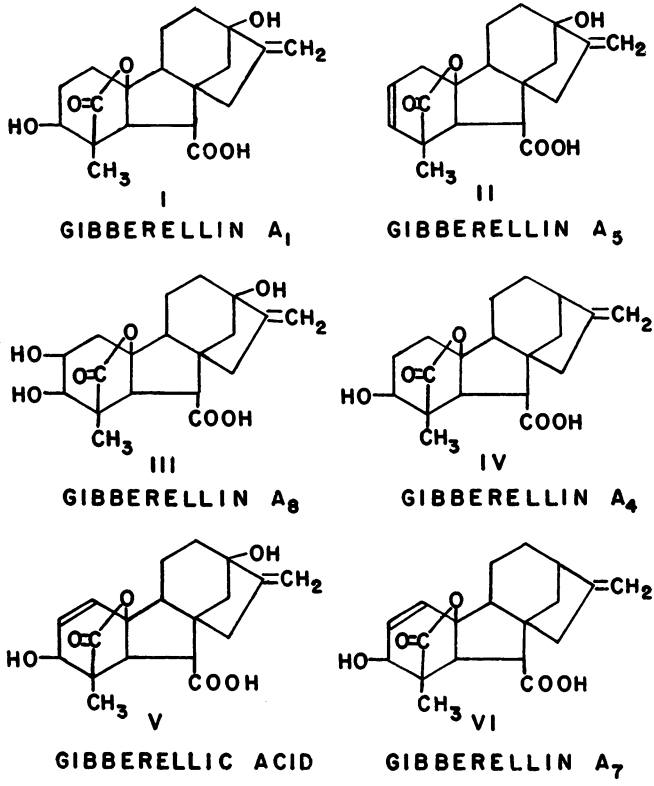
A gibberellin-like substance has been partially purified from acetone-water extracts of *Lupinus succulentus* seed and pods. Its chromatographic properties suggest a relatively polar gibberellin, perhaps identical with or closely related to the recently discovered gibberellin A_8. Purification of the gibberellin-like substances of *Echinocystis macrocarpa* endosperm led to the fractionation of two biologically active substances. Although there are insufficient data on which to base an identification, the chromatographic and fluorescence properties of Echinocystis Factor I most closely resemble those of the recently reported gibberellin A_7. The chromatographic and fluorescence properties of Echinocystis Factor II resemble most closely those of gibberellic acid, but small differences suggest that these substances are not identical.

Several years ago our laboratory investigated the presence of substances with gibberellin-like biological activity in extracts of flowering plants, using the growth response of dwarf mutants of maize as a bioassay (*9*). Approximately one third of the crude extracts prepared from seed or fruit of *Angiosperms* gave evidence of such substances. A number of the active extracts were then chromatographed on paper and the active zones located by bioassay. The results suggested the presence of a family of gibberellin-like substances, since several of the R_f values of active substances differed significantly from one another and from those of the then known fungal gibberellins—gibberellins A_1 and A_2 and gibberellic acid. Subsequently, gibberellin A_1 (I) and gibberellin A_5 (II), a compound which has not yet been found in fungal filtrates, were isolated and identified as natural constituents of bean seed [from *Phaseolus vulgaris* (*11*) and *Phaseolus multiflorus* (*6, 7*)]. MacMillan, Seaton, and Suter (*5*) have recently reported the isolation and identification of two additional new gibberellins, A_6 and A_8 (III), from extracts of *Phaseolus multiflorus* seed. In this paper we describe the progress made in the identification of the gibberellin-like substances in extracts of two other plant species.



Gibberellin-like Substance of* Lupinus succulentus *Dougl.

The gibberellin-like substance in acetone-water (1:1) extracts of the young seed and pods of *Lupinus succulentus* Dougl. did not migrate as far as gibberellic acid in any of a number of solvent systems used for paper chromatography. The ratio of the distance of migration of this gibberellin-like substance to that of gibberellic acid in the same solvent system, the R_{ga} value, was 0.44 in *n*-butyl alcohol–1.5*N* ammonium hydroxide (3:1) (upper phase), 0.83 in pyridine–*n*-amyl alcohol–water (35:35:30) (upper phase), and 0.79 in *n*-butyl alcohol–glacial acetic acid–water (19:1:6) (upper phase). This behavior was unique among the gibberellins and plant extracts tested and suggested the presence of a more polar gibberellin in lupine extracts. Recently gibberellin A_8 (III) has been isolated from bean extracts (5) and shown to have three hydroxyl groups. Since the other known gibberellins have only two or less hydroxyl groups and the same functional groups otherwise, A_8 is the most polar of the gibberellins. Although there is no more direct evidence than R_{ga} values at present, it may develop that the lupine gibberellin-like substance is identical or closely related to gibberellin A_8. To gain further evidence on this point a large quantity of lupine seed and pods has ben extracted and the gibberellin-like substance extensively purified by solvent partitioning and charcoal adsorption and chromatography. However, the best fractions available are still impure, so that little more of its properties has been learned to date.

Gibberellin-like Substances of* Echinocystis macrocarpa *Greene

Both the endosperm and cotyledons of the wild cucumber, *Echinocystis macrocarpa* Greene, have been shown to contain gibberellin-like substances. The

R_{ga} value was determined as 1.9 in *n*-butyl alcohol–1.5*N* ammonium hydroxide (3:1) (upper phase) and 1.3 in pyridine–*n*-amyl alcohol–water (35:35:30) (upper phase). Somewhat later it was shown that gibberellin A_4 (IV), a metabolite sometimes found in culture filtrates of *Gibberella fujikuroi* (*10*), gives these same R_{ga} values in these solvent systems. Furthermore, Bukovac and Wittwer (*1*) and Lockhart and Deal (*3*) demonstrated that a number of species of *Cucurbitaceae* were much more sensitive in their response to A_4 than to gibberellic acid or the other fungal gibberellins. These findings prompted the investigation of the chemical nature of the gibberellin-like substance of *E. macrocarpa*, a member of the family *Cucurbitaceae*.

Purification

The following scheme was followed in purifying gibberellin-like substances from *E. macrocarpa*.

Approximately 2 liters of viscous endosperm extracted from immature seed was adjusted to pH 3 by the addition of sulfuric acid. This suspension was extracted directly several times with ethyl acetate. Most of the biologically active material was removed to the ethyl acetate phase, as determined by bioassay with dwarf mutants of maize (*8*). The combined extracts were concentrated and extracted in turn with 5% aqueous sodium carbonate solution to remove acidic substances. All the biologically active material was removed to the aqueous phase. After this fraction had been acidified to pH 3, it was again extracted with ethyl acetate, which removed the biologically active substances to the ethyl acetate phase. The residue from the ethyl acetate layer (2.5 grams) was then chromatographed on a charcoal-Celite (1:2) column developed with increasing concentrations of acetone in water.

Fractions brought off the column with elutants ranging from 80% acetone in water to pure acetone (108 mg. of solids) contained most of the biologically active material. The solids in these fractions showed a yellow fluorescence when dissolved in concentrated sulfuric acid. Gibberellic acid (V) shows a similar fluorescence under these conditions, whereas gibberellin A_4 and other fungal gibberellins do not.

One-hundred-microgram portions of the biologically active solids from the charcoal column were chromatographed on paper using *n*-butyl alcohol–1.5*N* ammonium hydroxide (3:1) (upper phase) as the developing solvent. One chromatogram was tested for the presence of fluorescent materials after treatment with concentrated sulfuric acid and a second was tested for the presence of biologically active materials, using the dwarf maize mutant assay. Two zones of biological activity were located, both of which were correlated with weakly fluorescent zones on the chromatogram. The slower-moving component behaved like gibberellic acid in its fluorescence and was only slightly displaced from it in position. The faster-moving component was very similar in position to gibberellin A_4; however, A_4 does not fluoresce under these conditions.

The presence of a gibberellic acid-like material in this fraction was surprising, as there had been no indications of such a substance in crude extracts. It seemed possible that it had arisen from the faster-moving component as an artifact of chromatography. However, when the faster-moving component was eluted from a chromatogram developed with the *n*-butyl alcohol–ammonium hydroxide solvent system and rechromatographed in the same solvent system, there was no evidence of the formation of the slower-moving component from the faster one.

The active solids from the charcoal column were subjected to a fractional

precipitation by progressively increasing the concentration of petroleum ether (Skellysolve B) in a solution of the active material in ethyl acetate–acetone. Seventy-nine milligrams of white, amorphous material which showed some biological activity and fluorescence in sulfuric acid was precipitated first. An apparently crystalline fraction which had biological activity and fluoresced in sulfuric acid solution was obtained next. This fraction yielded 20 mg. of solid on recrystallization from ethyl acetate–petroleum ether mixtures. A quantitative bioassay on maize mutants showed that this material was only about 2% as active as gibberellic acid on a weight basis. A paper chromatographic study revealed that both active zones were still present. Therefore, this fraction was subjected to further purification.

Eight milligrams of the crystalline fraction was chromatographed on a column of anhydrous silicic acid developed with increasing concentrations of ethyl acetate in chloroform by a gradient elution technique. Fractions 6 and 7 contained 2.8 mg. of a biologically active white solid which showed a weak fluorescence in sulfuric acid. This material is referred to in subsequent discussion as Echinocystis Factor I. Fraction 13, eluted with a higher concentration of ethyl acetate in chloroform, contained 2.9 mg. of a biologically active, white solid which also showed a weak fluorescence in sulfuric acid. This material is referred to subsequently as Echinocystis Factor II.

Properties of Echinocystis Factors I and II

It is not known at present whether these preparations of Echinocystis Factors I and II represent pure substances. The relatively weak fluorescence and biological activity of each preparation make it seem likely that they are still impure.

Echinocystis Factor I behaves like gibberellin A_4 on paper chromatography. No accurate quantitative measurements of its biological activity on the various dwarf mutants of maize have been made as yet, but it appears to be of a low order of magnitude in this assay as compared with gibberellic acid on a weight basis. The activation and emission spectra of Echinocystis Factor I in concentrated sulfuric acid have been measured in an Aminco-Bowman Spectrophotofluorimeter. The characteristic maxima are recorded here, with those of gibberellic acid for comparison.

	Fluorescence Maxima mμ	
	Activation	*Emission*
Echinocystis Factor I	305, 465	510–515
Echinocystis Factor II	305, 330, 435	510–515
Gibberellic acid	305, 435	510

The second activation maximum for Echinocystis Factor I is approximately 30 mμ higher than that of gibberellic acid. The intensity of fluorescence is only about 3% that of gibberellic acid. Microspectrophotometric techniques for the determination of the acid equivalent weight (*11*) and of ethylenic double bonds (*11*) give a ratio of 0.75 double bond per acid equivalent in this fraction.

Echinocystis Factor II behaves like gibberellic acid on paper chromatography. It also seems to have a low biological activity on dwarf mutants of maize when compared with gibberellic acid on a weight basis. It gives a weak fluorescence in sulfuric acid solution which is qualitatively very similar to that of gibberellic acid. The only detectable difference is the presence of an extra-weak activation maximum in the Echinocystis Factor II spectrum at 330 mμ. The intensity of the

flourescence of the Echinocystis Factor II sample is only about 3% that of gibberellic acid. Microspectrophotometric tests suggest the presence of 0.90 ethylenic double bond per acid equivalent in this sample.

Discussion

The data currently available are an insufficient basis for definite conclusions regarding the structures of the gibberellins from *Echinocystis*. Echinocystis Factor I most closely resembles in its chromatographic and fluorescence properties gibberellin A_7 (VI), recently reported as a fungal metabolite by MacMillan *et al.* (2). That it might be identical with A_7 is an interesting possibility, since cucurbits are reported (2, 5) to be even more sensitive in their response to gibberellin A_7 than to gibberellin A_4, which in turn is much more effective than the other fungal gibberellins (1, 3). If, in fact, these are the same, the sample of Echinocystis Factor I available for study must still be impure, since the fluorescence intensities of gibberellin A_7 and gibberellic acid are of the same order of magnitude (4). Final identification of Echinocystis Factor I must await the preparation of a sample of known purity and a study of its properties.

Echinocystis Factor II closely resembles gibberellic acid in its chromatographic and fluorescence properties, but the small differences noted suggest that they are not identical. The fact that there is no chromatographic evidence of Echinocystis Factor II in the crude extract used as starting material suggests that it may have arisen from Echinocystis I during the purification procedure, although there is no direct evidence to support this hypothesis.

Acknowledgment

The authors thank Fung Liu for valuable technical assistance throughout this work.

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RECEIVED October 14, 1960. Research supported in part by National Science Foundation Grant 3526 and U. S. Public Health Service Grant 7065.

Endogenous Gibberellins in Resting and Sprouting Potato Tubers

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Because gibberellic acid can induce sprouting of potatoes (on or off the plant) at any time after tuber initiation, the possible role of endogenous gibberellins in the control of tuber dormancy was investigated. Freshly harvested Red Pontiac potato tubers were stored at 20° C. and sampled at 12-day intervals until sprouts appeared. The 1000-gram (fresh weight) samples of the peel layer were extracted and concentrated by vacuum, charcoal adsorption, solvent partitioning, and paper chromatography. The concentrated eluates from R_f 0.25 to 0.46 exhibited "gibberellin-like" activity as revealed by the Phinney maize bioassay, with dwarf strains 1, 3, and 5 which respond differently to various gibberellins. The concentration of endogenous gibberellins remained low during rest but increased up to 30-fold (approximate final concentration 3 μg. per kg. fresh weight) compared to a sample taken 12 days before. The similar response of strains d_1, d_3, and d_5 to the extracts suggests that gibberellin A_5 was not the active substance.

Gibberellin A_3 is known to shorten the rest period of certain plants. Normally, in seeds and tissues which experience rest, growth is slowed or arrested for a time (rest period), even though environmental conditions for growth seem optimal. After the rest period is past, growth is accelerated or renewed, unless environmental conditions are not suitable (*6*).

Gibberellin A_3 has been shown to shorten the rest period of certain trees, shrubs, and seeds (*5, 7, 10*), and is the only substance known to stimulate elongation of resting epicotyls of tree peony (*Paeonia suffruticosa* Haw.) seedlings (*1*). In the tubers of the potato (*Solanum tuberosum* L.), the rest period lasts from the time of tuber enlargement to 5 to 17 weeks after harvest, depending upon age,

variety, and storage temperature (*6*). Preharvest foliage sprays containing gibberellin A_3 induce sprouting of tubers attached to the mother plant within 1 to 2 weeks (*11*, *25*). Solutions containing low concentrations of gibberellin A_3 markedly shorten the rest period of freshly harvested tubers (*17*, *21*). Newly initiated tubers (1 to 2 cm. long) on plants grown in sand culture are induced to sprout when as little as 0.01 mg. of gibberellin A_3 per liter is added in the nutrient solution (*22*). Thus, gibberellin A_3 stimulates sprouting of the potato tuber on or off the plant at essentially any time from the beginning of tuber enlargement to the end of rest. These findings raised the questions: Do gibberellin-like substances occur naturally in potato tubers? Do the levels of these endogenous gibberellins change during and after emergence from rest?

Such an association would be of great interest in view of the effects on rest of low concentrations of applied gibberellin A_3. Smith and Rappaport (*22*) and Okazawa (*17*) almost simultaneously reported the occurrence of native gibberellin-like substances in potato tubers. Preliminary evidence for a 20-fold higher level of endogenous gibberellins in sprouting tubers than in freshly harvested tubers was presented by Smith and Rappaport (*22*). Okazawa (*17*) found "gibberellin substances" in all parts of the potato plant tested: the highest activity in the most actively growing area (sprout > stem tips > tubers > middle part of stem > leaf). The present report describes in greater detail the occurrence and changes of endogenous gibberellins in potato tubers during and after the rest period.

Materials and Methods

Extraction and Bioassay. One thousand grams of potato peelings, including buds, were macerated with 1500 ml. of acetone-water (85:15 v./v.) and allowed to stand with intermittent stirring for 24 hours at 0° C. The extract was concentrated and partially purified by charcoal adsorption and solvent partitioning with ethyl acetate, according to the method of West and Phinney (*28*). The concentrated extract was streaked on Whatman 3-mm. chromatographic paper ($22\frac{1}{2} \times 18\frac{1}{4}$ inches) and developed in a chromatographic cabinet by a descending solvent system containing a mixture of acetone, *tert*-amyl alcohol, *n*-butyl alcohol, ammonium hydroxide (specific gravity 0.90), and water (25:25:25:10 to 100) (*14*). The chromatogram was removed when the solvent front reached 35 cm. from the starting line.

Gibberellin A_3 was spotted on each chromatogram as a reference standard and its R_f (0.33 to 0.38) was detected by fluorescence in 3% sulfuric acid, and by dipping the chromatogram in a mixture of acidic potassium permanganate and potassium periodate, according to Mitchell (*14*). A 5-cm. strip (±2.5 cm. from center of R_f of gibberellin A_3, R_f 0.26 to 0.46) was then eluted by macerating it in a blender with 95% ethyl alcohol. The concentrated eluates were dissolved in 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) in water, and tested for activity by the Phinney maize bioassay using dwarfs 1, 3, and 5 (*16*) with two successive daily applications of 0.05 ml. each. Dwarf 5 was the primary bioassay plant, because it responds similarly to gibberellins A_1, A_2, A_3 A_4, and A_5. Replications of a minimum of eight single plants were grown in a randomized block or split plot design. Gibberellin activity was determined from a standard curve, such as one shown in Figure 1. The elongation of the first (or first and second) internodes was calculated as: log (average length of treated internodes −control internodes).

Results

Freshly harvested Red Pontiac potato tubers were stored at 20° C. and sampled at 12-day intervals until sprouts appeared (four times). Sprouting was determined by visual inspection.

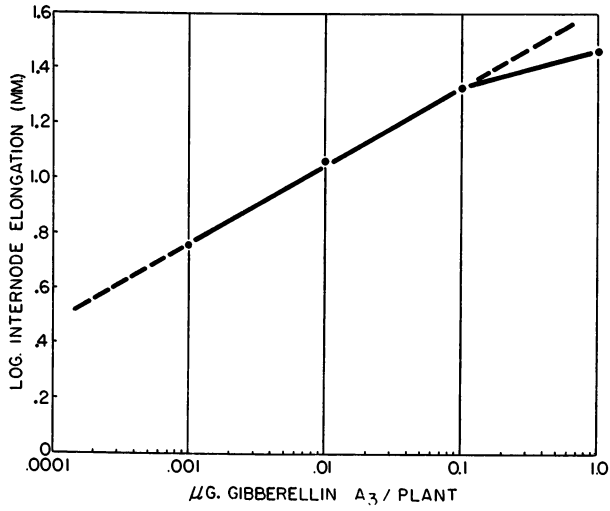

Figure 1. Standard curve showing response of first internode of d_5 maize mutant to applied gibberellin A_3

Internode elongation = log (height of 1st internode treated — height of 1st internode control)
Solid line indicates actual response of d_5.

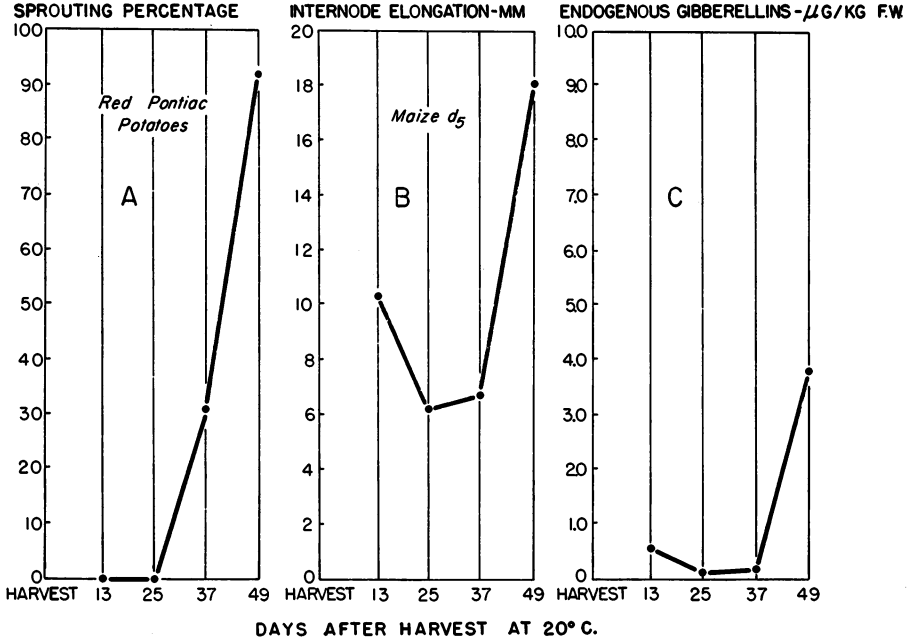

Figure 2. Relation between sprouting of Red Pontiac potato tubers (A) *and level of endogenous gibberellins in potato peels and buds during and after rest* (C) *as measured by internode elongation of maize mutant d_5* (B)

Figure 2 shows the elongation of the first internode of dwarf 5 (*B*) and the calculated changes in level of gibberellin-like substances (*C*) in relation to sprouting (*A*). The data of Figure 2, *C*, show that the level of endogenous gibberellins

remains low during rest, but increases strikingly near the onset of sprouting. Endogenous gibberellin-like substances increase some 30-fold after sprouting begins. The content was significantly higher after 37 days at 20° C. That other dwarf maize mutants respond alike to potato gibberellins was shown by applying extracts for each of the four sampling dates to dwarfs 1, 3, and 5 (Figure 3). Phinney and West suggested that these might be used to help distinguish between certain gibberellins. Elongation of the three maize mutants was similar for any one sampling date. The apparent inhibition from extracts of potatoes sampled 25 and 37 days after harvest (Figure 3) was not shown to be statistically significant.

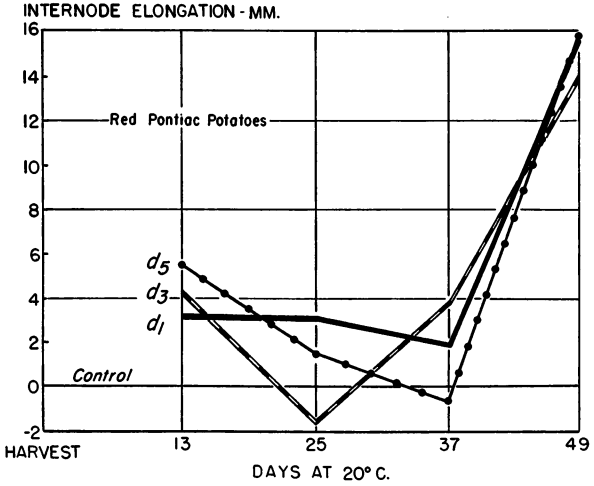

Figure 3. Gibberellin-like activity in peels of Red Pontiac potatoes during and after rest as revealed by internode elongation of maize mutants 1, 3, and 5

Discussion

The Phinney dwarf maize bioassay showed that the level of endogenous gibberellins in peelings of freshly harvested tubers remained low during the rest period of Red Pontiac potatoes and increased markedly near the time that rest terminated. The response of dwarfs 1, 3, and 5 to extracts of the peelings was the same. West and Phinney (28) showed that bean factor II (gibberellin A_5) stimulated internode elongation in d_5 about 20 times as much as in d_1. Therefore, the extract probably does not contain gibberellin A_5. The like response of dwarf maize mutants 1, 3, and 5 to potato extracts is interesting because they are known to respond about equally to gibberellins A_1 and A_3.

Visible sprouting is the terminus of a series of events which involve the change from a resting to a nonresting condition. The foregoing experiments do not make clear whether the increase in level of gibberellin-like substances precedes or is a consequence of sprouting. This may be due to difficulty in determining when sprouting actually begins in potato tubers. Once sprouting commences, it may take upwards of 25 days for a population of Red Pontiac potato tubers to sprout completely at 20° C., as in Figure 3, *A*. At any given time a percentage of the population (short of 100%) could not be clearly identified as sprouting or nonsprouting. Therefore, in current investigations, attention is given to methods for determining more precisely when sprouting begins.

Physiological phenomena like rest and dormancy have been popularly explained as the consequence of changes in level of endogenous substances, such as inhibitors and auxins. In several species, including *Solanum tuberosum*, auxin was shown to increase as rest terminates, whereas an inhibitor (Inhibitor β complex) remained high in level during rest and decreased with natural sprouting, or after sprouting was induced by ethylene chlorohydrin (*9*). This inhibitor is not specific, however: Buch and Smith (*3*) showed that it prevents elongation of Avena coleoptiles, but does not inhibit sprouting in the potato tuber. The Inhibitor β complex in potato, while now of dubious significance, may still have an important role in controlling the availability of adenosine triphosphate necessary for the synthetic reactions associated with growth (*13*).

Considering the dual effects of auxin on growth (both stimulation and inhibition as a function of concentration), and the failure of the Inhibitor β complex to prevent sprouting in potato tubers, it is naive to assume that only one growth regulator system is operative. Nevertheless, it is of interest here to summarize the effects of applied fungal gibberellins on potato and to speculate on the possible significance of endogenous gibberellins in the rest period.

It is reasonable to assume that the bud primordia of potato tubers are well differentiated during stolon elongation. Certainly, the potato tuber has well-developed vegetative buds very early after tuber initiation (*20*). They are thus capable of producing normal sprouts, but fail to do so for a period of several months. Young (1 to 2 cm.) and older tubers can be induced to sprout on or off the plant by low concentrations of gibberellin A_3. Elongation of stolons (underground stems that bear new tubers) and inhibition of tuber enlargement on the new plants result from treatment of potato plants or "seed pieces" (used for propagation) with increasing concentrations of gibberellin A_3 (0.5 to 10 mg. per liter) (*17, 24*). This effect is also seen on begonia plants, which produce aerial tubers. The interaction of indole-3-acetic acid and gibberellin A_3 in controlling growth of underground stems of potato was reported by Booth (*2*), who employed very high concentrations, however. Thus it seems that stolon elongation and tuber set are antagonistic phenomena and that gibberellin A_3 promotes vegetative growth, both stolon and bud. Okazawa (*17*) reached a similar conclusion after finding that gibberellin stimulated stolon elongation and prevented tuber "set." Indeed, the application of gibberellin A_3 directly to growing sprouts stimulates elongation (*19*). In view of the acceleration of sprouting by applied gibberellins A_1 (*18*) and A_3, and the marked increase in native gibberellin content near the onset of sprouting, the simplest working hypothesis to explain the role of endogenous gibberellins in rest of potato would be that the gibberellin available is insufficient for normal cell enlargement and division during the rest period. As rest terminates, gibberellin content increases and with other stimulators, like auxins, accelerates division and elongation of the sprout. This would explain the very slow growth of buds on tubers during rest (*4*) and the rapid elongation of sprouts when rest terminates. This is in accord with Haber's statement (*8*) that arrested growth in seed and perhaps other plant parts "is some subtle block that specifically prevents the initiation of cell expansion." However, for cell expansion in dormant imbibed lettuce seed, at least, Haber concluded that gibberellin was not the primary limiting factor.

That gibberellin alone would produce normal cell division and enlargement in potato buds remains to be seen, as does a clear demonstration that the rise in level of endogenous gibberellins actually precedes sprouting. Whether the increase in gibberellin precedes the reported increase in auxin as rest terminates is also of

interest, in view of Galston's suggestion that gibberellin acts by sparing auxin. Still another possible insight into how gibberellin acts was provided by Lockhart (*12*), who reported that gibberellin A_3, like IAA (*23*), is active in softening cell walls. He showed that gibberellin A_3 increased cell wall plasticity of Alaska pea stem segments in the light (where it stimulates stem elongation) but not in the dark (where it does not stimulate stem elongation of intact plants). Yoda and Ashida (*26*) also found that gibberellin A_3 had no effect on the plasticity of pea stem segments in the dark. The consequent relaxation of wall pressure results in water uptake by the cell and, therefore, in cell enlargement. The necessary cell divisions for sprout growth might be explained in part by the work of Sachs, Bretz, and Lang (*21*), who showed marked increases in mitotic figures in the subapical region of stems of rosette plants 24 hours after treatment with gibberellin A_3.

The speculative nature of these proposals is underlined by the failure of applied gibberellin A_3 to shorten rest in certain species, and actual extension of dormancy in others. Thus, in grape (*Vitis vinifera*) leafing out in the spring is delayed two weeks by foliar sprays applied the previous fall (*27*); the nature of this prolonged dormancy remains to be elucidated. Dormancy in aerial tubers of begonia is actually prolonged by gibberellin A_3 (*15*). However, elongation of buds on these tubers is dependent upon cold exposure, which gibberellin often imitates. In this case, therefore, gibberellin does not replace the cold requirement. Sprouting in potato is not conditioned qualitatively by cold, and is therefore different from sprouting in begonia. Gibberellin A_3 does not seem to shorten rest of certain bulbs and corms (onion and gladiolus) that experience rest. Generally, species not affected by gibberellins have qualitative cold and/or day length requirements for renewed growth. How different gibberellins—those now being elaborated and those yet to be discovered—will act on these species remains to be seen. Probably the action of gibberellins in shortening rest of potato tubers will be related to the interaction with other endogenous stimulators and inhibitors.

Acknowledgment

The technical assistance of Herman Timm is gratefully acknowledged. Seeds for bioassay were generously provided by B. O. Phinney, Department of Botany, University of California, Los Angeles.

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RECEIVED October 20, 1960. Investigation supported in part by grants from Merck & Co., Inc., Rahway, N. J., and the Public Health Service (RG 6682).

Gibberellin, Auxin, and Growth Retardant Effects upon Cell Division and Shoot Histogenesis

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With the advent of the gibberellins the problem of shoot histogenesis, stem tissue formation, has received considerable attention. Apparently a major role of the gibberellins is "regulation" of cell division in the subapical tissues which are responsible for the formation of nearly all the cells of the mature stem. The auxins, too, are intimately involved in the "control" of mitotic activity in these regions and several unknown cell division factors may play an important role in stem formation. As yet there is no clear path toward understanding the mode of action of growth substances. Auxin- and gibberellin-induced mitotic and enzymatic activities appear nearly simultaneously with increased cell wall plasticity, and it seems unlikely that these effects are simply the results of prior auxin effects upon the cell wall. The number and variety of responses elicited by growth substances seem to preclude a simple, master reaction as their cause. Thus, the nature of the tissue treated and the balance of the different kinds of growth substances deserve special consideration.

Histogenesis usually has the connotation of tissue origin or formation, rather than tissue growth, and for this reason it is concerned primarily with cell number rather than cell size. According to this view, in studies of histogenesis, cell division (unless otherwise noted, cell division refers to mitosis as well as cytokinesis) is of greater interest than cell expansion. Yet cell expansion and division are generally overlapping, concurrent events in many tissues. Most growth substances, including the auxins, gibberellins, kinins, and some growth retardants, affect cell division as

well as expansion. A rather thorough discussion is presented here of the relationship between the two processes.

Shoot Histogenesis

A very good place to begin a study of stem formation is in a rosette plant, one that in some part of its life cycle does not have a stem, although after certain treatments it may form a fully elongate shoot. An analysis of stem formation in such plants awaited the discovery and isolation of the gibberellins which cause many rosette plants to form an elongate stem, regardless of prior or current environmental conditions (*16*). In some cases gibberellin replaces the requirement

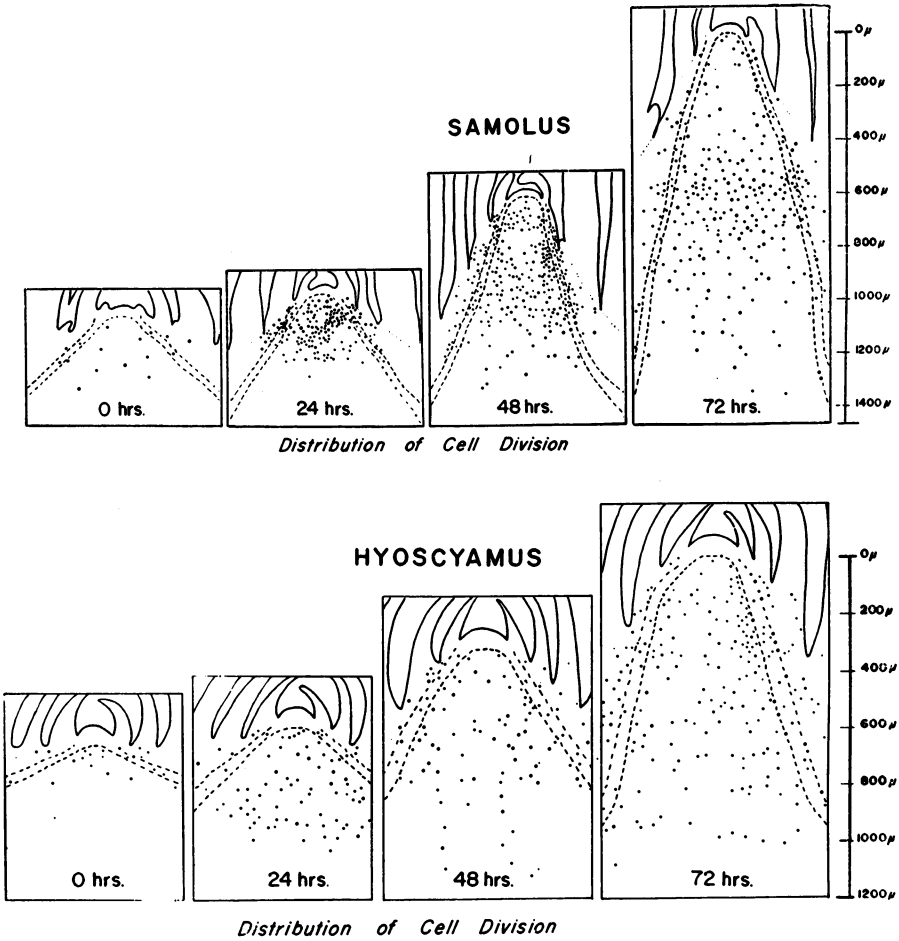

Figure 1. Number and position of mitotic figures in median 64 microns (8 median longisections, 8 microns per section) of apices of Samolus *and* Hyoscyamus *following application of gibberellin*

Each mitotic figure indicated by dot. Pith tissue bounded by apical meristem at top and vascular tissue on sides. Observations for cortical tissue confined to area bounded by outer edge of vascular tissue and line connecting leaf bases. Boundaries for vascular tissue and lower limit of apical meristem . . . indicated by dashed lines.

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for a cold treatment (vernalization), as in *Hyoscyamus niger* var. bienn., and also that for extended day lengths (long day), as in *Samolus parviflorus*. Gibberellins occur not only in fungi but in higher plants as well (*1, 14, 16, 34*) and are probably responsible for the normal control of bolting (*10, 11*) in many rosette plants.

How do gibberellins incite stem formation? Shortly after the first application of gibberellin (in this paper gibberellin refers only to gibberellin A_3, gibberellic acid) to *Samolus* or *Hysocyamus*, and for several days or weeks thereafter as the treatment continues there is a considerable increase in cell division activity in the subapical tissues (*12, 19, 20*, Figure 1). It is this induced subapical mitotic activity and not that of the apical meristem which is responsible for the formation of practically all of the cells of the elongate shoot. Apparently the major difference between the bolting and rosetted plant lies in the extent of mitotic activity in the subapical tissues, including the epidermis, cortex, vascular strands, and pith. Cells also expand as a result of gibberellin treatment, but they expand equally in the untreated plants; thus, mitotic activity is not obviously related to cell size and the effect of gibberellin must be more directly concerned with the inception of mitosis or preservation of mitotic capability. In fully vernalized, bolting *Hyoscyamus* there is still considerable response to exogenous gibberellin; indeed, the stems of gibberellin-treated vernalized plants are generally twice as long as those of untreated vernalized plants. The difference in height is due in part to greater cell length and in part to greater cell number in the gibberellin-treated stems (Figure 2). Although flower initiation and development are the same in both groups, apparently the untreated, vernalized plants do not produce enough gibberellin to support maximum cell division and cell expansion below the apical meristem. For this reason gibberellin-induced flower initiation and shoot growth deserve separate consideration in questions concerning the distribution and mode and site of action of growth substances.

In plants with an elongate stem (caulescent plants), cell divisions extend far into the subapical region of the growing shoot (*21*), often to 1 cm. and sometimes

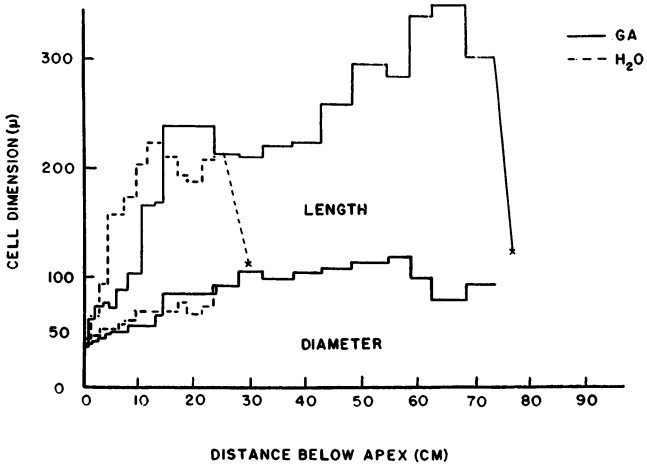
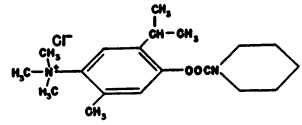


Figure 2. Pith cell dimensions in shoots of Hyoscyamus niger *var. bienn. 5 weeks after removal from cold treatment*

———— *Measurements from plants having received 10 μg. GA daily*
X marks base of stem.

as much as 10 cm. (Table I, A and B). Gibberellin treatment in such plants has little effect upon mitotic activity in the subapical region. However, certain quaternary ammonium carbamates—in particular 4-hydroxy-5-isopropyl-2-methyl-phenyl-1-trimethylammonium chloride-1-piperidinecarboxylate (Amo-1618)—



greatly reduce stem elongation in many caulescent plants (35) without seriously interfering with other growth processes (leaf formation, etc.) and this effect is counteracted by application of gibberellin (3). A cytohistological study of the effects of Amo-1618 (Figure 3 and Table I) alone, combined with, or followed by gibberellin, revealed that Amo suppresses the subapical mitotic activity of chrysanthemum shoots completely without materially affecting that of the apical meristem proper, and that gibberellin prevents or reverses this effect, maintaining or restoring the normal level of subapical cell division (22).

Apparently there exists in plants in general a rather clear-cut division of labor in the growth of the stem; the apical meristem is the site of shoot organogenesis (leaf initiation and tissue pattern determination) but its direct contribution to stem growth is small; the great majority of cells that actually constitute the mature

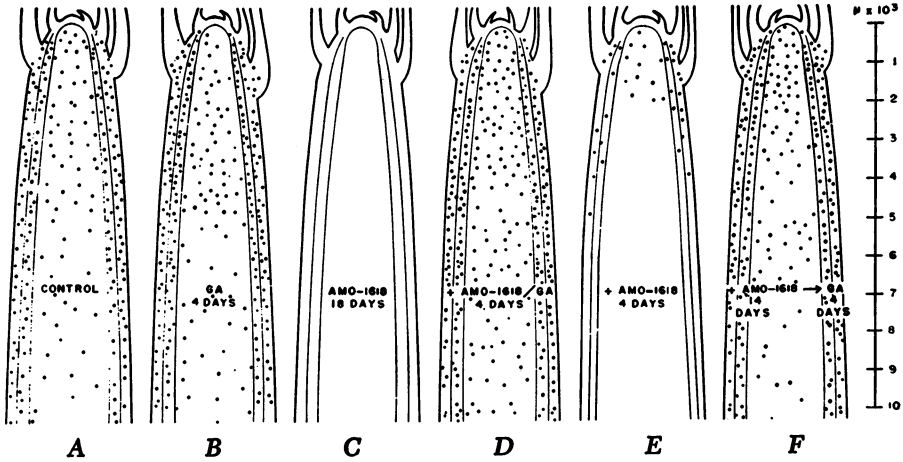


Figure 3. Number and position of mitotic figures in median 60 micron (6 median longisections, 10 microns per section) of apical portions of shoots of Chrysanthemum morifolium *var. Crystal Queen*

A. *Control*
B. *Treated with GA, collected 4 days after treatment*
C. *Treated with Amo-1618, collected 4 days after treatment*
D. *Treated with Amo-1618 plus GA, collected 4 days after treatment*
E. *Treated with Amo-1618, observed 18 days after treatment*
F. *Treated with Amo-1618, after 14 days, followed by GA, observed 4 days after application of GA*

Each dot represents transverse mitotic figure. Pith tissue bounded by apical meristem at top and vascular tissue on sides. Boundaries for vascular tissue (and lower limit of apical meristem) indicated by thin lines. Observations for cortical tissue confined to area bounded by outer edge of vascular tissue and line connecting leaf bases. Most leaf bases and vascular traces not shown

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Table I. Apical and Subapical Meristematic Activity

			Transverse Cell Divisions per 60 μ Pith Tissue		
		Treatment	*Subapical meristem*	*Apical meristem*	*Lowermost mitotic figure, μ below Apical meristem*
A.	*Xanthium pennsylvanicum*	Control	180	9	>10,000
		MH	0	0	...
		MH + GA	0	0	...
B.	*Chrysanthemum morifolium*[a]	Control	100 ± 25	8	>11,000
		GA 14 days	100 ± 20	8	>11,000
		Amo 14 days	0	6	...
		Amo + GA 14 days	100 ± 20	6	>11,000
C.	*Mathiola incana* var. Avalanche	Control	27	7	4,000
		GA	53	8	8,000
D.	*Gerbera jamesonii*[a]	Control	180 ± 20	..	>40,000
		Decap	15	..	2,000
		Decap + GA	100 ± 20	..	>40,000
		Decap + IAA	100 ± 20	..	>40,000
		Decap + GA + IAA	180 ± 20	..	>40,000
E.	Bamboo	Control	700	..	>40,000

[a] Errors quoted are standard errors.

A. 2-week-old vegetative plants treated with 100 μg. daily (applied to apical cluster of leaves) of maleic hydrazide (MH) or 100 μg. each of MH and gibberellin (GA). Apical pieces examined after 7 days.
B. Data taken from experiments illustrated in Figure 3.
C. Apical pieces collected from control and GA-treated plants (sprayed weekly with 100 μg. of GA per liter) 3 weeks after beginning of treatment.
D. 5 cm. scapes decapitated and treated with lanolin pastes containing GA, IAA, GA + IAA, or H_2O. Collected 2 days after decapitation and a 30 micron slice including all stem tissues examined.
E. 5-cm. shoot collected and 1000 micron wide column of pith tissue examined. Since pith tissue was frequently ruptured, mitotic figures recorded represent only a fraction of those present. Moreover pith tissue is considerably less active than the more peripheral tissues, indicating that total subapical mitotic activity in growing bamboo shoots may attain extraordinary proportions.

stem are formed by cell division activity in the subapical region (Table I), which can be considered as an intercalary meristem in its own right, and the site of shoot histogenesis. Moreover our results strongly suggest that a major function of gibberellin in the plant is the regulation of this subapical meristem. Amo-1618 seems to function as a gibberellin antagonist, at least in this particular action of gibberellin; thus the role of native gibberellin antagonists deserves consideration in studies of shoot development (*4*).

For additional information concerning shoot histogenesis we have turned to still another type of stem, the scape, the sole function of which is to support and transport nutrients to a flower. Since from the very earliest stages the apical meristem is in the reproductive, flower-producing state, there are no leaves or axillary buds appended to the scape. Yet the structure of scapes—e.g., those of the tulip, dandelion, and English and Transvaal daisies—is precisely the same as for other leaf-bearing stems, only simplified by the absence of a branched vascular system. But of particular importance is the fact that in these plants the apical meristem contributes only to flower development; thus, essentially every cell of the elongate scape is derived from cell divisions in the subapical region. Direct cytological evidence has been obtained for a zone of meristematic activity which, at some stages during scape development, extends 5 cm. or more below the expanding flower bud; thus, even with regard to the cytological details of its development, a scape is very much the same as a leaf-bearing shoot (Table I, D).

Of greater interest has been the discovery, for the Transvaal daisy (*Gerbera jamesonii*), that if the flower bud is removed by decapitation at any stage during

scape development, elongation stops immediately, and, as expected, cell division and expansion cease as well. Apparently something(s) diffusing down from the flower bud into the subapical tissues is necessary for normal cell division and expansion, and hence, for scape development. What does the flower bud produce? As yet no analyses have been made, although a lanolin paste containing 0.1% each of gibberellin and auxin applied to decapitated scapes supports nearly the same elongation as if the flower bud were attached. Scape growth is reduced if gibberellin and auxin are applied separately, although the response to either substance is still considerable. There is definitely no synergistic effect; in fact, the response to simultaneous application is less than the sum of response to either alone. Cell division and elongation proceed normally in the gibberellin-auxin scapes for about 6 days following decapitation (Table I, D), after which time cell division declines rapidly (mitosis is likewise inhibited, since binucleate cells have never been observed) and only cell expansion continues for another 10 to 14 days. Apparently then, there is still another shoot growth substance(s) necessary for normal cell division and probably produced in the developing flower bud. In the development of the structurally related tulip scape, gibberellin is without effect, whereas auxin, though promoting scape elongation, supports cell expansion alone; here again is evidence for still another factor(s) required for normal scape development which includes both cell division and expansion.

An auxin effect similar to that for *Gerbera* has been found for stem elongation in rosette plants. Completely defoliated plants do not bolt or bolt to a very slight extent when supplied with gibberellin alone, yet a small amount of auxin added to the gibberellin promotes much greater shoot growth (*23*). If leaves less than 10 mm. long are left attached to the plant, the auxin effect disappears; apparently both gibberellin and auxin are required for stem formation, the auxin normally being supplied by the young leaves. In defoliated plants, too, stem formation involves subapical meristematic activity as well as cell expansion, both of which are considerably promoted by the combined treatment with gibberellin and auxin. Thus, in this case too, auxin must play some role in mitosis, perhaps similar to the one it plays, alone or in combination with kinetin, in promoting cell division in tobacco pith tissue cultures (*15*). A similar example of a dual gibberellin-auxin requirement is that for pea stem growth (*31*); in this case gibberellin-promoted shoot elongation is greatly reduced if the apical meristem, including the terminal cluster of leaves, is removed. The inference is clear that the apical portion supplies the auxin required for optimal response to gibberellin. However, the situation is not clearly in favor of a role of auxin in mitosis, since the total growth observed may have been a result of cellular expansion.

Even with gibberellin and auxin the growth of the defoliated or decapitated plants is significantly less than that of the intact ones, suggesting that, as with *Gerbera*, still other growth factors must be accounted for. In two other cases dual roles of auxin and gibberellin in mitosis are clearly indicated.

Cellular proliferation in citron mesocarp tissue, cultured in vitro, is greatly promoted in the presence of gibberellin and auxin (*24*).

In dormant *Acer* shoots the cambium is activated in striking fashion by treatment with gibberellin and auxin (*32*).

Although in the latter case gibberellin appears to be primarily responsible for the increased mitotic activity and auxin for differentiation of the newly formed cells, auxin may also be involved in the primary cambial activation mechanism (*25*). After the discovery of the gibberellins several studies showed conclusively that auxin and gibberellin have separate, sometimes additive and sometimes syner-

gistic, effects upon stem growth (7). For example, in pea stems the effect appears to be synergistic—i.e., auxin and gibberellin applied simultaneously give a greater response than the sum of the growth effect of either applied alone. There is an interesting complication in etiolated pea stem sections, where the effects of auxin and gibberellin appear to be additive; younger tissues show a greater response to gibberellin, whereas older sections respond more to auxin. This phenomenon has not been described for other plant tissues and the cellular basis for the differential response is unknown—e.g., is gibberellin more active at an early stage of cell expansion and auxin more active in the later stage? Petiole growth in the sweet potato is stimulated by gibberellin, yet if the leaf blade is removed, the gibberellin effect is greatly reduced. However, if the auxin is applied at the cut petiole tip in place of the detached leaf, the petiole now responds to gibberellin. In these cases growth is probably the result of cell expansion alone.

Several different kinds of growth substances must now be incorporated into our picture of the control of shoot growth. Alkyl lipides, such as methyl linoleate (27), and long-chain aliphatic alcohols, such as octadecanol (30), have significant growth-promoting properties at relatively low levels. Since the methyl linoleate–promoted growth has been demonstrated in the presence of optimal concentrations of gibberellin, auxin, and several other pea-stem section growth factors, it is apparent that such lipides are truly new shoot growth substances. The much greater promotive effect of gibberellin on pea stem sections in the intact plant as compared with the excised stem pieces, suggests that still other unknown factors are involved in shoot growth, perhaps related to the much discussed, hypothetical caulocaline (2, 33).

Growth and Mode of Action of Growth Substances

So far little has been said regarding the general nature of cellular growth and the mode of action of growth substances—truly the central problems for current and future research. At high levels of organization growth is recorded simply as an increase in volume, yet at the cellular level we are quick to recognize that cell expansion (increase in volume) and cell division (increase in number) are two separate processes, both contributing to the growth of a tissue, organ, and organism. There are several reasons why we should emphasize the differences between cell expansion and cell division:

There is a limit to the size a cell may attain, and ultimately growth is limited by the number of cells available for expansion.

The orientation of cells and thus the direction of growth are determined sometime prior to or during mitosis.

Cell division is ordinarily limited to the meristems, whereas cell expansion occurs over a much greater part of the plant—i.e., cells normally differ in their capacity to divide or expand.

Cell expansion may at times require little more than "wall softening" and increased water uptake, whereas cell division and mitosis is always preceded by cytoplasmic and nuclear syntheses and involves complex chromosomal movements.

Hence, differences between cell division and expansion suggest that factors specific to one or the other process may act entirely independently of one another.

The original proposal concerning the mode of action of auxin stated that auxin made cell walls more plastic, thus inducing a water-diffusion-pressure deficit and causing the cells to expand (7). Much evidence has since been gathered in support of this hypothesis. For example, in Avena coleoptiles it has been established that the auxin-induced increase in plasticity precedes, or coincides with, the

auxin-induced promotion of growth (*28*). In fact it has been suggested that the primary site of auxin action—i.e., the locus of the master, auxin-controlled reaction—is in the cell wall and that all other auxin-induced effects are the results of this initial effect upon the cell wall.

What are some other auxin-induced phenomena? Considering only the cellular or molecular effects, one finds that indoleacetic acid, believed to be the major auxin in plants, may induce the formation of an oxidase which can destroy auxin, preserve the selective permeability of cell or vacuolar membranes, induce the formation of a peroxidase, which is perhaps active in lignification and xylogenesis, increase the rate of protoplasmic streaming and decrease cytoplasmic viscosity, and induce mitosis (*7*). Many of these auxin effects are observed within the same time intervals as that of increased wall plasticity and it is difficult to understand how they may be simply the results of a change in cell wall properties. It is often implied that a cell is "triggered" so that of necessity mitosis will begin as soon as a cell attains a certain critical size; in this view the control of cell expansion is an indirect method of controlling cell division. There are numerous reports that there is no strict dependency of cell division upon cell size (*5, 17, 18, 22*), and hence some other relationship between expansion and division and another type of mitotic trigger must be sought.

A certain amount of evidence shows that methyl group metabolism may be affected as a result of auxin treatment—generally increased pectin-methyl esterification is observed (*7*). Though pectin-methyl esterification may be correlated with increased wall plasticity, there are as yet no obvious connections between general methyl group availability on the one hand and permeability, peroxidation, and mitosis on the other.

No matter how unsatisfactory it seems from the standpoint of working with the simplest hypothesis, one must consider the possibility that there is no master, auxin-controlled reaction; rather several reactions may separately or in concert account for the various auxin effects. In this view it is not surprising that the type of response evoked by auxin is very dependent upon the tissue in question.

Approximately the same arguments concerning the "master, gibberellin-controlled reaction" can be advanced. Recently it has been demonstrated that gibberellin increases the plasticity of pea stems (*13*), and perhaps in such systems, where growth is due primarily to cell expansion, the total gibberellin effect may be explained by this action. Yet other studies show that gibberellin induces mitotic activity at least as rapidly as it does pea-stem plasticity (*20*); for this and other reasons cited for auxin, it is difficult to accept the thesis that gibberellin-induced cell division is the result of a prior effect upon the cell wall.

In several cases cell expansion and division have been separated. For example, maleic hydrazide, a powerful mitotic inhibitor, is completely without effect upon cell expansion in wheat seedling or lettuce seeds (*8, 9*)—the cells expand even in the presence of maleic hydrazide at levels preventing mitosis and, moreover, still retain the capacity to respond to gibberellin. The argument has been advanced that in these cases ". . . the growth-stimulating effects of gibberellin are caused solely by a direct effect upon cellular expansion; thus the apparent effects on cell division are results, and not causes, of such growth stimulation (*9*)." Two assumptions underlying this statement must be proved before it can have general validity: There is only one mechanism limiting mitosis and only one gibberellin-controlled reaction. For an alternative explanation of the maleic hydrazide–gibberellin effects, one need only suppose that several independent mechanisms must be completed before a cell is ready to enter mitosis (*6*), only one of

which is blocked by maleic hydrazide and another is promoted by gibberellin. In such cases, even if there were a master gibberellin-promoted step common to both cell expansion and mitosis, maleic hydrazide, though blocking mitosis, would not be expected to affect cell expansion. Other studies with maleic hydrazide and gibberellin in no way conflict with this latter interpretation (*21*, see Table 1, A).

There are several examples of mitosis and/or cell division in the absence of cell expansion—the early pregastrula cleavages of amphibian embryos; the septation of free-floating nuclei in liquid endosperm; and the internal divisions of large, free-floating cells in liquid culture (*26, 29*). In such cases there must be specific cytokinetic or mitotic factors, the action of which need not be related in any way to cell expansion.

Thus, the picture emerging is rather complex: Several factors, not necessarily closely related chemically, may limit cell expansion and/or cell division; and the response of a tissue to an exogenous growth substance may depend not only upon the endogenous level of that compound but probably upon the levels of all other "growth factors" at the time of treatment.

Acknowledgment

Much of the work and many of the ideas reported in this paper are the result of close, continuing cooperation with Anton Lang, and both of us are indebted to Charles Bretz and Joan Roach for excellent technical assistance.

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RECEIVED October 20, 1960.

Relationship of Gibberellic Acid to Enzyme Development

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The addition of 1 to 3 mg. of gibberellic acid per kg. of barley during the early stages of germination has marked effects on the enzymes of barley: increase in respiration, α-amylase, diastatic power, protease, cellulase, catalase, and transaminase systems, and decrease in the dextrin-maltose ratio. Stimulation by gibberellic acid is not limited to the embryo alone, but separated endosperm liberates more reducing sugar upon treatment. Treatment of bean seedlings with gibberellic acid increases phosphatase. The total carbon content of wheat seedlings is increased by gibberellic acid addition, but increase in photosynthesis could not be demonstrated. Recent work on endogenous gibberellic acid in germinating barley kernels points out the distinct possibility that the biological form of gibberellic acid may be a bound or complex form.

In the more than 30 years since the potentialities of the plant auxins were discovered and widely recognized as natural growth promoters in plants, no plant hormones have excited as much botanical and horticultural interest as the gibberellins. In all this time, despite numerous and able research efforts (*8*), the primary mechanism of action of the auxins has not been elucidated. This failure is undoubtedly due to the tremendous difficulties which stand in the way of such investigations; these include the extraordinarily low concentrations of auxins that produce the growth effects being measured and the probable subtlety of mechanism involved. The same is true of the gibberellins, although active research on the role of these compounds in plant physiological processes has not been under way for as long a time.

An examination of the differences in the physiological responses to auxins and gibberellic acid allows for a differentiation between these two classes of plant growth regulators. It can be seen from Table I (*5*, *8*) that distinct differences exist, in that gibberellic acid does not undergo polar transport and has no effect on rootlet growth or leaf abscission. Gibberellic acid has a pronounced effect on germination, bolting, and flowering, whereas auxin does not. The effect of gib-

berellic acid seems to be a general response by the plant rather than the more localized effects produced by auxin.

Table I. A Summary of Major Differences between Auxins and Gibberellins (8, p. 240)

		Auxin	*Gibberellin*
1.	Transport polar	Yes	No
2.	Promote root initiation	Yes	No
3.	Inhibit root elongation	Yes	No
4.	Delay leaf abscission	Yes	No
5.	Inhibit lateral buds	Yes	No
6.	Induce callus formation	Yes	No
7.	Promote epinastic responses	Yes	No
8.	Promote growth of intact plants, especially of the dwarf variety, and of monocotyledonous leaves	No	Yes
9.	Promote seed germination and breaking of dormancy	No	Yes
10.	Promote bolting and flowering in nonvernalized biennials and in long-day plants	No	Yes

A brief review of the reported metabolic effects of the gibberellins suggests that these compounds exist in the growing plant in an interrelationship with the other plant-growth regulators. The responses observed when gibberellic acid is added to a plant may be the result of direct action of gibberellic acid on the plant enzymes or on other plant-growth regulators. No clear-cut linkage of the gibberellins with a metabolic pathway has yet been established, but some of the observed responses to this compound are:

1. Alteration of the carbohydrate constituents (*6, 12, 26, 29*)
2. Reduction of chlorophyll content (*6*)
3. Dislocation of nitrogen metabolism (*7, 31*)
4. Promotion of respiration in seeds (*12, 15, 28*)
5. Variation in the level of certain enzymes (*12, 28*)
6. Reversal of maleic hydrazide inhibition (*6, 13, 14*)

Although the effects of the gibberellins have for the most part been observed in higher plants, growth-promoting effects on liverworts (*2*), mosses (*27*), algae (*23*), and ferns (*16*) have been reported. Some reports state that fungi and bacteria are not influenced by purified gibberellins. But applications of gibberellin modified the appearance of plants infected by fungal and viral diseases; the effect could be on the plant itself, rather than on the disease organisms. Gibberellic acid is apparently destroyed by soil microorganisms (*6*), but its application to soil is reported to have modified the microbial population to favor growth of the nitrogen-fixing bacteria *Azotobacter* (*18*). The effects of gibberellins on mushrooms and yeast have been briefly reported (*26*). This information, and data which indicate that hormonal levels of gibberellin promote the growth and respiration of the BCG strain of *Mycobacterium tuberculosis* (*10*), suggest that work on microorganisms should not be abandoned.

It is surprising that so little basic work concerning the relationship of gibberellic acid to enzyme function and synthesis has been reported. The field of study is very fertile and should be extremely rewarding for an individual who wishes to investigate some of the basic plant growth–regulating processes.

One of the earliest reports of the use of gibberellic acid on germinating grains appeared in 1940. Hayashi (*11*) reported that gibberellin stimulated the germination of barley, wheat, and rice grains, and in so doing, increased the amount of amylase in the germinated barley and wheat grains. The addition of gibberellin

to a malt solution did not activate the amylases. Because the amylolytic power of malt increased during the earlier period of germination, Hayashi felt that it might be suitable for the manufacture of malt.

Yabuta and Hayashi (*30*), extending these investigations, found that the agent increased the saccharifying activity per unit amount of dry matter, although the malting loss of the treated material was higher than that of the control, and concluded that the promotion of saccharifying activity was due to stimulation of the mechanism of enzyme formation in the early stages of germination. Although much of the early work with barley was performed with mixtures of gibberellins and what are now known to be degradation products, it does not detract from the value of the work. In their first work Munekata and Kato (*19*) were using gibberellins A (a mixture of A_1, A_2, and A_3) and B (probably allogibberic acid). When barley was steeped in a solution containing 10 p.p.m. of gibberellin B, or sprayed with this solution, or a combination of both treatments was applied, an increase in the α-amylase content and elongation of rootlets were observed in the early stages of germination. The effect was lost by the fifth day of germination. Gibberellin B showed only a slight effect on the growth of acrospires and the activity of β-amylase and protease at the same stage of germination. Gibberellin A had a definite effect at a concentration range of 0.5 to 5.0 p.p.m.; the mixture increased the growth of acrospires, α-amylase activity, β-amylase, protease, and catalase. These effects markedly differed according to the variety of barley used in the test. The increase in enzyme yield was more significant if the gibberellins were added before the malting process was half completed. The results obtained by Munekata and Kato (*19*) are listed in Table II. The barley they used is a much poorer germinating variety than those commonly used in the United States.

Table II. Effect of Gibberellin Concentration in Mixture on Enzyme Formation

	1.0 p.p.m.	*5 p.p.m.*	*20 p.p.m.*	*H_2O*
72 hours α-Amylase[a]	210	240	280	100
β-Amylase[a]	22.2	20.4	19.2	15.0
Protease[a]	165	105	110	80
96 hours α-Amylase	300	390	310	350
β-Amylase	21.9	24.0	22.5	21.3
Protease	200	145	195	140
138 hours β-Amylase	...	34.9	35.7	30.8
Protease	...	274	210	252
Catalase[b]	...	267	270	170

[a] Units developed by author and unknown to reviewer.
[b] Ml. of 0.01*N* $KMnO_4$ required to titrate 10 ml. of 0.25*N* H_2O_2 plus 2 ml. of malt extract.

Nielsen and Bergqvist (*20*) investigated the action of gibberellic acid on the germination of barley, wheat, timothy, rape, and peas. One hundred grams of seeds (fresh weight) were soaked for 48 hours in 150 ml. of distilled water, or in solutions of gibberellic acid at different concentrations (1, 3, 10, 30, and 100 p.p.m.). Thin layers of seeds were placed in large Petri dishes in order to secure an adequate supply of air, and were kept in the dark at 25° C. After soaking, the seeds were transferred to glass containers through which carbon dioxide–free air was blown and respiratory carbon dioxide formed was absorbed in soda lime in the usual way. The production of carbon dioxide was measured at different times up to 50 hours, calculated from the end of the soaking period. From each measurement the carbon dioxide formation during one hour was calculated.

For all the seeds investigated, respiration was stimulated by treatment with

gibberellic acid. Rape seeds treated with a 10-p.p.m. solution of gibberellic acid showed about 10% increase in respiration. With all the other seeds the stimulation was greater, especially for barley and wheat. Barley seeds treated with a 10-p.p.m. solution of gibberellic acid gave a respiratory value after 24 hours that was twice as high as the control. Even a 1-p.p.m. solution gave a measurable stimulation. The data obtained from these experiments are presented graphically in Figure 1; only the three lower concentrations are listed, as concentrations above 30 p.p.m. showed little, if any, additional stimulation (*20*). About 4 hours after the seeds have been transferred to the containers the stimulation of respiration, caused by the added gibberellic acid, is manifest and continues to increase throughout the entire 50 hours.

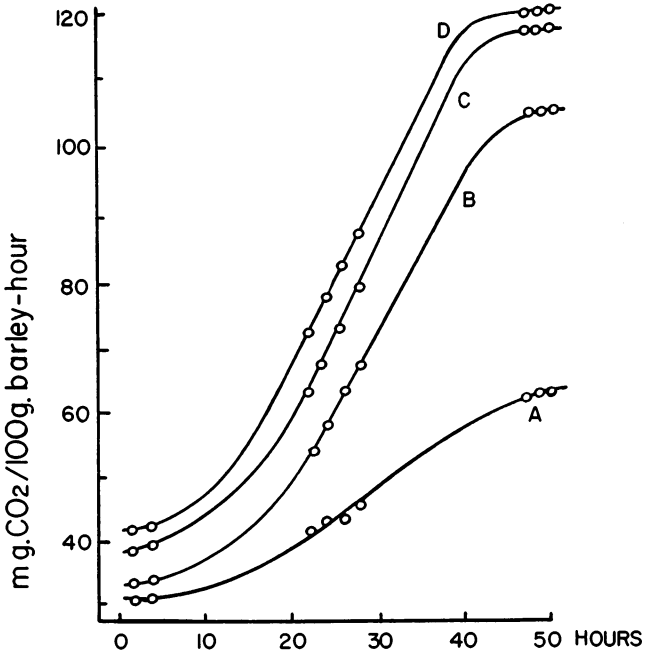

Figure 1. Respiration of 100 grams of barley (fresh weight) after treatment with gibberellic acid

A. *Control without gibberellic acid*
B. *1 p.p.m. gibberellic acid*
C. *3 p.p.m. gibberellic acid*
D. *10 p.p.m. gibberellic acid*

Figure 2 shows the respiration rate for barley 24 hours after completion of soaking in different concentrations of gibberellic acid (*20*). A marked stimulation of carbon dioxide evolution occurs between 0 and 3 p.p.m. of gibberellic acid, whereas the response is less at higher concentrations. The authors suggest that the evolution of carbon dioxide may be used for quantitatively determining gibberellic acid.

Sandegren and Beling (*25*) found that they could reduce the total amount of gibberellic acid required for maximum stimulation by first soaking the barley in water and then wetting it with a gibberellic acid solution (Table III). The final concentration per unit weight of barley was the same as other workers had used,

but the total amount was much less, since the ratio of water to barley in steep is much greater than that used during germination. Here again the data indicate the marked stimulation of enzyme systems resulting from the treatment with gibberellic acid. Their work was the first to show an increase in cellulase activity. The last column of Table III shows a reduction in viscosity which confirms the change in dextrin-maltose ratio (*19*). It further suggests that the synthesis of the entire group of enzymes involved in starch breakdown is stimulated by the addition of gibberellic acid.

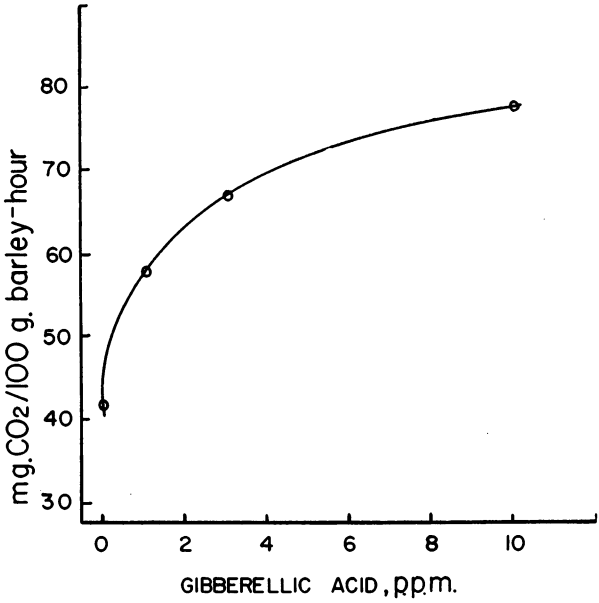

Figure 2. Respiration of 100 grams of barley (fresh weight) after 24 hours

Table III. Analysis of Malt Enzymes of Herta Barley

GA, Mg./Kg. Barley	H_2O,[a] %	*Kolbach No.*	*D. P.*	*α-Amylase*	*Protease*	*Cellulase*	*Wort Viscosity, Cp.*
0	5.4	34	385	32	110	330	1.686
2	5.3	48	465	51	115	435	1.541
3	5.0	50	450	51	115	510	1.515
4	5.2	48	470	52	120	540	1.535

[a] All analyses performed according to European Brewing Convention standard methods of analysis (*1*)

Bawden, Dahlstrom, and Sfat (*4*) reported on a considerable number of tests with gibberellic acid; the results demonstrated the growth-regulating properties of this compound. The addition of trace amounts of gibberellic acid to the common varieties of malting barley stimulated the acrospire growth and increased respiration, α-amylase, β-amylase, and protease. Even the highest concentrations of gibberellic acid were without effect on rootlet growth.

In view of the decided response of barley to this regulator, further investigation was necessary to determine the rate at which gibberellic acid affects some of these systems. Figures 3, 4, and 5 show the pronounced effect on increasing the

diastatic power, α-amylase, and protease (*4*). In these experiments, barley had been steeped to about 40% moisture and then gibberellic acid applied at a concentration of 1 mg. per kg. of barley, Samples were removed each day from the germination chamber of enzymatic analysis. There is an immediate and rapid stimulation of enzyme formation which continues higher throughout the course of the experiment.

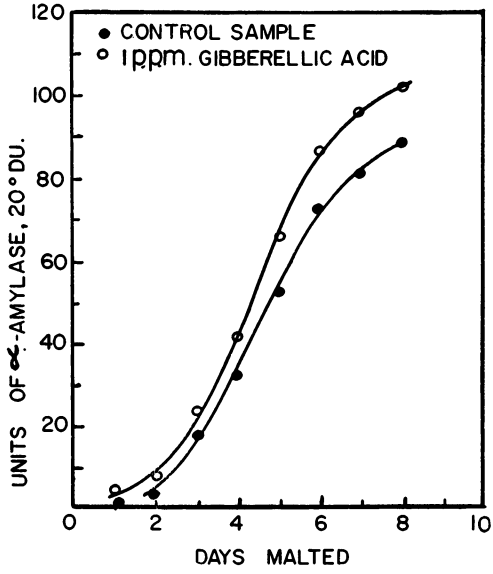

Figure 3. Effect of gibberellic acid on α-amylase

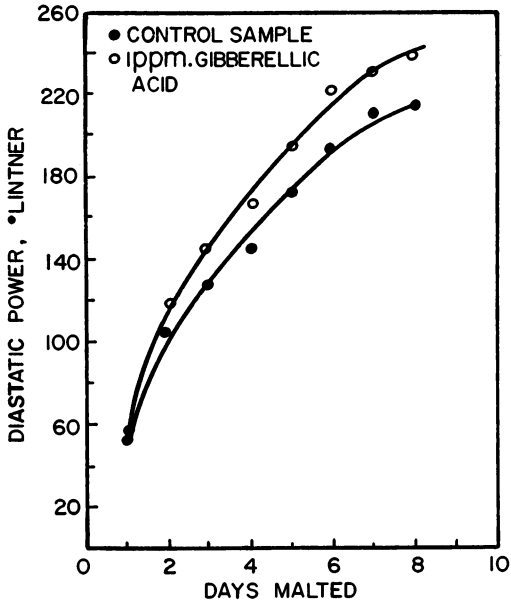

Figure 4. Effect of gibberellic acid on diastatic power

Paleg (*21*) in attempting to integrate the action of gibberellic acid with the normal physiological process of the barley kernel, removed the husks from the seeds, cut them in half transversely, and incubated them for 18 hours in Petri plates containing water. Following incubation, each sample was rinsed thoroughly with water, blotted, and placed in Warburg flasks containing solutions similar to those of the initial soaking period. Respiration experiments were conducted with

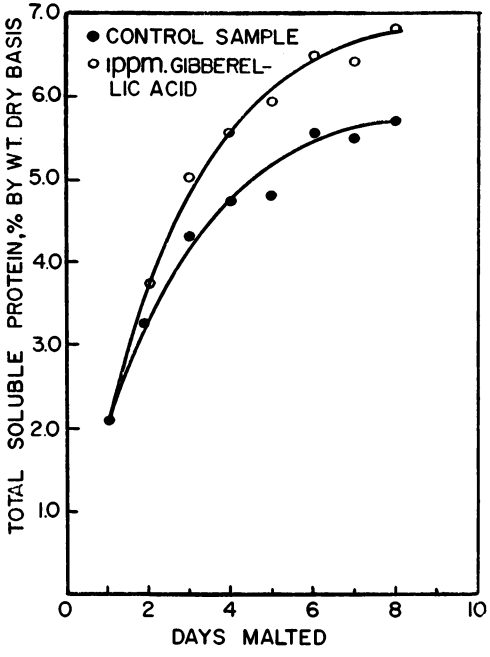

Figure 5. Effect of gibberellic acid on total soluble protein

potassium hydroxide in the center well to determine the rate of oxygen consumption by the endosperm. Rate of oxygen consumption was proportional to the concentration of gibberellic acid. As it has been shown (*3*) that endosperm tissue is essentially devoid of respiratory activity, Paleg examined the grains for bacterial contamination and found that the oxygen uptake was definitely associated with the presence of a very large number of an unidentified, rapidly reproducing strain of bacteria. Gibberellic acid did not stimulate the respiration of the endosperm in the absence of bacteria, but apparently produced or liberated something from the endosperm which enabled the bacteria to respire and reproduce readily. The flasks contained at most 200 μg. of gibberellic acid, so the observed respiration could not be a function of the oxidation of gibberellic acid. Bacterial contamination was prevented by sterilizing the seeds in 5% calcium hypochlorite solution for 20 minutes, removing the husks, and returning the seeds to the calcium hypochlorite solution for a further sterilization period of one hour. The seeds were then cut in half and placed in a solution containing streptomycin. The treated seeds were incubated at 24° C. for 22 hours with varying amounts of gibberellic acid. As a result of gibberellic acid treatment, the endosperm liberates reducing

sugars into the surrounding medium (Figure 6). Heating the kernel destroys all response to gibberellic acid treatment and production of reducing sugars. Chromatographic identification of the sugars liberated showed glucose, fructose, maltose, sucrose, and two spots similar to maltotriose and maltotetrose.

The carbohydrate composition of barley grain at maturity and during malting has been investigated several times with different varieties. In each case, the almost complete absence of maltose in the ungerminated grain has been noted; hence in Paleg's experiments (*21*) the levels of reducing sugars obtained must be caused by the enzymatic dissolution of starch.

If gibberellic acid is acting upon the endosperm during the presoak to produce more amylase, then the medium surrounding the endosperm should be capable of hydrolyzing starch more efficiently. If the action of gibberellic acid increases the number of molecules of starch that one enzyme molecule will hydrolyze, the water solution around untreated endosperms should hydrolyze starch more rapidly after gibberellic acid is added. Table IV gives the results of such an experiment (*21*). The presence of gibberellic acid in the assay induces almost no increase in amylase activity, but its presence during the endosperm incubation results in a large amount of amylase activity in the solution surrounding the endosperm. These results substantiate the hypothesis that the observed maltose release is due to the activity of amylase and that gibberellic acid increases in the number of active amylase molecules derived from the endosperm itself, in the complete absence of the embryo.

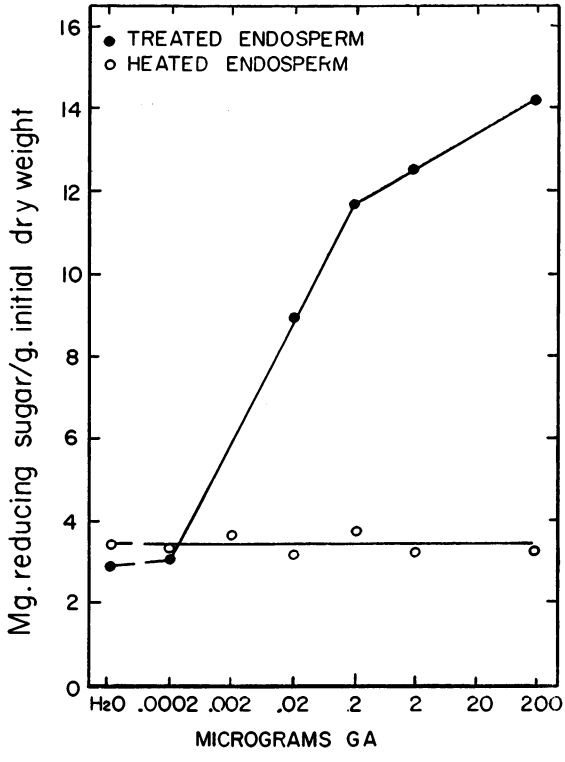

Figure 6. Effect of gibberellic acid on sugar release by barley endosperm

Table IV. Effect of Gibberellic Acid on Liberation and Activity of β-Amylase Derived from Endosperm

Endosperm Treatment[a]	*Assay Treatment*[b]	*Reducing Sugar Produced in 20 Min., Mg./0.5-Ml. Aliquot (as Maltose)*
GA	H_2O	6.59
H_2O	H_2O	0.55
H_2O	GA	0.72

[a] 200 μg. of GA/3 ml. solution added to endosperm.
[b] 100 μg. GA added to assay solution about three times amount that theoretically might have been carried over from solution of GA-treated endosperm.

In examining the action of gibberellic acid, it is necessary to depart momentarily from the work with barley and look at a few enzyme systems in other plants. Gibberellic acid stimulates several enzyme systems in bean seedlings. The results obtained by Weller *et al.* (*28*) are listed in Table V. The plants were treated with 10 μl. of an aqueous solution containing 10 μg. of gibberellic acid; 96 hours after treatment, the plants were harvested and separated into leaves, stems, and shoots. Soluble enzyme preparations were prepared from the desired part and activity was compared with the corresponding untreated control. The data from the enzyme determinations, expressed on a fresh weight basis, indicated that only traces of α-amylase were present, β-amylase was increased in the roots and phosphatase in the leaves and stems, and there was little or no action on pectin methyl-esterase and no apparent increase in phosphorylase.

Table V. Effect of Gibberellic Acid on Certain Enzymes in Various Tissues of Bean Plants

	Leaves		*Stems*		*Roots*	
Enzymes	*Treated*	*Not Treated*	*Treated*	*Not Treated*	*Treated*	*Not treated*
α-Amylase	Trace	Trace	Trace	Trace	Trace	Trace
β-Amylase[a]	81.3	89.4	43.5	54	98.7	72.1
Phosphorylase	Trace	Trace	Trace	Trace	Trace	Trace
Phosphatase[b]	4.5×10^3	3.35×10^3	5.13×10^2	4.69×10^2	Trace	Trace
Pectin methyl esterase[c]	0.52	0.48	0.16	0.22	26.4	59.4

[a] Mg. soluble starch converted to maltose by enzyme per g. tissue in 1 hr. at 25° C. and pH 4.5.
[b] Mg. phosphorus liberated from β-glycerophosphate by enzyme per g. tissue in 1 hr. at 25 C. and pH 5.8.
[c] Mg. methoxyl group liberated from pectin by enzyme per g. tissue in 1 hr. at 25° C. at pH 7.0.

Sandegren (*24*) reported that addition of gibberellic acid to germinating barley increased the transaminase systems, glutamic-oxalacetic and glutamic-pyruvic, but gave no quantitative data. The workers at the Barley and Malt Laboratory in Madison, Wis., have, for several years, been working to correlate the transaminase activity of the barley variety with its germination energy. Thus far, no clear correlation seems to exist. In view of the fact that gibberellic acid does stimulate the transaminase systems and germination, it will be interesting to see if the compound is of value in clearing the picture.

Brian *et al.* (*6*) reported an increase in the total carbon content of wheat seedlings (roots, whole plants, and shoots) and attributed it to an increase in photosynthesis caused by gibberellic acid. However, Haber *et al*, could not demonstrate an increase in photosynthesis. These workers (*9*), by following the rate of $C^{14}O_2$ fixation after allowing basal ends of detached leaves to bathe in a gibberellic acid solution, found that total $C^{14}O_2$ fixed per leaf was unchanged by a 2-hour treatment with gibberellic acid Examination of autoradiograms of the

chromatograms of extracts from oats, barley, and peas showed no significant difference in the distribution of Carbon-14 among the soluble products within any one species.

The variety of enzyme syntheses which are stimulated by the addition of gibberellic acid does not preclude the interaction of gibberellic acid with these enzymes, but it suggests that the mechanism of stimulation is more deep-seated. The stimulation must then occur at the level of synthesis of the enzymes. This immediately suggests that all enzymes are synthesized at one locus or that gibberellic acid is converted to several active forms, each form stimulating the synthesis of a specific enzyme or group of enzymes. It is not difficult to postulate the interconversion of gibberellins A_1, A_3, and A_5; and A_2 and A_4. This has, in fact, already been done by Phinney and West (22).

$$\text{Gibberellin } A_5 \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{gibberellin } A_1 \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \text{gibberellin } A_3$$

$$\text{Gibberellin } A_4 \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{gibberellin } A_2$$

It is conceivable that several more than the five known gibberellins can and probably do exist. It might be, then, that rather than gibberellin stimulating one locus that synthesizes enzymes, a specific gibberellin stimulates the locus of synthesis of a specific enzyme or closely related systems of enzymes.

Recently, Lazer, Baumgartner, and Dahlstrom (*17*) were able, by an isotope dilution derivative labeling procedure, to demonstrate that gibberellic acid occurs naturally in germinating barley, probably in a bound or derivative form, and this may well be the biologically active form. In two separate analyses for endogenous gibberellic acid, these workers found a larger than expected spread, even considering possible sample variations. There was no indication of the presence of any measurable amount of gibberellin A_1 in either sample. On the other hand, the radiochromatograms of the two samples showed a marked difference between the radiopeaks at R_{ga} 0.65 and R_{ga} 2.33 (Figure 7). Both samples had been treated identically, except for the time element. As a result, the materials in the first analysis had been exposed to an acidic pH for a longer time. This suggested that the gradual hydrolysis of a natural derivative, or precursor, might have influenced the results. A third analysis was carried out, in which one half of the material after extraction was allowed to stand at room temperature (pH 2.5) for 24 hours and the other half was processed in about 8 hours. The corresponding radiochromatograms are shown in Figure 8. The sample with the shorter exposure to the acidic pH (sample IIIb) showed a marked radiopeak at R_{ga} 0.65, but contained little or no gibberellic acid. The radiopeaks at R_{ga} 1.25 and R_{ga} 2.33 were absent. On the other hand, the sample which had been allowed to stand at the acidic pH (sample IIIa) showed a high concentration of gibberellic acid (45 p.p.b.), but little or no radioactivity within the R_{ga} 0.65 range. It remains to be determined whether we are dealing with a pH-sensitive enzymatic reaction or simply an acid effect. Since the existence of a complex is strongly suggested by the radiopeak at R_{ga} 0.65, the question arises as to whether gibberellic acid itself is the biochemically active material, or whether the bioactivity is associated with a natural derivative, or complex in the sense of an enzyme-coenzyme system.

In a previous study, barley was treated with tritium-labeled gibberellic acid and then analyzed for residual gibberellic acid after the end of the germination period. An 80% recovery of the added gibberellic acid suggested a surprising

stability of the material. It may well be that the stability was caused by a binding of the gibberellic acid by the plant tissue, rendering it less susceptible to degradation. In view of these results, further experiments will have to be examined with this possibility in mind.

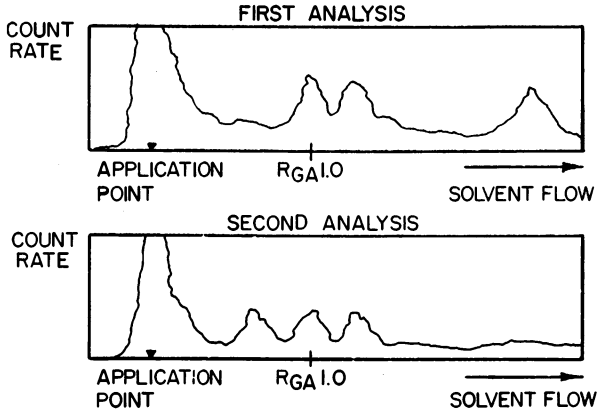

Figure 7. Radiochromatograms of gibberellin methyl-C^{14} esters
First and second analysis

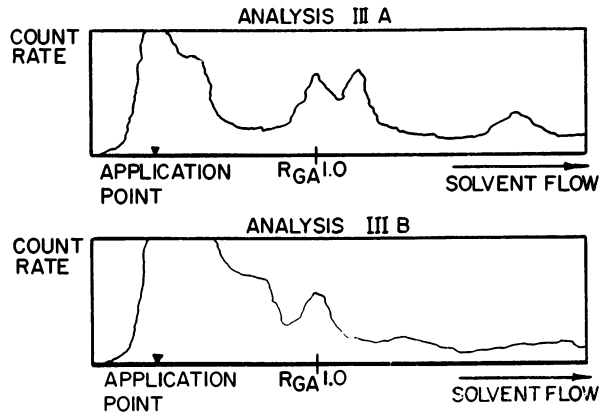

Figure 8. Radiochromatograms of gibberellin methyl-C^{14} esters
Third Analysis

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RECEIVED October 13, 1960.

Gibberellins and Flower Formation

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Application of gibberellin results in flower formation in numerous plants in which this process is normally dependent on exposure to low temperature or long days. Gibberellins are the first chemicals known to have a promotive effect on flower formation in a consistent, reproducible manner and a well-defined pattern. When flower formation is induced by the appropriate physical factors, the endogenous gibberellins of the plant seem to undergo quantitative as well as qualitative changes. It is thus probable that they perform some function in the physiological regulation of flower formation in plants.

The discovery of the effects of gibberellin on flower formation is closely linked with older studies on the physiology of this process, particularly its regulation by certain external or environmental factors.

In many plants, flower formation is spontaneous—i.e., under exclusive regulation by internal factors. In other plants it is controlled by certain, highly specific and precise environmental conditions. Thus, some plants initiate flowers after having undergone exposure to a period of cold. In nature, these "cold-requiring" plants usually flower in spring or early summer, after having been subjected to the low temperatures of winter; if given an artificial cold treatment (and subsequently a temperature and light regime favorable to flowering) they may flower at any time of the year.

In other plants, flower formation depends on day length or photoperiod. Two principal groups of photoperiodic plants may be recognized. "Long-day plants" flower when the days are relatively long—i.e., when day length exceeds a certain, minimal value which may vary from one plant to another; "short-day plants" exhibit the opposite behavior, flowering in relatively short days—i.e., when photoperiod remains below a certain, maximal length. Under natural conditions, long-day plants flower mostly in summer, when days have become sufficiently long; short-day plants in fall, when day length drops below the critical maximum. More recently, plants with a dual day length requirement have been described. They stay vegetative if grown on continuous long day or continuous short day but flower

if exposed, either first to long then to short days (long-short-day plants) or vice versa (short-long-day plants). Most cold-requiring plants also have a dual environmental requirement, flowering if the low-temperature treatment is followed by a long-day regime.

The phenomenon of cold requirement with regard to flower formation is called "vernalization"; that of day length control of the process, "photoperiodism." The conditions conducive and nonconducive to flower formation in a given plant type have been termed inductive and noninductive, and the exposure of cold-requiring and photoperiodic plants to inductive temperatures and photoperiods is called thermoinduction and photoinduction, respectively. In cold-requiring and photoperiodic plants alike the need for induction may be absolute—i.e., the plant will fail to form flowers altogether unless given inductive treatment; or it may be facultative—i.e., flowering will ultimately occur without induction, although with greater or lesser delay.

Effects of Applied Gibberellin on Flower Formation

Cold-Requiring and Long-Day Plants. The first impetus for the study of the effects of gibberellin applications on flower formation came from a peculiarity common to the majority of cold-requiring and long-day plants. These plants, when maintained under the noninductive temperature or photoperiod regime, not only fail to form flowers but also do not have an elongate stem, growing in the habit of so-called rosettes. The following account refers only to this type of plants; the response of cold-requiring and long-day plants having a caulescent type of growth —i.e., forming an elongate stem—even when grown under noninductive conditions will be briefly discussed later, in another context.

Gibberellin treatment was known to promote stem growth in caulescent plants. It was of obvious interest to determine whether it would affect stem growth in the "stemless" rosette plants as well, and it was found that such treatment results, first, in active cell division in the subapical region of the rosette axis, leading to the formation of an elongate stem, and second in flower formation. This review is not the appropriate place to attempt an enumeration of all cases in which this result has been obtained, as this would be of interest only to the plant biologist specifically concerned with gibberellins and flower formation. The following selected lists of plants in which flower formation has been caused or hastened under noninductive conditions indicate the extent of the phenomenon, while Figures 1 and 2 illustrate the effect.

Cold-requiring plants. *Arabidopsis thaliana, Beta vulgaris* (sugar beet), *Brassica oleracea* (cabbage and kale), *Brassica napus* (rape), *Brassica rapa* (turnip), *Centurium minus, Cichorium endivia* (endive), *Daucus carota* (carrot), *Digitalis purpurea* (foxglove), *Hyoscyamus niger* (henbane), *Oenothera biennis* (evening primrose), *Petrosilenum crispum* (parsley), *Solidago virgaurea* (goldenrod), and others (*5, 6, 8, 10, 13–15, 20, 22, 24, 38, 43, 44*).

Long-day plants. *Anethum graveolens* (dill), *Arabidopsis thaliana, Brassica juncea* (mustard), *Brassica pekinensis* (Chinese cabbage), *Cichorium, Crepis leontodontoides, Crepis tectorum, Hyoscyamus niger, Lactuca sativa* (lettuce), *Matthiola incana* (stocks), *Nicotiana sylvestris* (wild tobacco), *Petunia hybrida, Polemonium caeruleum, Raphanus sativus* (radish), *Rudbeckia bicolor, Rudbeckia hirta, Rudbeckia speciosa, Samolus parviflorus, Spinacia oleracea* (spinach), etc. (*10, 11, 13, 14, 24, 27, 29–31, 43, 44*). (*Arabidopsis, Cichorium,* and *Hyoscyamus niger* occur in both cold-requiring and straight long-day strains.)

Flower formation is usually obtained only after repeated applications of relatively substantial gibberellin doses. In lettuce, two treatments of 20 μg. each,

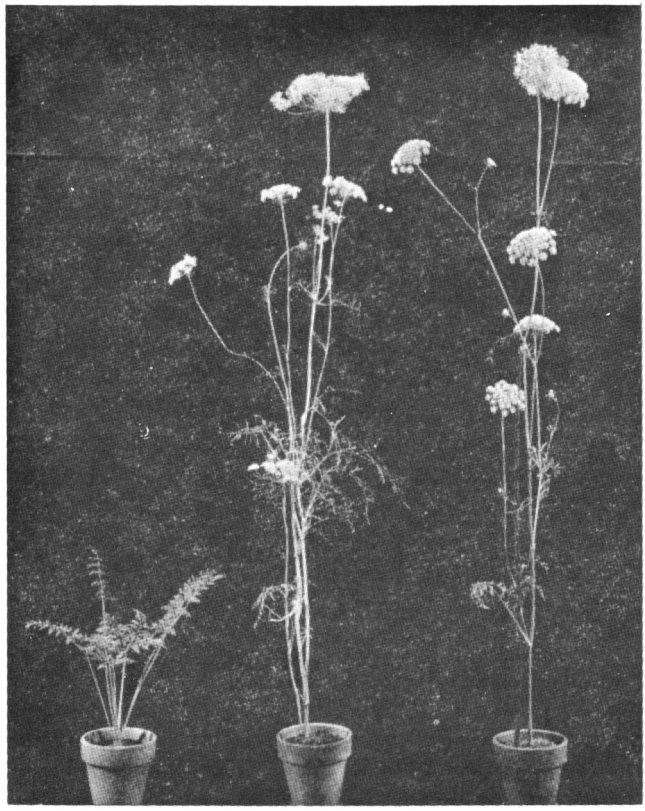

Figure 1. Effect of gibberellin on flowering in carrot (variety Early French Forcing), a cold-requiring plant (24)

Left. No cold, no gibberellin
Right. 8 weeks of cold treatment
Center. No cold, 10 μg. of gibberellin daily
All plants grown on long days

Figure 2. Effect of gibberellin on Samolus parviflorus, *a long-day plant* (24)

Left to right. Plants treated with 0, 1, 2, 5, 10, and 20 μg. of gibberellin daily, all grown on 9-hour short days

given with a weekly interval, may be sufficient (*43*); other plants require more extended treatment periods and some very prolonged ones. The minimal total dose varies accordingly. In *Samolus parviflorus* (see Figure 2), 20 μg. (1 μg. daily for 3 weeks) produce a substantial response, and about 100 μg. yield the maximal one. In parsley, on the other hand, a total of about 1 mg. (5 μg. daily over 6 months) was needed and the response was still incomplete, only part of the plants having initiated microscopic flowers (*24*). Even the *Samolus* doses are very much higher than those needed for many vegetative responses, particularly on stem growth in dwarf plant varieties, where 0.001 μg. may cause significant increases. Some cold-requiring plants—e.g., sugar beet—furthermore yield a good flowering response only if the gibberellin treatment is given at temperatures only slightly above the inductive range or is combined with a subthreshold thermoinductive treatment (*5, 15*).

Most cold-dependent plants also possess a long-day requirement. The gibberellin treatment of these plants was usually conducted under long-day conditions, so that only the temperature-controlled block to flower formation was present. If the treatment was given under short-day conditions, only stem elongation was obtained; it is evidently difficult to overcome the simultaneous presence of two blocks to flower formation.

Both some cold-requiring and some long-day rosette plants have refused to form flowers in response to gibberellin treatment. Examples are *Geum urbanum, Reseda, luteola,* and *Scrophularia vernalis* among the former and *Lactuca scariola* and *Mimulus luteus* among the latter (*13, 14, 30, 31*). In some of these plants, gibberellin application resulted in stem elongation without flower formation (*Lactuca scariola, Mimulus, Scrophularia*); in others, however, even the rosette growth habit was not broken (*Geum, Reseda*). In some of the negative cases the duration of the treatment, the dose, or the mode of application may have been inadequate. Another possibility which merits close examination is inadequacy of the gibberellins employed in the experiments. Almost all attempts at flower induction by gibberellin treatment were made using either gibberellin A_3 (gibberellic acid) or, in a few cases, mixtures of gibberellins A_1 and A_3. Experiments with lettuce indicate that these two gibberellins are more effective in promoting flower formation in this plant than are A_2 and A_4 (*6, 7*). On the other hand, the activity of different gibberellins in vegetative growth responses is different and may exhibit certain specificities depending on the genetic character of the treated plant. Thus, gibberellin A_4 is the most active in stem growth in cucumber (*7*), while gibberellin A_3 is more active in other plants; the activity of gibberellins A_1 through A_3 on the maize dwarf mutants *d*-1, *d*-2, *d*-3, *d*-5, and *an*-1 is of the same order of magnitude, but gibberellin A_5 (bean factor II) is much less active on *d*-1 than on the other mutants (*41*). It is therefore conceivable that similar differences exist with regard to the flower-inducing activity of the various gibberellins in different plants and that the absence of a flowering response in certain plants may have been due to the use of the "wrong" gibberellin.

However, it is not very probable that all cases in which gibberellin application failed to cause flower formation in cold-requiring and long-day plants are attributable either to the use of inadequate gibberellins or to inadequacies of the treatment. In small-grain cereals (wheat, rye, barley, etc.) which include both cold-requiring and long-day varieties, gibberellin may inhibit flower formation when applied to young plants but promote it when applied to older ones (*9*). It appears that in plants like these the effect of gibberellin on flower formation is profoundly different from that in plants such as *Hyoscyamus,* carrot, lettuce, *Samolus,* etc.

Short-Day and Long-Short-Day Plants. In short-day plants, in contrast to cold-requiring and long-day plants, there is no single, unequivocal case of induction or promotion of flower formation by gibberellin application under noninductive conditions—i.e., in long days (*10, 11, 24, 30*). When the treatment was given under short-day conditions, flowering was promoted in some species [*Perilla, Pharbitis, Xanthium,* and others (*16, 28, 33, 36*)] but markedly delayed and inhibited in others [*Kalanchoë blossfeldiana,* strawberry (*19, 40*)]. It thus appeared that in their flowering response to applied gibberellin short-day plant fell into two distinct and opposite classes. Recently, however, it has been shown that the response in *Kalanchoë* may be positive—i.e., flower formation may be enhanced—if the short-day treatment is optimal (*39*). Thus, the flowering response of short-day plants to gibberellin may depend on the intensity of photoinduction and the opposite responses which have been observed in different short-day species may have been caused by differences in this factor.

In some long-short-day plants (species of *Bryophyllum*) grown under short-day conditions gibberellin treatment resulted in prompt flower formation (*3, 19*); when the plants were grown under long-day conditions no flowering response was elicited (*19*). Thus, applied gibberellin seems capable of substituting for the long-day part of photoinduction in these plants but not for their short-day photoinduction.

Endogenous Gibberellins and Flower Formation

Applied gibberellin is capable of inducing or promoting flower formation in numerous plants. This is the first major instance of chemical promotion of flower formation. Flower formation may be delayed and inhibited by various chemical treatments, but enhancement of flower formation by such treatments has been successful in only few and isolated cases and sometimes only on the borderline between inductive and noninductive conditions. The gibberellins are the first class of compounds which cause flower formation in numerous plants grown under strictly noninductive temperature or light regimes, this effect, in any given species, being consistent and reproducible. Moreover, the effect exhibits a definite pattern. Gibberellin treatment is capable of substituting for vernalization or for long-day photoinduction but apparently incapable of substituting for short-day photoinduction, although it may increase the latter's effectiveness. The question thus arises whether gibberellins play some specific part in the flowering mechanism of plants—that is, the internal processes which result in flower formation—or whether they are a picklock which because of its shape is capable of opening a closed door but has not been specifically designed for that particular door. In other words, is the gibberellin effect on flower formation in cold-requiring and long-day plants a physiological or a pharmacological effect?

As long as gibberellins could be considered as products of a mold, this question was highly legitimate. But at about the same time as the effect of gibberellin on flowering was discovered gibberellin-like substances were demonstrated to be present in seed plants; today it is evident that gibberellins—including at least one of the gibberellins originally found in *Fusarium*—are of very general if not ubiquitous occurrence in these plants (*1, 32, 42*). Effects of application of seed-plant gibberellins on flower formation are similar to those of *Fusarium* gibberellins. Thus, crude preparations of gibberellin-like materials from the endosperm of immature seeds of wild cucumber (*Echinocystis macrocarpa*)—a material particularly high in such substances (*35*)—caused flower formation in cold-requiring

Hyoscyamus niger and in *Samolus parviflorus* (*26;* see Figure 3) and extracts from immature bean seeds induced flower formation in *Bryophyllum* (*4*). Other authors went a step further, showing that gibberellin-like materials prepared from *Rudbeckia* cause flower formation in this same plant species (*12, 17*); gibberellin-like materials from *Pharbitis* seeds promoted the flowering response of this short-day plant to inductive photoperiod conditions (*34*). There is thus no question that gibberellins are normal, physiological materials of seed plants and that these native or endogenous gibberellins are capable of causing or promoting flower formation in plants, including those plants in which they are actually found.

Figure 3. Short-day-grown Samolus parviflorus *plants* (26)

Left. Treated with endosperm of wild cucumber (Echinocystis macrocarpa)
Right. Treated with gibberellin A_3

Furthermore, when a plant, by proper inductive treatment, is caused to switch from vegetative growth to flower formation, this transition is accompanied by striking changes in the endogenous gibberellin composition. Thus, when extracts from vegetative plants of a long-day *Hyoscyamus* variety were tested on three different dwarf mutants of maize, no activity was found on mutant *d*-1 and some activity in the R_f range of 0.3 to 0.4 was found on mutants *d*-3 and *d*-5. Extracts from plants which had been exposed to long days and had just initiated microscopic flower buds showed activity at R_f 0.3 not only on *d*-3 and *d*-5 but also on *d*-1. The activity of this region on the former two mutants was increased, and it seemed—although this finding is based on a single experiment and thus may need further confirmation—that these extracts contain activity at R_f 0.6—i.e., some new, faster-moving material active only on *d*-5 (*23;* see Figure 4). Similar results—i.e., both quantitative and qualitative changes in the gibberellin composition closely associated with onset of flower formation—have been found in *Rudbeckia* and in a cold-requiring and a short-day variety of chrysanthemum (*18*). Such changes are no absolute proof for a close, causal connection but suggest some physiological relation between flower formation and gibberellin metabolism of the plant.

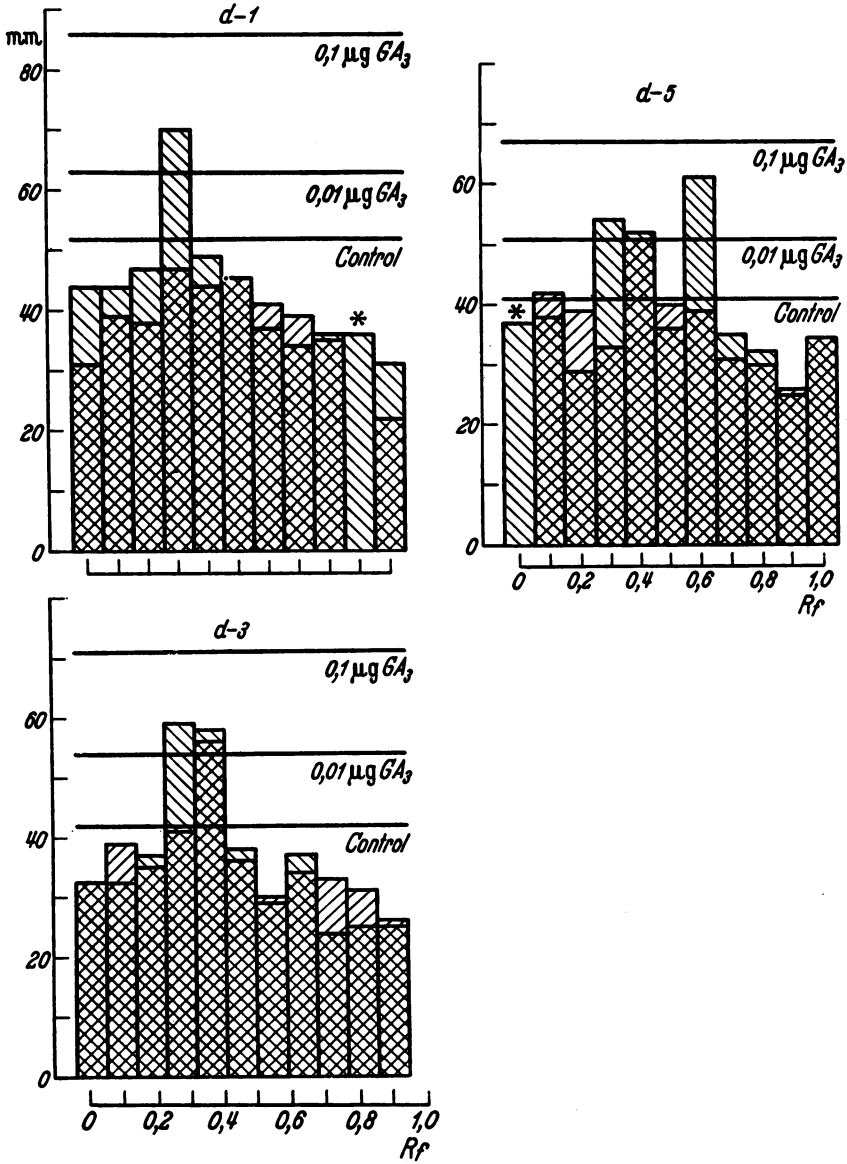

Figure 4. Histogram of extracts from young leaves of vegetative and photoinduced plants of Hyoscyamus niger, *tested on d-1, d-3, and d-5 maize mutants* (23)

Ordinate. Sum of lengths of first and second leaf sheaths of test seedlings. Horizontal lines indicate value of tallest untreated control seedling and responses to 0.01 and 0.1 µg. of gibberellin A3

* *Test plants killed or severely injured*

Function of Gibberellin in Flower Formation

Can we say anything more precise about the function of gibberellins in flower formation? Most plant biologists feel that flower formation is controlled by specific, hormone-like substances, so-called flower hormones. Attempts at ex-

tracting and identifying these materials have so far been without success, but we have a substantial body of physiological information, mainly derived from grafting experiments, on certain features of the flower hormones. By graft union with a photoinduced long-day plant it is possible to cause flower formation in a non-induced short-day plant, and vice versa. This suggests that the flower hormones of the two principal photoperiodic plant types are interchangeable and hence, in all probability, identical. On the other hand, similar experiments between cold-requiring and certain photoperiodic plants indicate that the flower hormones of these plant types are *not* identical.

The name "florigen" has been proposed for the flower hormone of photoperiodic plants, and "vernalin" for that of cold-requiring plants (for further information, see *21*). However, gibberellin, while causing flower formation in noninduced cold-requiring and long-day plants, has no comparable effect in non-induced short-day plants, rendering it improbable that gibberellins are identical with "florigen." Some cold-requiring (and some long-day) plants fail to form flowers in response to gibberellin treatment. We have considered the possibility that this failure is due to the use of gibberellins not active in flower formation in these particular plants. But as long as this possibility has not been conclusively proved, it also seems premature to identify gibberellins with "vernalin." It rather seems that in many cold-requiring and long-day plants, but not in some others nor in short-day plants, gibberellins are factors which limit the formation of flower hormones, but are not the flower hormones themselves.

Short-day plants, in which gibberellin application fails to cause flower formation under long photoperiods, do not grow in the rosette habit, having an elongate stem under both inductive and noninductive conditions. A few cold-requiring and some long-day plants also exhibit this type of growth, and in these plants—e.g., species of *Calamintha, Circaea, Urtica* and others, also certain pea varieties—gibberellin is ineffective in causing or promoting flower formation (*2, 13, 14, 30*). On the other hand, in some cold-requiring and long-day plants the main axis never elongates—as it does in *Hyoscyamus, Samolus*, lettuce, etc.—flowers being produced on lateral shoots only. Such plants (*Geum urbanum, Plantago major*) are also nonresponsive to gibberellin with regard to flower formation (*14, 25*). It thus may appear that gibberellins are not flower but stem-growth hormones.

In plants in which stem growth is inhibited by lack of the proper gibberellin, the block to flower formation may be secondary and will be released once stem growth becomes possible. In plants in which stem growth is not blocked at all, or is blocked in some other manner and therefore cannot be released by applied gibberellin, gibberellin does not cause flower formation. The assumption that the primary action of gibberellin is concerned with stem growth is strengthened by the effects of gibberellin in the subapical region of shoot meristems (*37*) and also by the fact that in gibberellin-treated cold-requiring and long-day plants stem elongation usually precedes flower formation, whereas in thermo- and photoinduced individuals the two processes proceed almost simultaneously. However, unless one assumes that these species require specific gibberellins, the above interpretation does not account for those plants in which gibberellin treatment causes stem elongation but no flower formation; it is clearly a hypothesis and its validity depends on the outcome of further experimental work.

Conclusions

The precise function of gibberellin in flower formation is by no means clear and may be indirect in nature. On the other hand, gibberellins are the first

materials which provide a substantial degree of chemical control of flower formation. They occur in plants, and their effect on flower formation shows a rather clear-cut relation to the action of certain, specific environmental factors which control flower formation in the same plants in which this process can be also induced by gibberellins. Environmentally induced flower formation is accompanied by changes of the gibberellin composition of the plant. It is thus reasonable to hope that further studies will provide new insights into the physiology of flowering and the manner in which gibberellins regulate plant growth.

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RECEIVED September 8, 1960.

Gibberellin Modification of Flower Sex Expression in *Cucumis sativus* L.

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Flower sex expression of cucumbers can be modified by treating seedlings with several growth-regulating substances. With the exception of gibberellin, chemicals tend to reduce the number, or suppress the development, of staminate flowers, and increase the number or accelerate the development of pistillate flowers. Gibberellin, in contrast, increases the number of staminate flowers in monoecious cucumbers and results in the formation of staminate flowers on gynoecious cucumbers which otherwise would produce only pistillate flowers. In promotive effects on staminate flower formation and vegetative extension, GA_4 is approximately tenfold more active than GA_3.

A model nodal sequence in appearance of flower types may be ascribed to a monoecious cucumber plant, grown at a given environment. Currence (*4*) reported that as the monoecious cucumber plant develops, the flower sex gradually changes from staminate to pistillate. The first nodes formed are staminate (staminate stage), followed by a series of nodes which are truly monoecious (a mixed stage bearing both staminate and pistillate flowers), and finally successive nodes which produce exclusively pistillate flowers (pistillate stage). The number of nodes preceding the appearance of the first pistillate flower has been used as a criterion of flower sex expression (*24*). The fewer the nodes preceding the first pistillate flower, the shorter the staminate stage and the earlier the mixed stage. The consistent appearance of pistillate flowers at successive nodes denotes the pistillate stage. In some varieties the pistillate stage is attained only under environmental conditions most favorable for pistillate flower formation.

Flower sex expression is subject to genetic, environmental, and chemical control (*6, 14, 20, 26, 27*). Alteration of the length or duration of any of the flower sex stages, but not necessarily the order of appearance, has been modified by environment. The staminate stage has been extended by long photoperiods (*6, 26*) and the appearance of the pistillate stage accelerated by low temperatures, short days (*3*), and low light intensities (*26*). Numerous chemicals (Table I)

when applied to young cucumber seedlings, modify sex expression. To the authors' knowledge, with the exception of the gibberellins, all chemicals shortened the duration of the staminate stage and hastened the appearance of the mixed and pistillate stages.

Table I. Some Chemicals Shown to Alter Flower Sex Expression in *Cucumis sativus* L.

Effect and Chemical	*Authority*
Hasten appearance of pistillate flowers	
Acetylene	(*16*)
Carbon monoxide	(*5, 17*)
Methylthionine chloride	(*19*)
Allyl trimethylammonium bromide	(*18*)
2,4-Dichlorophenoxyacetic acid	(*12*)
Maleic hydrazide	(*3, 28*)
3-Indoleacetic acid	(*11, 14*)
1-Naphthaleneacetic acid	(*8, 12–14*)
N-p-Chlorophenylphthalamic acid	(*3*)
2,3,5-Triiodobenzoic acid	(*11*)
Delay appearance of pistillate flowers	
Gibberellin A_3	(*27*)
Induce staminate flowers on gynoecious types	
Gibberellin A_3	(*21*)

Table II. Effect of Photoperiod and Gibberellin A_3 on Flowering of *Cucumis sativus* L., var. Burpee Hybrid

[Modified after Wittwer and Bukovac (*27*)]

Treatment	*No. of Staminate Flowers Produced Preceding First Pistillate Flower*	*Days from Seeding to Anthesis of First Pistillate Flower*	*Nodes Preceding First Pistillate Flower*
9-hour photoperiod			
Control	22.7	51.4	9.7
Gibberellin[a]	35.8[b]	60.9[b]	14.7[c]
18-hour photoperiod			
Control	20.5	55.9	10.5
Gibberellin[a]	27.8	58.4	10.4

[a] Four applications of GA_3 at 10-day intervals beginning at emergence of first true leaf.
[b,c] Values significantly different from control at $P = 0.01$ and 0.05, respectively.

Studies reported herein are primarily concerned with the effects of gibberellin in extending the staminate stage of monoecious cucumbers and with the formation of staminate flowers on gynoecious cucumbers.

Experimental

Modification of Sex Expression in Monoecious Cucumbers. The effect of gibberellin on flower sex expression of monoecious cucumbers was first reported in 1958 (*27*). The duration of the staminate stage was extended and the pistillate stage was delayed in the variety Burpee Hybrid grown at a 9-hour photoperiod (Table II). Fifty micrograms of gibberellin A_3 (GA_3) were applied four times at 10-day intervals beginning with the unfolding of the first true leaf. The number of staminate flowers produced before anthesis of the first pistillate flower was increased 58% above the control by treatment with GA_3. The increase in number of staminate flowers following GA_3 treatment of plants grown at an 18-hour photoperiod was not significant. Gibberellin did not significantly alter the number of

days from seeding to anthesis of the first staminate flower or the node at which it appeared at either the 9- or 18-hour photoperiod. Similarly, no marked differences were noted between gibberellin-treated and nontreated plants at the 18-hour photoperiod in days from seeding or number of nodes formed before the appearance of the first pistillate flower (Table II).

Galun (8) has recently reported similar results for monoecious cucumbers differing in the normal duration of the staminate stage. He demonstrated a close relationship between gibberellin-induced vegetative elongation and modification of flower sex expression (8).

Pistillate flowers are usually formed earlier (at a lower node) when plants are grown under a short photoperiod. A similar but less striking effect can be induced by a brief (2- to 3-week) exposure of cucumber seedlings, beginning at cotyledon expansion, to a short photoperiod, followed by an extended photoperiod (3).

Foliar applications of gibberellin (100 p.p.m.) to young pickling-type cucumber seedlings (Wisconsin SMR-12) during 2 weeks of short-day exposure (9 hours daily) markedly reduced the effect of the short photoperiod of hastening pistillate flower formation (Table III). In this respect, the effects of gibberellin were opposite to that of a short photoperiod and simulated the effects of a long photoperiod. The number of days from seeding to anthesis and nodes preceding the appearance of the first staminate flower were not significantly altered by gibberellin (Table III).

Table III. Effect of Photoperiod and Gibberellin A_3 Treatments during Seedling Development on Flowering of *Cucumis sativus* L., var. Wisconsin SMR-12

	Days from Seeding to Anthesis		
Treatment	*First staminate flower*	*First pistillate flower*	*Nodes preceding first pistillate flower*
9-hour photoperiod			
Control	84.2	83.9	9.7
Gibberellin	87.7	91.0	10.6
18-hour photoperiod			
Control	79.9	82.6	10.6
Gibberellin	83.9	86.8	10.8
L.S.D. $P = 0.05$	4.1	6.0	1.8
$P = 0.01$	5.4	7.9	2.4

Modification of Sex Expression in Gynoecious Cucumbers. The ultimate effect of a chemical on sex expression would be a complete reversal of flower sex. To demonstrate such a reversal one would have to replace, in part or in its entirety, the initial staminate stage with pistillate flowers or the terminal pistillate stage with staminate flowers. In monoecious cucumber plants an extension or reduction in the duration of either the staminate or pistillate stage may not be equivalent to reversal of sex expression, since the exact transition between the various stages is not sufficiently precise. The consistent production of pistillate flowers at the beginning of the staminate stage, or of staminate flowers late in the terminal pistillate stage, would be valid evidence of flower sex reversal in monoecious cucumbers. Suitable plant material for demonstrating the chemical reversion of flower sex would be cucumber plants which produce exclusively staminate or pistillate (gynoecious) flowers.

An inbred line of cucumber, MSU 7-13–5, homozygous for the gynoecious

character has been developed by crossing a gynoecious segregate found in the Korean variety, Shogoin (PI 220860), with Wisconsin SMR-18 (*20*). When grown under a wide variety of temperatures and photoperiods it produces only pistillate flowers. Underdeveloped pistillate flowers occur usually at the first and second nodes and functional pistillate flowers at each succeeding node. Functional staminate flowers have been induced on the gynoecious cucumber, MSU 713–5, by two or three weekly applications of GA_3 (1500 p.p.m.) with the initial treatment during the expansion of the first true leaf (*21*). The minimum effective dosage necessary to cause the formation of staminate flowers may be related to plant age, field and greenhouse culture, and accompanying photoperiod and temperature regimes.

The promotion of staminate flower formation in the above-described gynoecious cucumber was followed in five groups of greenhouse-grown plants by momentarily dipping one of several sequential leaves of the main axis, when approximately 5 to 8 cm. in diameter, into a solution of 100 p.p.m. of GA_3. These included the first, second, third, and fourth true leaves. Staminate flowers were formed only on plants where the first true leaf was treated. Apparently with an increase in plant maturation the effectiveness of a given dosage of gibberellin in the promotion of staminate flower formation was reduced.

Although the gynoecious flowering behavior of MSU 713–5 is not altered by photoperiod alone, photoperiod has an effect if it is combined with appropriate gibberellin treatment that in turn will modify flower sex expression (Table IV). Single foliar applications of GA_3 at 2×10^{-3} and 5×10^{-3} *M* to MSU 713–5 grown at 9- and 18-hour photoperiods resulted in staminate flower production on only 70 and 80% of the treated plants, respectively. Repeated applications at the 9-hour photoperiod and both single and repeat applications at the 18-hour photoperiod caused every treated plant to produce some staminate flowers (Figure 1). No staminate flowers were formed on plants not treated with gibberellin, irrespective of photoperiod. On plants that were induced to form staminate flowers the various gibberellin treatments caused no differences in the number of days from seeding to first anthesis or the number of nodes preceding the appearance of the first staminate flower.

Photoperiod enhances the promotive effect of gibberellin on staminate flower formation in the gynoecious cucumber not by increasing the number of nodes which

Table IV. Effect of Single and Repeat Application of Gibberellin on Expression of Staminate Flowers on a Gynoecious Cucumber (MSU 713-5)

Gibberellin Treatment		*Days from Seeding to Anthesis of First Staminate Flower*	*Nodes Preceding First Staminate Flower*	*% of Plants Bearing Staminate Flowers*
Molar concn.	*No. of applications*			
9-Hour Photoperiod				
0	0	No staminate flowers produced		
2×10^{-3}	1	51.7	6.9	70
2×10^{-3}	2	50.4	8.0	100
5×10^{-3}	1	51.7	7.6	80
5×10^{-3}	2	50.1	7.9	100
18-Hour Photoperiod				
0	0	No staminate flowers produced		
2×10^{-3}	1	51.1	7.6	100
2×10^{-3}	2	51.1	7.0	100
5×10^{-3}	1	50.4	7.5	100
5×10^{-3}	2	49.6	7.4	100

Figure 1. Effect of gibberellin on flower sex expression of gynoecious cucumber, MSU 713–5

Left. Control, pistillate flowers occur at each successive node
Right. Staminate flowers following treatment with GA$_3$

produce staminate flowers but by increasing the number of staminate flowers at each node (Table V). Comparable numbers of nodes (4.1 and 4.9 at the 9- and 18-hour photoperiods, respectively) of gibberellin-treated gynoecious plants bore staminate flowers. However, a much higher proportion of the nodes bearing staminate flowers on the gibberellin-treated plants at the 18-hour photoperiod were entirely staminate (4.4 as compared with 2.4), and fewer nodes bore both staminate and pistillate flowers. The ratio of nodes with staminate flowers only to nodes with both staminate and pistillate flowers was 1.4 for the 9-hour photoperiod compared to 8.8 for the 18-hour photoperiod. Thus, while photoperiod alone did not

Table V. Effect of Gibberellin and Photoperiod on Promotion of Staminate Flower Formation on Gynoecious Cucumber (MSU 713-5)

Treatment	*Total Nodes Producing Staminate Flowers*	*Nodes with Staminate Flowers Only (S)*	*Nodes with Staminate and Pistillate Flowers (S + P)*	*Ratio, S/(S + P)*
9-hour photoperiod				
Control		No staminate flowers produced		
Gibberellin	4.1	2.4	1.7	1.4
18-hour photoperiod				
Control		No staminate flowers produced		
Gibberellin	4.9	4.4	0.5	8.8

alter flower sex expression in the gynoecious cucumber, MSU 713–5, it modified the flower sex response induced by gibberellin.

Comparative Activity of Gibberellins A_3 and A_4 and Some Esters of Gibberellin A_3. Quantitative differences among GA_1, GA_2, and GA_4 have been demonstrated over a wide concentration range in monogenetic dwarf maize mutants (22) and perhaps there are qualitative differences for the induction of seed-stalk elongation in heading-type lettuce (2). These results suggest that there are differences in the physiological roles of the various gibberellins. In our studies (2) GA_4 was the most active gibberellin in inducing vegetative extension in the cucumber (Figure 2). This pronounced activity warranted evaluation of gibberellin A_4 on flower sex expression. In tests where the relative activities of GA_3 and GA_4 were compared in the promotion of staminate flowers on the gynoecious cucumber, MSU 713–5, GA_4 was approximately ten times more effective than GA_3. The results were expressed either as the per cent of plants which formed staminate flowers or as the number of staminate flowers produced per plant.

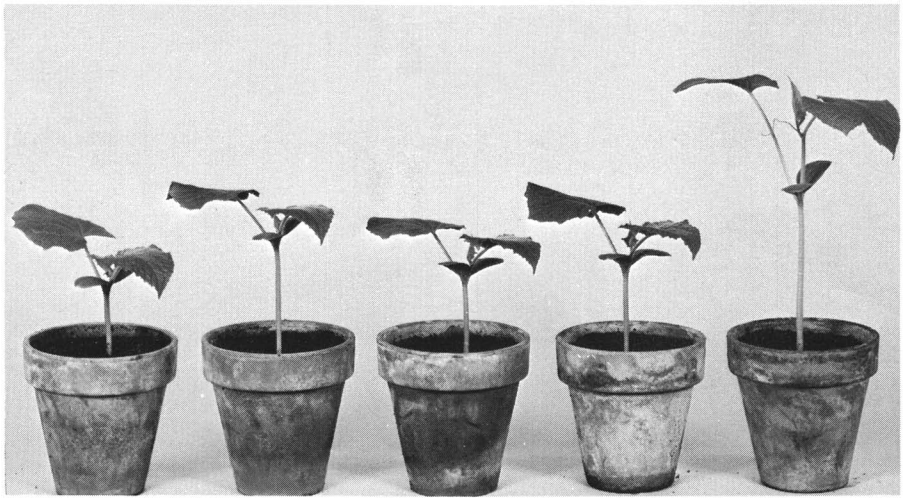

Figure 2. Response of a monoecious cucumber (var. Burpee Hybrid) to different gibberellins

Left to right. Control, 10 μl. of 3 × 10^{-3}M solutions of GA_1, GA_2, GA_3, and GA_4

Esterification of the carboxyl group of GA_3 resulted in compounds with no biological activity when applied to test systems in which hydrolysis to the free acid could not readily occur (1, 2, 23). The butyl Cellosolve ester was as active as the free acid as assayed by the elongation of bean epicotyls or the parthenocarpic growth of tomato ovaries (2).

In the present studies the relative biological activities of GA_3, the methyl ester, the butyl ester, and butyl Cellosolve ester of GA_3 for inducing vegetative extension and promoting staminate flower expression were determined. Gynoecious cucumber plants (MSU 713–5) were sprayed with 10^{-3} *M* solutions of each gibberellin preparation during expansion of the first true leaf. All treatments were repeated during the expansion of the fifth true leaf.

The most pronounced internode elongation occurred on plants treated with the butyl Cellosolve ester of GA_3. No growth promotion was observed with either

the methyl or butyl gibberellates. Internode elongation resulting from treatment with GA_3 was intermediate (Figure 3). The comparative response from GA_3 and the butyl Cellosolve ester of GA_3 expressed as per cent of treated plants that produced staminate flowers and the number of staminate flowers, according to nodal distribution, is recorded in Table VI. The butyl Cellosolve ester was the most active in promoting the formation of staminate flowers. Two distinct areas on the main axis (three to four nodes for each) of staminate flowers were produced —one following each application of the butyl Cellosolve ester. Plants treated with GA_3 produced only one area (four nodes) of staminate flowers which followed the first leaf application. No staminate flowers were formed following the second application (fifth leaf) of GA_3 (Table VI). Only two of ten plants treated with methyl gibberellate produced staminate flowers and there was only one per plant. No staminate flowers were produced on plants treated with butyl gibberellate.

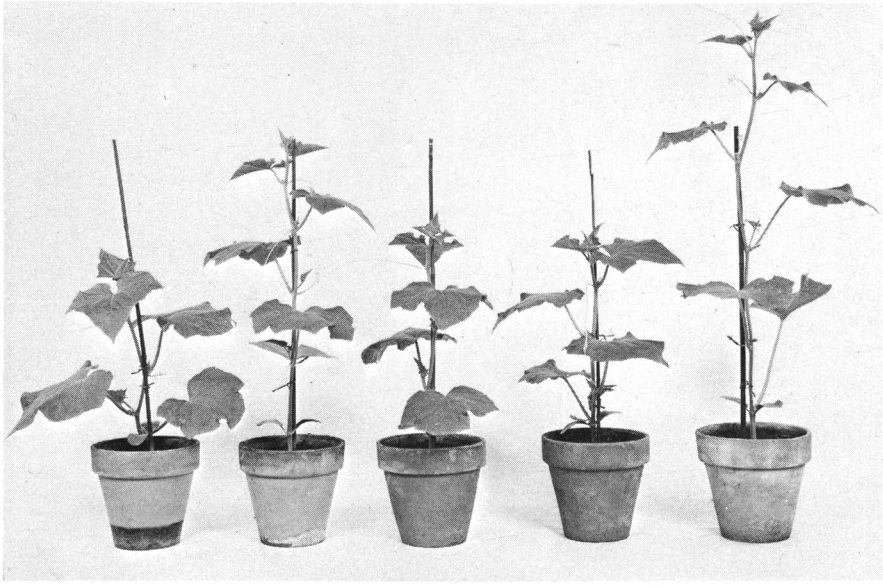

Figure 3. Comparative response of a gynoecious cucumber (MSU 713–5) to foliar applications of 1 × 10^{-3}M solution of GA_3 and several esters of GA_3

Left to right. Control, GA_3, methyl ester, butyl ester, and butyl Cellosolve ester

Discussion

Inspection of flower primordia in the Japanese cucumber, Somohanhiak, revealed that both staminate and pistillate primordia are present in each flower, and sex expression at anthesis is apparently dependent upon the relative growth rates of the two types of primordia (*10*). Perhaps this is also true for other cucumber varieties. Thus, environmental or chemical alterations in flower sex expression probably fall under the category of regulation which is an effect on development or maturation, rather than induction or differentiation.

The promotion of staminate flower formation in cucumbers by gibberellin is not surprising if one considers the effects of gibberellin as similar to those of a long photoperiod. Extension of the staminate stage in monoecious cucumbers by gibber-

Table VI. Comparative Activities of Gibberellin A_3 and Butyl Cellosolve Ester of Gibberellin A_3 on Promotion of Staminate Flower Formation on Gynoecious Cucumber (MSU 713-5)[a]

	Gibberellin A_3		*Butyl Cellosolve Ester of A_3*	
Node No.	*% of plants bearing staminate flowers*	*No. of flowers*	*% of plants bearing staminate flowers*	*No. of flowers*
1	0.0	0	0	0
2	0.0	0	0	0
3	12.5	0.1	12.5	0.1
4	75.0	1.6	100	4.1
5	87.5	1.3	87.5	3.8
6	62.5	1.0	37.5	1.5
7	0	0	0	0
8	0	0	0	0
9	0	0	25.0	0.9
10	0	0	50.0	1.3
11	0	0	12.5	0.3
12	0	0	0	0

[a] Foliar spray 10^{-3} *M* at expansion of first true leaf and repeated at expansion of fifth true leaf.

ellin is similar but more pronounced than that of a long photoperiod. Gibberellin induction of staminate flowers on the gynoecious cucumber can also be modified by photoperiod. Thus, gynoecious cucumber plants respond to long photoperiods through an intensified "maleness," providing a staminate stage can be induced with gibberellin. Photoperiod alone, however, provides no control over staminate flower formation. Thus, gibberellin stands alone as an external variable that can induce the formation of staminate flowers on the MSU 713–5 gynoecious cucumber.

The course of floral morphogenesis in the cucumber appears to be regulated by endogenous levels of plant growth substances. Before the possible role of gibberellin in flower sex expression of the cucumber was discovered, Heslop-Harrison (9) suggested that development of staminate primordia of monoecious plants was favored by a low auxin level and the pistillate primordia by a high auxin level at the differentiating apex. Data of numerous experiments have supported this hypothesis in which exogenous applications of auxins to cucumber seedlings have promoted the formation of pistillate flowers. Heslop-Harrison (9) has reviewed in detail the possibilities of experimental modification of sex expression in flowering plants.

Galun (7) failed to correlate the native growth substance content of cucumber plant parts with flower sex expression. The amounts of extractable growth substances in the stem tips and young leaves were not significantly different in plants that varied widely in "maleness" and "femaleness." The most striking difference was a greater concentration of a growth inhibitor extracted from old leaves of strongly male plants. The nature of the inhibitor and its possible role in flower sex expression remain to be elucidated.

The opposite effects of auxin and gibberellin on flower sex expression suggest the interesting possibility that sex expression is simply a matter of a ratio of endogenous auxin-type and gibberellin-type growth substances. This ratio may be altered not only by varying either one of the two groups of growth substances, but by varying or applying levels of any substances that are antagonistic to, or enhance, the activity of either. Thus, differences or lack of differences in endogenous levels of either auxin or gibberellin need not necessarily be an argument against their role in flower sex expression of the cucumber. It would be interesting to determine if the gibberellins modify sex expression through auxin metabolism or more directly

by altering the rate of development of staminate or pistillate primordia. Straus and Epp (25) recently found GA_3 to be a specific growth factor in tissue cultures of *Cupressus funebris* derived from staminate cones. No data are yet available on the tissue requirements from pistillate cones.

The differential activity among the gibberellins on vegetative extension and alteration of flower sex expression in the cucumber is difficult to explain in light of the similarity of their molecular structures. Nevertheless, GA_4 is far more active than GA_1, GA_2, or GA_3, and MacMillan reported that GA_7 is as active, or more so, than GA_4 on stem elongation in cucumbers (*15*). Perhaps it will be as active in modifying sex expression. In view of these findings, the role of gibberellin in sex expression deserves further attention.

Acknowledgment

The authors sincerely appreciate the cooperation of C. E. Peterson, Michigan State University in generously supplying seeds of the inbred line MSU 713–5, and of Y. Sumiki for supplying gibberellins A_1, A_2, and A_4, without which these studies would not have been possible.

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RECEIVED October 13, 1960. Journal Article 2695 from the Michigan Agricultural Experiment Station.

Growth of Grapes in Relation to Gibberellin

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The responses of grapes to exogenous gibberellins all essentially occur naturally under suitable environmental conditions. Endogenous gibberellins have been found in berries of seedless grapes, and in all pollen tested. Gibberellins in addition to auxins probably play an important role in fruit set and development. Gibberellin can act as a pollenicide when applied at prebloom or bloom stages. Berries respond less to GA_2 than to GA_1, GA_3, or GA_4. The phytotoxicity of GA_3 is far greater to seeded than to seedless varieties of *Vitis vinifera* L. Exogenous gibberellins prolong bud rest. The rest period induced by short days has been broken by gibberellin for certain relatively short-day-insensitive varieties. Commercial uses for gibberellins on grapes include elongation of cluster parts to decrease rotting, enlargement of berries, and increasing berry set. Other possible uses are under study.

Soon after the striking effects of gibberellin on certain plants were demonstrated, we began testing gibberellin on grapes at the University of California at Davis (*33*, *34*, *36*, *41*). Experiments on grapes were also carried out in many experimental stations throughout the world (*5*, *16*, *17*, *19*, *25*, *49*). Most work to date has been on the *Vitis vinifera* L. species (also referred to as the Old World grape, the European, and, more recently, the California grape). However, in the eastern United States experiments have been performed on the *Vitis labrusca* L. (*10*, *28*) and in Germany on *Vitis riparia* and other varieties (*4*, *6*, *9*). The physiological responses of grapes to exogenous gibberellin were all essentially natural. Fruit set, berry enlargement, shoot elongation, internode elongation, elongation of cluster parts, formation of shot berries, and hastening of flowering all take place naturally under suitable environmental conditions.

Gibberellin A_1, gibberellin A_2, gibberellin A_3 (gibberellic acid), and gibberellin A_4 are referred to as GA_1, GA_2, GA_3, and GA_4, respectively. Grapes have been shown to be equally responsive to potassium gibberellate (KGA_3) and GA_3 (*40*). 4-Chlorophenoxyacetic acid is abbreviated as 4-CPA, and 3-indoleacetic acid as IAA.

One of the most striking effects of exogenous GA_3 on grape shoots is to increase their rate of elongation. For example, when young Zinfandel shoots 2 to 3 inches long were treated with foliage applications at 1 to 1000 p.p.m. they showed increased growth within several days (Figure 1). Within 2 or 3 days of treatment, foliage became slightly yellowish, but one week later all shoots except those sprayed with 1000 p.p.m. became a more normal green. The greater length of shoots was the result of elongation of intermodes. Although length was greatly increased, the curve of growth was not altered (Figure 1). There were no stem curvatures or callus formation, which often result from auxin application (35).

Young clusters as well as shoots are elongated by sprays of GA_3 applied 2 weeks or more preceding bloom time. In one experiment at Davis Zinfandel vines were sprayed with 0 to 50 p.p.m. of KGA_3 when the shoots were 7 to 13 inches long and clusters $1^1/_2$ to 3 inches long. At harvest, on September 8, the rachis and pedicels of sprayed clusters were much elongated, and some shot berries were formed (small seedless berries that fail to enlarge) (Figure 2).

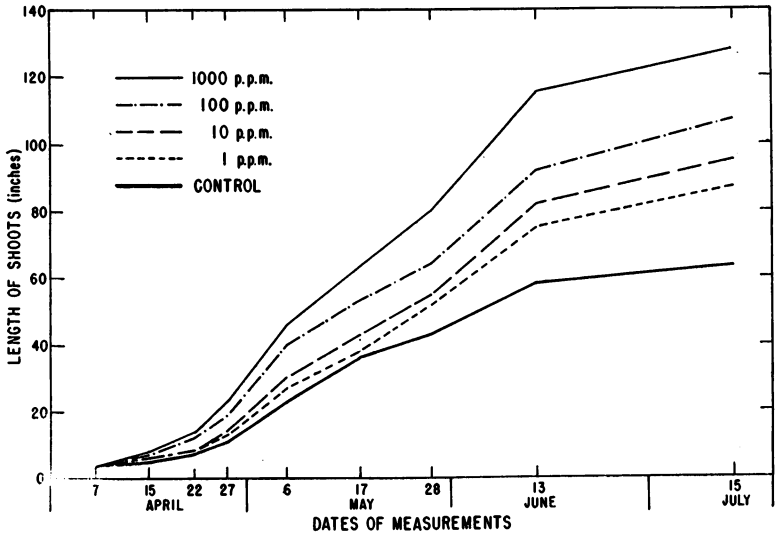

Figure 1. Elongation of Zinfandel shoots sprayed with KGA_3 on April 7, 1957

Even 1 p.p.m. concentration resulted in longer shoots; shoots sprayed at 1000 p.p.m. were about twice the length of untreated controls on July 15

The sprays also hastened flowering by 3 or 4 days. For example, on May 19 calyptras that had fallen from the flowers sprayed with 0, 10, 25, or 50 p.p.m. of KGA_3 were, respectively, 0, 75, 80, and 85%. An advance in flowering from exogenous gibberellin had been noted in other plants (32).

The size of berry in seedless grape varieties is greatly increased by gibberellin treatment near the time of bloom or berry shatter. In one experiment fruit of Thompson Seedless was sprayed at the full-bloom stage at 0, 10, 100, or 1000 p.p.m. of KGA_3. At harvest treated berries had greatly enlarged, in proportion to the concentration of compound used (Figure 3). Berries were also much elongated, a typical effect of gibberellin on seedless varieties of *Vitis vinifera*.

When seeded vines are treated with relatively high concentrations of gibber-

ellin, bud break may be delayed in the following spring and some buds may be killed (*38*). This prolonged bud rest occurs normally in some vineyards, and appears much like prolonged rest induced by gibberellin. At present, the role of gibberellin in bud rest, if any, is unknown.

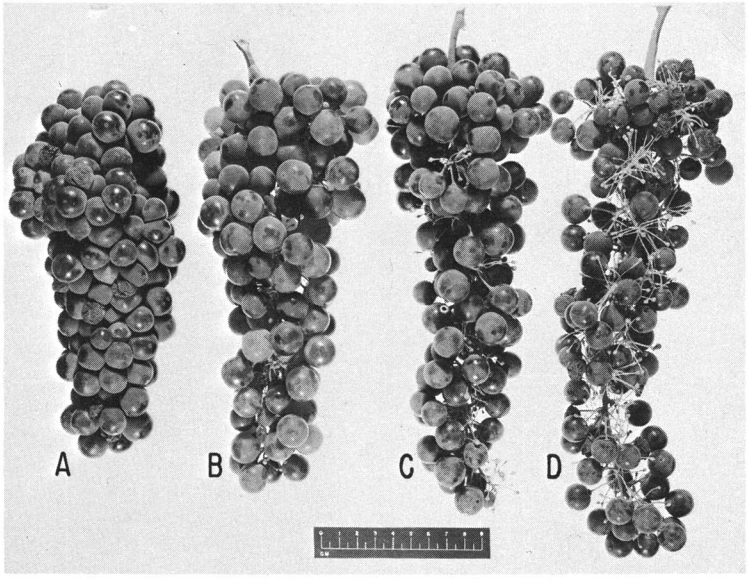

Figure 2. Zinfandel grape clusters at harvest, from vines sprayed April 28 with KGA_3

A. Unsprayed
B. 10 p.p.m.
C. 25 p.p.m.
D. 50 p.p.m.
Note elongation of treated clusters and formation of shot berries

Endogenous Gibberellin and Auxin

The fact that effects caused by gibberellin all take place naturally under suitable environmental conditions suggested that hormones physiologically similar to the gibberellins were present in grapes. Furthermore, grapes had been shown to be very responsive to several plant-growth regulators.

For these reasons Coombe, doing graduate work in the Department of Viticulture and Enology at Davis, surveyed the presence of gibberellin in grape berries and pollen, correlating it with the stage of development of the berries (*13*). He measured growth and development from anthesis to maturity of certain parts of the fruit and seed of two seeded (Muscat of Alexandria and Emperor) and three seedless (Thompson Seedless, syn. Sultanina; Black Corinth; and Seedless Emperor) varieties of *Vitis vinifera*. These were compared with changes in the sugar concentration of the juice and the levels of auxins and gibberellins in partially purified extracts of berries. Auxins were tested with Nitsch's Avena first internode test bioassay, and gibberellins were tested for with Phinney's d-1 dwarf corn for a bioassay.

Both seeded and seedless varieties of grapes have a double-sigmoid growth

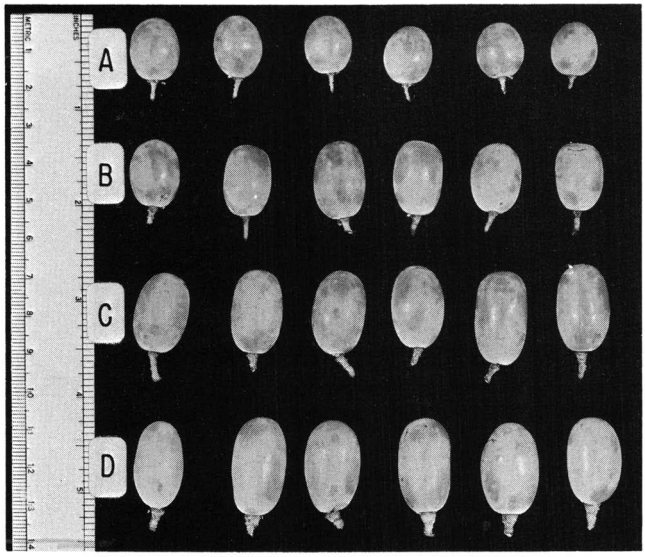

Figure 3. Close-up of berries of Thompson Seedless 89 days after being sprayed with KGA_3 at full bloom on May 29

A. 0 p.p.m.
B. 10 p.p.m.
C. 100 p.p.m.
D. 1000 p.p.m.
Berries larger and more elongated and pedicels thicker as concentration increases

curve (*48*). The fruit of all varieties studied by Coombe (*13*) clearly showed a two-humped rate curve. The curves for Emperor and Seedless Emperor are shown graphically in Figures 4 and 5. In the two seeded varieties, the first growth cycle was paralleled by a rise and fall in meristematic activity in the seeds and in the auxin content of the berries. In the seedless berries, the first growth cycle was greater than would be expected from the berry's auxin content and the seed's meristematic activity.

Auxins in seeded Emperor rose from a low level just after anthesis to a high level during the first few weeks (Figure 4). In Seedless Emperor (not in other seedless varieties) auxins are at a low level after anthesis (Figure 5). However, there is a second smaller rise in auxin content at about time sugaring begins.

Pollen (stamens) in all varieties shows gibberellin activity (Figure 6), but only berries of the seedless varieties show the presence of gibberellin. Gibberellin was found only during the first 14 days after anthesis, not subsequently. Coombe (*13*) suggests that gibberellins may be involved in the growth mechanism during cycle 1 of seedless berries and that the level of naturally occurring gibberellins may be an important factor limiting their growth. The difference in gibberellin activity between seedless and seeded berries may be correlated with their responsiveness to exogenous GA_3. Seedless (but not seeded) berries ordinarily enlarge after treatment with GA_3 (*42*).

Coombe's data (*13*) also indicate that the sugaring of berries rapidly increases about when the second stage of berry growth begins. The second growth cycle

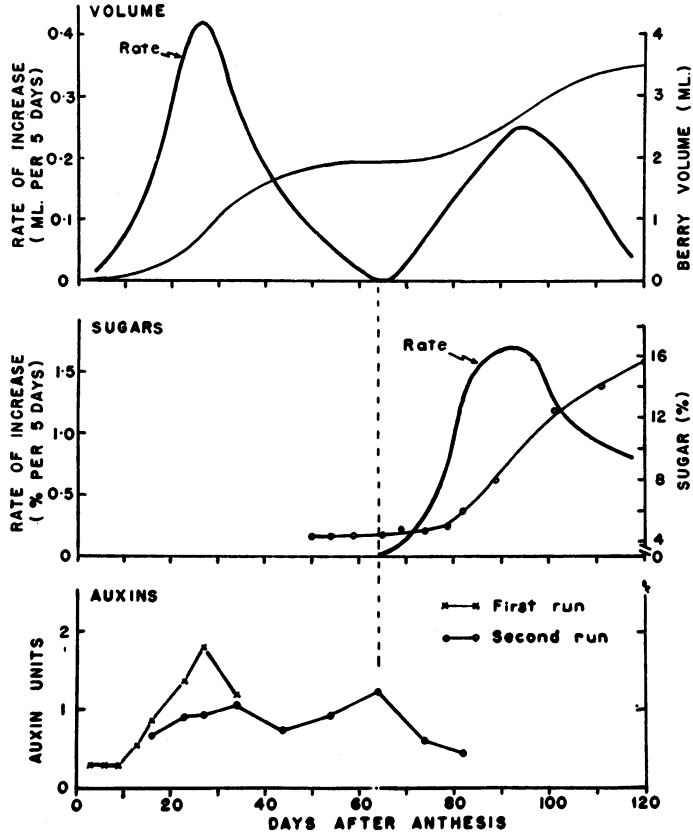

Figure 4. Changes in berry volume, juice sugar concentration and auxin concentration in Emperor grape (13)

could not be correlated with morphological, auxin, or gibberellin changes but could be related to the influx of sugars into the berries (Figures 4 and 5). It is Coombe's theory that sugaring causes this growth cycle by an osmotic attraction of water. Perhaps as sugar moves into cells of the flesh, water also moves in to adjust diffusion pressure deficits (*13*).

Coombe showed that the activity of grape extracts was much greater on d-5 dwarf corn than on d-1 (*13*). Two different compounds have been isolated from bean seeds—bean factor 1 behaved like GA_1, but bean factor II was ten times as active on d-5 as on d-1 (*24, 45*). Coombe believed that the activity in grapes may be due in part to the presence of bean factor II or other gibberellin-like compounds.

Effect of Prebloom Treatment on Pollen Germinability and Fruit Development

It has been demonstrated in California (*29, 30, 33, 42, 43*) and elsewhere (*2, 5, 27, 46*) that prebloom sprays of GA_3 result in a loosening of compact-clustered varieties (Figure 2). Clusters elongate and/or more shot berries are formed. Why shot berry formation is stimulated is unknown. Gibberellin application may injure the pistil or stamens, or both. In this experiment the effect of

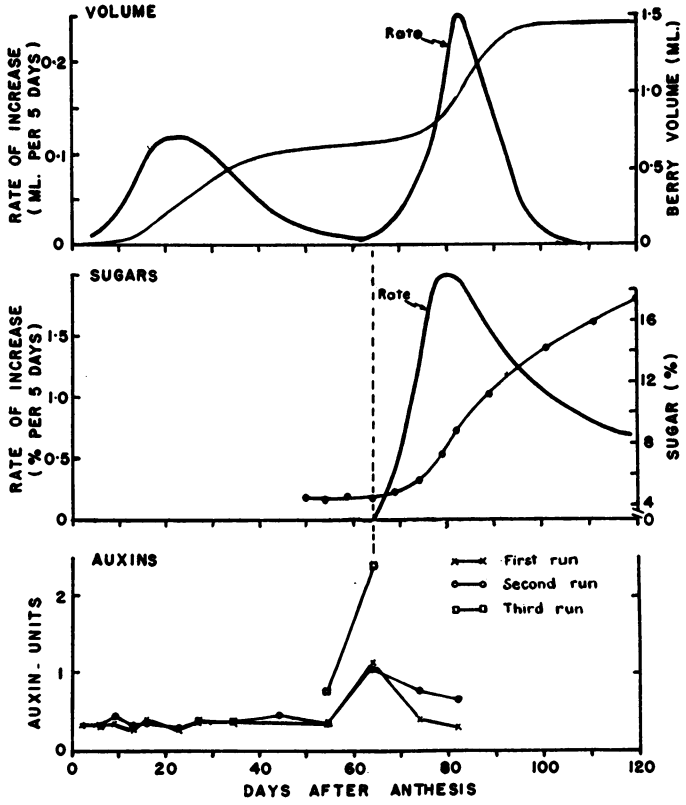

Figure 5. Changes in berry volume, juice sugar concentration, and auxin concentration in Seedless Emperor grape (13)

KGA_3 on pollen germinability was studied, using the hanging-drop procedure as described by Olmo (22), and on development of the fruit, including the number of hard seeds per berry, determined by cutting berries open with a razor blade. Small shot berries that failed to soften or color were not counted.

Treatments were made on April 16, when shoots were 8 to 10 inches and clusters $1^1/_2$ to $2^1/_2$ inches long. Only the basal clusters were dipped; the rest were removed. Pollen was collected on May 11 from flowering clusters treated with KGA_3 at 100 p.p.m., and on May 14 from flowers of the other treated plants and from the controls. The compound at 100 p.p.m. had hastened flowering markedly.

On October 28 the pollen was removed from storage, and tests of germinability were run in a 20% sucrose solution. Only pollen grains that produced pollen tubes more than three times the diameter of the pollen grain were considered to have germinated in this experiment. Three replicates of about 200 to 300 pollen grains each were counted. The average germinations of pollen from clusters previously dipped in 0, 1, 5, 10, 25, or 100 p.p.m. of KGA_3, were, respectively, 90, 90, 50, 17, 7, and 0%. Thus, treatment with KGA_3 reduced germinability markedly.

On November 2 pollen from undipped clusters was placed in solutions containing 20% sucrose plus 0, 1, 5, 10, or 25 p.p.m. of KGA_3. Germination was,

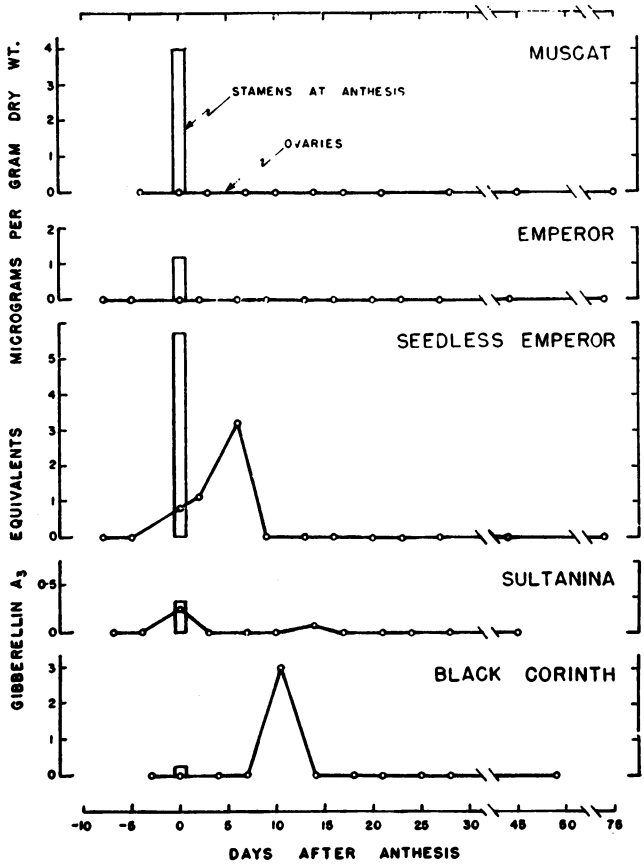

Figure 6. Gibberellin activity of extracts of grape stamens and ovaries of different varieties at different ages

As measured by d-1 maize bioassay (13)

respectively, 33, 21, 19, 1, and 1%. This indicates that KGA_3 acts as a pollenicide, which may explain its thinning action when applied at full bloom (*12, 43*).

Clusters were harvested on August 25. The data show that clusters were lengthened by application of 5 p.p.m. or more KGA_3. Average berry weight usually appeared lower in the same treatments, and degree Balling reading tended to be higher.

Treatment with KGA_3 at 100 p.p.m. resulted in more berries with one seed, at the expense of berries with three or four seeds. Lower concentrations showed no definite trends. When the average weight per berry in each seed class was determined (Figure 7), it was positively correlated with the number of seeds per berry. Within a given seed class, however, there were no significant differences in weight.

Lavee (*18*) found, on the other hand, that seeded berries in the Queen of the Vineyard grape also are enlarged by gibberellin to a limited extent. He reported that gibberellin compensates for the lack of seeds, but has no further effect, once the berry has a sufficient number of seeds.

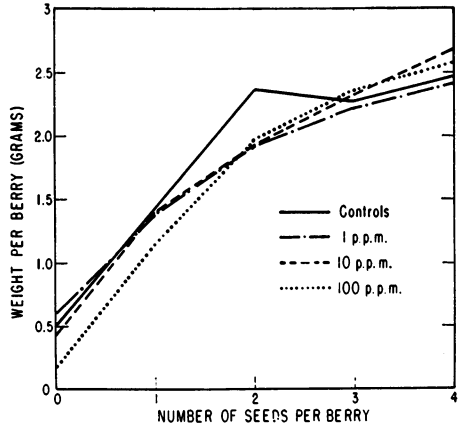

Figure 7. Effect of prebloom KGA_3 applications on weight per berry

Berries are heavier as number of seeds increases, but KGA_3 applications resulted in no significant trends within a seed class. L.S.D. at 5% is not significant for weight per berry in a given seed class, and is 0.25 for weight per berry between seed classes at a given concentration

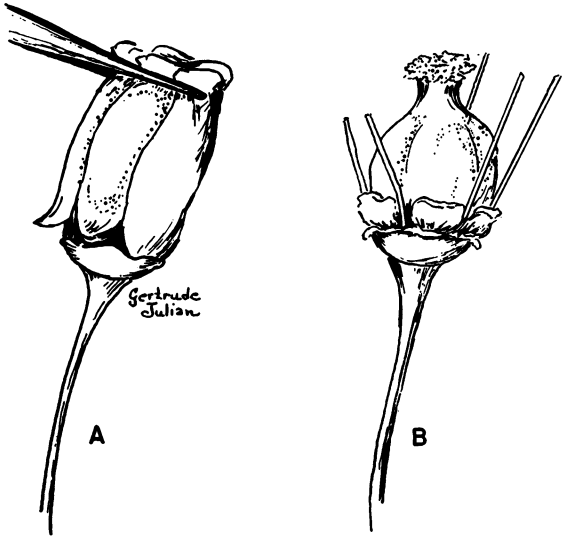

Figure 8. Emasculating a Tokay grape flower by removing calyptra and stamens with forceps

Response of Emasculated Flowers to KGA_3 and 4-CPA

In the following experiments the pollen, a rich source of auxin and gibberellins, was removed from the flowers so that the influence of different treatments on the ovaries could be studied. The flowers of *Vitis vinifera* are usually self-pollinated—i.e., the pollen usually falls on the stigma of the same flower. Emasculation experiments were performed as soon as five to ten calyptras had fallen from the

lower cluster. The open flowers were removed, and the remaining ones emasculated by pulling off the calyptra and the stamens with forceps (Figure 8). To eliminate or minimize cross pollination, flowers were emasculated when there was little or no wind. Clusters were dipped immediately after emasculation. After the spray dried, the clusters were bagged for about 10 days to prevent pollination while the stigmas were yet receptive.

Black Corinth is parthenocarpic. The stimuli of pollination and girdling are thought to be required to induce set, the process being referred to as "stimulative parthenocarpy" (*21*, *23*). A test was made to determine whether fruit would set with or without girdling and without pollination, and whether KGA_3 or an auxin, 4-CPA, could replace pollination and/or girdling. Girdling, also called "ringing," removes a complete ring of bark $^1/_8$ to $^1/_4$ inch wide from the cane below the fruit that it is intended to affect. As a result the carbohydrate and other materials produced in the leaves will accumulate in the parts above the wound, including the clusters or blossoms of fruit, and materially influence their development.

Twelve Black Corinth clusters were emasculated on May 11. The plants were girdled or the clusters dipped in KGA_3 or 4-CPA. There were three replicate clusters per treatment. One series of emasculated clusters on ungirdled canes was bagged but not dipped. Three unemasculated clusters on ungirdled canes and three on girdled canes were tagged for observation, but were not bagged. On May 21 one cluster was dead on ungirdled vines that had been emasculated and undipped; the others were small. The emasculated undipped clusters on girdled vines had set some berries about $^1/_8$ inch in diameter. Emasculated clusters on ungirdled vines dipped in KGA_3 at 20 p.p.m. had set some berries about $^5/_{16}$ inch in diameter, and emasculated clusters dipped in 4-CPA at 20 p.p.m. had produced an irregular set of berries $^1/_{16}$ to $^3/_{16}$ inch in diameter.

Fruit was harvested on July 21, when emasculated clusters on ungirdled canes were dead and dry (Figure 9, Table I). Berries in emasculated clusters treated with KGA_3 had set some very large berries, but clusters treated with 4-CPA were similar to those on girdled canes. Berries in unemasculated clusters on girdled canes were about half as large as those treated with KGA_3. The number of berries per cluster was smallest following KGA_3 treatment.

Table I. Data at Harvest (July 21, 1959) for Black Corinth Grapes Emasculated and/or Treated at Bloom Stage[a]

Treatment	*Concentration, P.P.M.*		*No. of Berries per Cluster*	*Weight per Berry, G.*
	KGA_3	*4-CPA*		
Emasculated				
Not girdled	0	0	Clusters dead and dry	
Cane-girdled	0	0	323	0.31
Not girdled	20	0	153	1.10
Not girdled	0	20	268	0.30
Not emasculated				
Not girdled	0	0	487	0.11
Cane-girdled	0	0	667	0.59

[a] Averages of three replicate clusters.

In Thompson Seedless, pollination and fertilization occur but the embryo soon aborts. Stout (*31*) called this condition stenospermy. To determine whether girdling, KGA_3, or 4-CPA would induce berry development following emasculation in this variety, clusters were emasculated on May 13. Four clusters

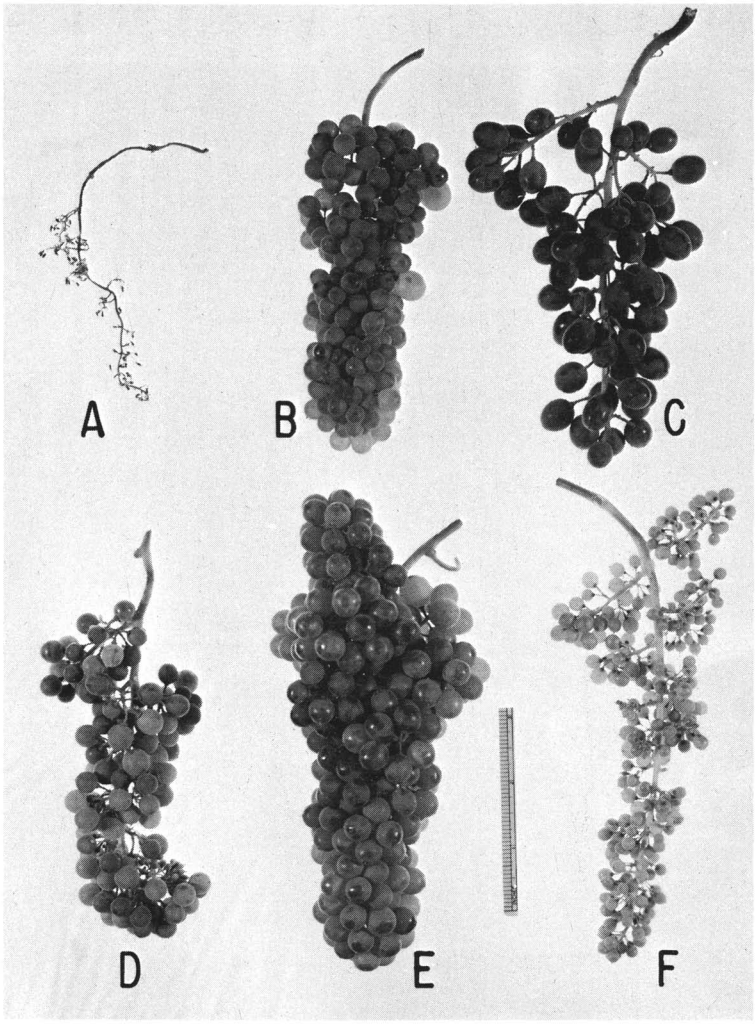

Figure 9. Black Corinth clusters 71 days after treatment

A. Emasculated, not girdled
B. Emasculated, girdled
C. Emasculated, not girdled, dipped in KGA_3 at 20 p.p.m.
D. Emasculated, not girdled, dipped in 4-CPA at 20 p.p.m.
E. Not emasculated, not dipped, girdled
F. Not emasculated, not dipped, not girdled
Girdling (B), KGA_3 (C), or 4-CPA (D) induced setting of berries in emasculated clusters; largest berries followed treatment with KGA_3

were bagged, two on girdled and two on ungirdled canes. Other clusters on ungirdled vines were dipped in KGA_3 at 20 p.p.m. or in 4-CPA at 15 p.p.m., two per treatment, and then bagged. Two unemasculated clusters on ungirdled canes were undipped and unbagged. Bags were removed on June 1.

Clusters were harvested on July 21, when undipped emasculated clusters on both girdled and ungirdled canes were dead and dry (Figure 10). The peduncle of one of the former, however, had been broken early in the season.

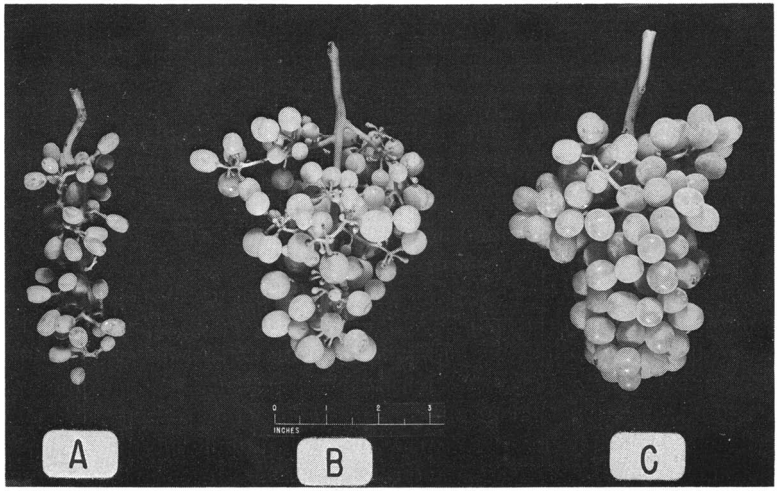

Figure 10. Thompson Seedless clusters 69 days after treatment

A. Emasculated, not girdled, dipped in KGA_3 at 20 p.p.m.
B. Emasculated, not girdled, dipped in 4-CPA at 15 p.p.m.
C. Not emasculated, not dipped, not girdled
Both KGA_3 (A) and 4-CPA (B) induced set of berries, though smaller than normal (C). Emasculated undipped clusters (not shown) were dead and dry on both girdled and ungirdled vines

Emasculated clusters dipped in KGA_3 at 20 p.p.m. had set small, elongated berries. The concentration used was too high, as indicated by a twisting of the rachis. One emasculated cluster dipped in 4-CPA at 15 p.p.m. was dead, except for the green rachis, but the other cluster had set many large berries besides numerous shot berries. The unemasculated control had produced a normal cluster. The stimulus of girdling was apparently insufficient to induce berry formation in this experiment, although both KGA_3 and 4-CPA were effective.

Seed development in Tokay is normally complete in a high percentage of ovules. Clusters were emasculated to determine whether KGA_3 or 4-CPA will induce berry formation in the absence of pollination and fertilization.

Cluster were treated on May 18, two per treatment. One series was emasculated and undipped, another was emasculated and dipped in 4-CPA at 5 p.p.m., and a third was emasculated and dipped in KGA_3 at 5 p.p.m. The controls were two unemasculated, undipped, unbagged clusters. Clusters were harvested on July 21 (Figure 11). One emasculated undipped cluster set no berries, but the other five berries. This may have been the result of pollen contamination or failure to emasculate all the flowers. Emasculated clusters dipped in 4-CPA set many berries of almost normal size. The unemasculated undipped control set a normal number of berries and those dipped in KGA_3 had set many berries of almost normal size. Thus, both KGA_3 and 4-CPA induced fruit set in this seeded variety.

Müller-Thurgau (*20*) long ago stated that elongation of berries in many varieties depends on fertilization. Since grape pollen is a rich source of gibberellin (*13*), this gibberellin may be involved in subsequent berry elongation. In our experiments treatment of emasculated Black Corinth and Thompson Seedless clus-

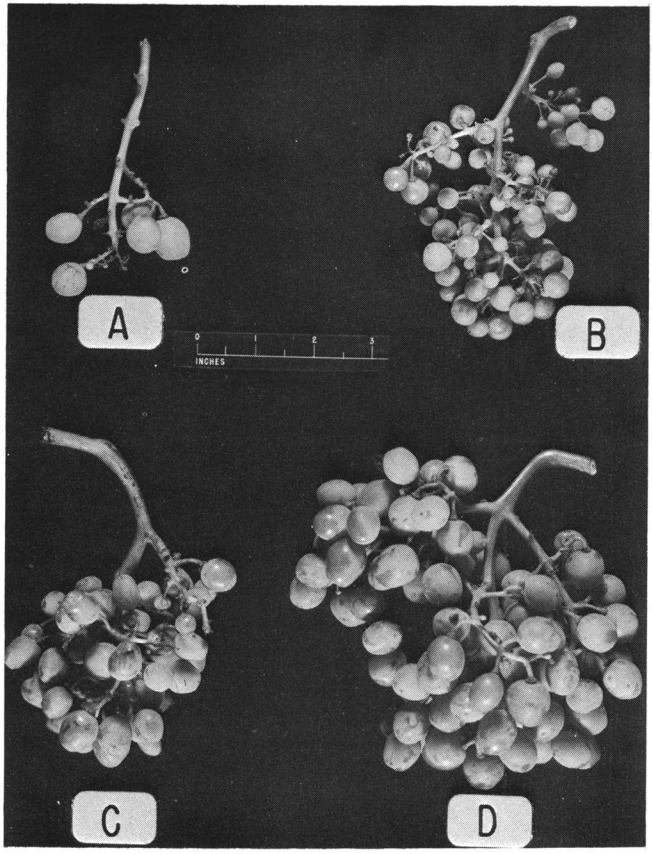

Figure 11. Tokay clusters 64 days after emasculation and dipping in 5 p.p.m. 4-CPA (B) or KGA_3 (C)

A. Emasculated undipped cluster
D. Unemasculated undipped cluster
Both regulators induced set in emasculated clusters, and berries of almost normal size were induced by KGA_3

ters with KGA_3 resulted in elongated berries, an effect also noted with unemasculated grapes (*30, 36, 42*).

The results show that the role of gibberellin in fruit set and development must be thoroughly investigated. It has long been the viewpoint that auxins produced in the seeds control fruit set and development.

Auxins have set several many-seeded fruits such as tomato, but have failed to set single-seeded fruit. Gibberellin has set many-seeded fruits, and Crane, Primer, and Campbell (*14*) showed that it can also set single-seeded fruits such as peach, almond, and plum.

Effect of GA_1, GA_2, GA_3, and GA_4 on Black Corinth and Thompson Seedless Fruit

Clusters of Black Corinth and Thompson Seedless were dipped in solutions of GA_1, GA_2, GA_3, and GA_4 at 0. 1, 5, or 10 p.p.m. All clusters of Black Corinth responded to all gibberellins, although the response appeared less to GA_2 than to

the other compounds. The results for Thompson Seedless were similar, including much less increase in berry size from GA_2. The significance of this difference is unknown, but is further evidence that biological materials may vary in sensitivity to different gibberellins (*11*, *24*).

In one experiment, when the increased fresh weight of shoot tips was used as a criterion of response, it was shown that GA_1 and GA_3 resulted in about equal shoot response (*39*). In this bioassay the apical 6 inches of Muscat of Alexandria shoots were sprayed with GA_1 or GA_3 at 25 p.p.m. Used as criterion for the response was the total fresh weight for growth 12 days after spraying.

Phytotoxicity of Gibberellin to Seedless and Seeded Varieties of Vitis vinifera

In the spring of 1958 delayed foliation and dead buds were noted at Davis, Calif., on certain seeded varieties that had received high concentrations of KGA_3 in 1957 (*37*, *38*). Observations were therefore continued for two more seasons to determine the damage and to see whether production would return to normal.

Since the five seeded varieties were similar in response, they are represented herein by Red Malaga, various vines of which were sprayed in 1957 with gibberellin at one of three stages (prebloom, full bloom, and after berry shatter). Shoots and clusters were counted in April of 1958 and 1959, and crop weights were measured in September or October. Since the results were essentially alike, regardless of spray date, only results of the full-bloom spraying are presented here (Table II).

Table II. Response of Red Malaga Grapes to KGA_3 Sprays

(Applied June 4, 1957)

KGA_3, Concn., P.P.M.	Shoot Count, No. per Vine		Cluster Count, No. per Vine		Crop Weight, Lb. per Vine	
	1958	1959	1958	1959	1958	1959
0	26.8	22.0	19	13	30.2	16.3
0.1	24.3	27.0	19	16	27.9	18.9
1	22.8	24.0	17	12	24.9	12.3
5	20.3	21.0	16	8	12.4	11.8
25	2.0	24.0	0	18	3.6	24.0
$d_{.05}$	5.3	N.S.	3.9	N.S.	8.5	4.0

KGA_3 applied in 1957 decreased the shoot and cluster count in 1958 on vines that received 25 p.p.m., and significantly decreased crop weight on vines that received 5 or 25 p.p.m. By 1959, however, recovery was complete. A high yield in 1959 on vines that received 25 p.p.m. in 1957 was probably a result of the low crop in 1958. Thus, injured vines had recovered within two seasons.

The seedless varieties used were Thompson Seedless and Black Corinth. On June 10, 1957 (after berry shatter), Thompson Seedless vines, four per treatment, were sprayed with KGA_3 at 0, 5, 20, or 50 p.p.m. The same vines were similarly sprayed in 1958 and 1959 at the same physiological stage of development. Table III shows the harvest data for 1957 and 1959. KGA_3 at 20 and 50 p.p.m. produced greatly enlarged berries in 1957 but had much less effect in 1959, probably because the crop was unusually heavy. The percentage of total soluble solids (degrees Balling) in 1959 was typical of other years. This experiment shows that gibberellin is nontoxic to Thompson Seedless grapes.

Table III. Response of Thompson Seedless Grapes to KGA_3 Sprays

(Applied in June 1957)

KGA_3, Concn., P.P.M.	Av. Weight Fruit per Vine, Lb., 1959	Av. Weight per Berry, G. 1957	Av. Weight per Berry, G. 1959	Degrees Balling 1957	Degrees Balling 1959
0	19.5	1.59	2.30	22.4	22.2
5	32.1	1.91	2.62	23.2	22.6
20	37.1	2.71	2.89	18.9	21.4
50	44.3	3.15	2.59	17.6	17.3
$d_{0.05}$		0.13		0.7	

One Thompson Seedless and one Black Corinth vine, sprayed after flowering in two consecutive years, respectively, showed no visible injury from KGA_3 at 1000 and 100 p.p.m. Thus, high concentrations are not toxic to the seedless varieties studied, although far lower concentrations are highly toxic to the seeded varieties studied. A generalization must await tests with more varieties.

The difference between seeded and seedless grapes in sensitivity to gibberellin probably relates to differences previously noted. Girdling shortly after flowering greatly enlarges seedless grapes but has little effect on seeded varieties (*15*). Thinning, in contrast, ordinarily increases berry size in seeded grapes but not much in seedless grapes (*47*). Gibberellin increases size of seedless fruit, but usually has little or no effect on seeded grapes (*42*). It is interesting, therefore, that natural gibberellins have been found in seedless, but not in seeded varieties.

Prolonging Dormancy in Vitis vinifera with Gibberellin

At Davis, Calif., grape buds developing on the basal portions of shoots normally enter a dormant stage about September. By the following April the buds usually begin rapid growth. Both a small amount of cold and a certain interval of time are required for dormancy to break. If the dormant buds could be made to grow in the autumn, the crop of the following year could be estimated. Since gibberellin has been reported to break the dormancy of buds in several species (*32*), it was tested for this purpose on grapes.

In one experiment, Zinfandel vines were sprayed with KGA_3 at 0, 10, 50, or 250 p.p.m. on September 6, 1957, two vines per treatment. At this time the foliage was still green and the percentage of total soluble solids of fruit was about 20. After the leaves had fallen, in November, buds in all treatments were still dormant. In January 1958, vines were pruned to 9 to 11 spurs, each bearing two to three buds (*47*). On April 19, control shoots were about 3 inches long (Table IV). The number of shoots decreased with increasing concentration of gibberellin, indicating that dormancy had been prolonged by KGA_3. Results were comparable in an experiment with the Tokay variety.

Table IV. Growth of Shoots on April 19, 1958, on Zinfandel Vines Sprayed Previous Autumn with KGA_3

KGA_3, Concn., P.P.M.	Av. Length per Shoot, Inches	No. of Shoots per Vine on Spurs	No. of Water Sprouts per[a] Vine
0	3.0	18	56
10	1.7	14	34
50	1.7	3	8
250	1.6	1	2

[a] Water sprouts refer to shoots arising from wood more than one year old.

In a second type of experiment, cuttings of Tokay canes about 16 inches long were taken from the vineyard on December 4, 1957, and placed in a greenhouse with their bases in solution containing 0, 0.01, 1, 10, or 100 p.p.m. of KGA_3. The solutions, about 3 inches deep, were changed each week. There were 30 cuttings per treatment. At later intervals the cuttings were examined to see whether buds had begun growth. A cutting was said to have initiated growth when green was visible on one or more buds of a cutting. The results (Table V) show that the higher the concentration of KGA_3, the longer the development was delayed. With one exception, no buds began growth by February 20 on cuttings placed in gibberellin in the range 1 to 100 p.p.m. This experiment was repeated with other sets of cuttings after they had been stored at about 32° F for 2, 4, or 6 weeks. The trends were similar.

Table V. Number of Tokay Cuttings in 30 That Showed Bud Growth after Being Placed in KGA_3 Solutions on December 4, 1957

KGA_3, Concn., P.P.M.	*Date of Readings*		
	Jan. 23, 1958	*Feb. 6, 1958*	*Feb. 20, 1958*
0	7	9	17
0.01	3	8	17
0.1	1	5	13
1	0	0	0
10	0	0	0
100	0	0	1

Gibberellin prolongs the rest of buds of *Vitis vinifera*, despite the fact that growth of shoots is greatly stimulated (*3, 7, 8, 26, 37*). Gibberellin may be of use for retarding bud break in the spring in regions of early frosts.

In some vineyards buds fail to break normally in the spring. This prolonged rest appears much the same as that caused by gibberellin applications. An interesting problem will be to study the role of gibberellin, if any, in naturally occurring prolongation of bud rest in grapes.

Translocation of Gibberellin

When young shoots were individually treated with gibberellin, the compound was not translocated to adjacent shoots, as judged by stimulation of growth (*43*). In one experiment, Ribier vines with shoots usually 7 to 9 inches long and clusters $1^1/_2$ to 3 inches long were used. Four shoots, including their clusters, were placed in waterproof plastic bags, and the whole vine was then sprayed with KGA_3 at 100 p.p.m. After the spray had dried, the bags were removed. Although sprayed shoots and clusters were greatly stimulated, unsprayed shoots on sprayed vines grew similarly to those on control vines, indicating that there was little or no translocation from one young shoot to another, at least not in amounts sufficient to cause measurable changes in growth.

Similar experiments were performed on older shoots 5 or 6 feet long. There was little or no translocation of gibberellin from one shoot to another (*43*).

Gibberellin readily translocates within a shoot. If one leaf is treated, the compound moves to the apical growing region and there stimulates growth. This fact has been used in studies of absorption and/or translocatability of different gibberellins in the grape (*39*).

In one experiment the basal leaf on each of 12 shoots of Carignane (about 6 inches long) was painted with KGA_3 at 100 p.p.m., and in a second series, the

youngest leaf with a width of $^1/_2$ inch or more was painted. Shoots and clusters were measured at subsequent intervals. The data showed that more KGA_3 reached the growing shoot apex by way of the basal leaf. For example, 25 days after treatment the average lengths of control shoots, shoots with apical leaf treated, and shoots with basal leaf treated were, respectively, 75.1 (7.5), 83.8 (6.7), and 91.9 (8.5) inches. Average cluster lengths are shown in parentheses. The L.S.D. at 5% was 6.6 for shoot length and 1.0 for cluster length.

Other experiments were run to show whether KGA_3 enters fruit through the leaves. To determine this, clusters were enclosed in waterproof plastic bags, and the foliage was then sprayed. After drying, the bags were removed. Response of clusters to KGA_3 was shown by increase in berry size or cluster elongation. In Zinfandel, a wine grape, much KGA_3 entered the fruit through the leaves. In Black Corinth and Thompson Seedless, however, the foliage was a relatively ineffective avenue of entry to the fruit. These differences may be explained by the earlier time of treatment for Zinfandel, and varietal differences may also affect translocation.

To test whether KGA_3 moved from one part of a cluster to another, the apical or basal portions of young Thompson Seedless and Black Corinth clusters were treated (*44*). Only sprayed berries enlarged, indicating little or no translocation of KGA_3 from one berry to another (Figure 12). When KGA_3 was applied to

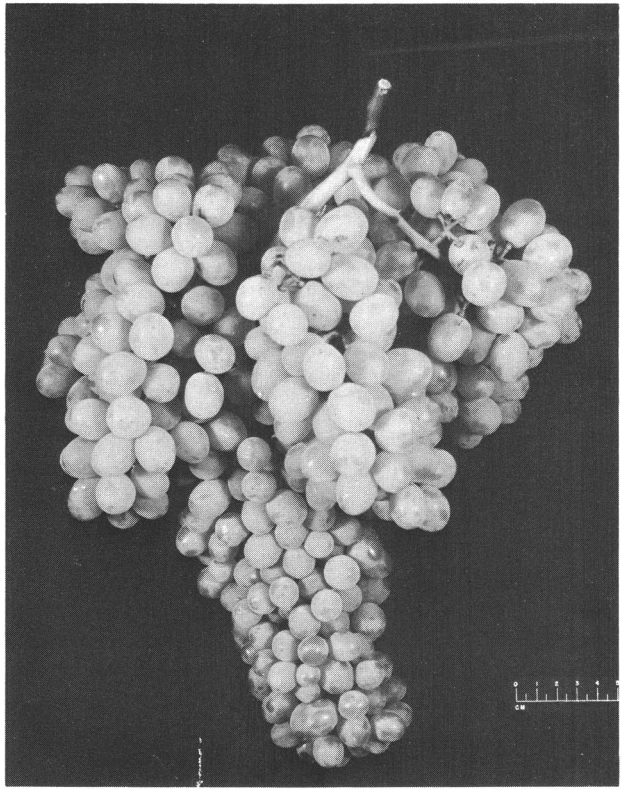

Figure 12. Thompson Seedless cluster 79 days after basal portion treated with KGA_3 at 100 p.p.m.

Only treated portion responded to KGA_3

various portions of individual berries, all treated berries enlarged and elongated symmetrically, which demonstrates that gibberellin moves readily within a treated berry.

Role of Gibberellin in the Photoperiodic Response

Little work on photoperiodic responses of grapes is known to the author. Alleweldt (*1*, *4*, *5*, *9*) has demonstrated with potted grape plants that certain varieties such as Riparia G 1, Rupestris St. George, and *Solonis robusta* respond to short-day conditions by dying of the shoot tip, and premature cessation of growth. Internode length and number were also reduced. This response to short-day conditions was manifested to a much smaller degree by varieties such as Riesling and Sylvaner.

A light break with a higher proportion of red light was more effective in bringing about a long-day effect than light with a lower proportion of long-wave rays. Applications of GA_3 to the short-day-sensitive varieties failed to break the rest period induced by the short day. With the relatively short-day-insensitive varieties, however, GA_3 completely overcame the short-day conditions. The recovery was judged by shoot extension and development of new leaves. Alleweldt (*6*) suggested that the basis for the photoperiodic reactions of the grape can be explained by assuming that synthesis of a system of growth inhibitors is induced by exposure of the vines to short days. Further, he stated that IAA plays an insignificant role in these reactions and is not responsible for the vine's entering the resting state, whereas GA_3 or similar substances act in the enzymatic processes of inhibitor synthesis or degradation.

Uses for Gibberellin on Grapes

Gibberellins show promise for several uses in grape production. Girdling has long been used to induce set in Black Corinth, from which the currants of commerce are made. In recent years, however, girdling in Black Corinth has been almost entirely replaced by spraying with the auxin 4-CPA(35). Gibberellin has also produced an excellent set in Black Corinth, but it remains to be seen whether it is superior to 4-CPA in this regard (Figure 13).

When Thompson Seedless grapes are girdled after the shatter of impotent flowers, berry size usually increases greatly. Since the 1920's, Thompson Seedless grapes in California have been girdled when used for table grapes because the public prefers them and will pay more for them than for smaller-berried clusters. In recent years 4-CPA has also been used on a limited scale for the same purpose. The use of 4-CPA on Thompson Seedless, however, has never been accepted as it has with Black Corinth. Gibberellin also greatly increases size of berry when the sprays are applied at the proper time for girdling (Figure 14). It can be applied either separately or in conjunction with girdling. In many cases gibberellin applied separately produces larger berries than those obtained with girdling only. The largest berries result from a combination of girdling and gibberellin.

Prebloom sprays of gibberellin greatly elongate the clusters (Figure 2). The flower clusters are elongated, so that clusters of compact varieties are loosened, and some shot berries are formed. The loosening of compact-clustered wine grapes should prevent, or at least decrease, the amount of rot that may develop.

Prebloom sprays of gibberellin also hasten flowering. Sprayed vines flower 3 or 4 days earlier than the unsprayed. In some varieties such as Zinfandel, ripen-

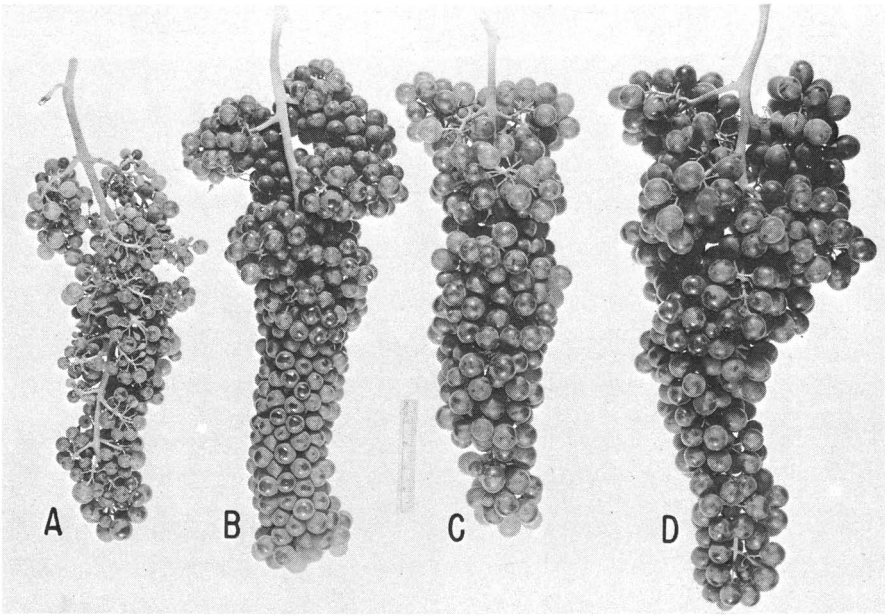

Figure 13. Black Corinth grapes 59 days after being sprayed on May 31 with KGA_3

A. Control
B. Girdled but unsprayed
C. 5 p.p.m.
D. 20 p.p.m.
Berries sprayed at 5 p.p.m. are larger than girdled but unsprayed berries, but those sprayed at 20 p.p.m. are largest

ing also may be hastened. The berries color and sugar up earlier. For example, when Zinfandel vines were sprayed on June 4 with 0, 1, 15, 25, or 40 p.p.m. of gibberellin, the respective approximate percentages of total surface of fruit that were colored on July 29 were 0.5, 1, 15, 25, and 40. At harvest on September 26 the respective percentages of sugar in the juice were 18.7, 19.1, 20.7, 20.9, and 22.1. In most varieties, however, gibberellin failed to hasten ripening.

When gibberellin is sprayed on seeded vines during the summer or fall, foliation is often delayed in the following spring. Such sprays may be valuable to retard bud break in the spring and protect against early frost injury. Further work must be done on this phase, however.

Residual Gibberellin in Grapes and Wine

It is generally believed that exogenous gibberellins rapidly disappear from the vine, and that little or none is present in fruit at harvest. Coombe (*13*) failed to find endogenous gibberellin in grapes at harvest. Zweig (*50*), working in the Coachella Valley desert of California, sprayed clusters of Thompson Seedless with GA_3 at 100 p.p.m., and found a small residue (0.021 p.p.m.) when he sampled the crop 34 days after treatment. It seems unlikely that exogenous gibberellin applied around bloom time or earlier would be toxic to humans, since it occurs naturally in the fruit of seedless grapes near bloom.

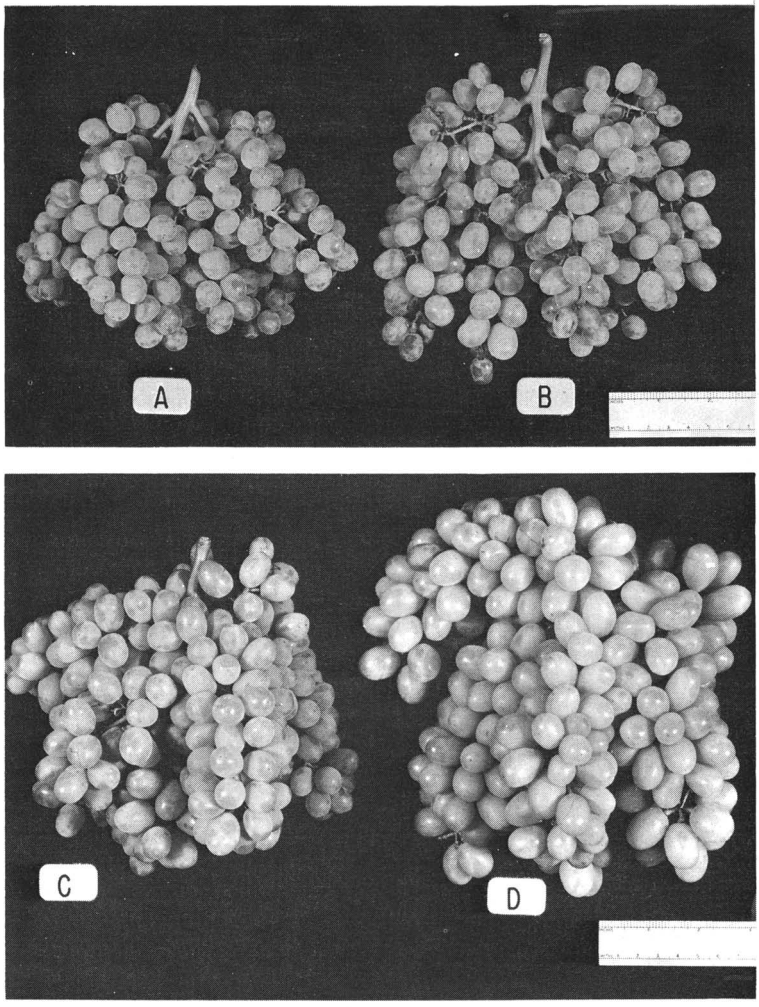

Figure 14. Thompson Seedless grapes 76 days after being sprayed on June 11 with KGA_3

A. Ungirdled, unsprayed
B. 5 p.p.m.
C. 20 p.p.m.
D. 50 p.p.m.
Berry size increases with higher concentrations, and some berry elongation occurs with higher concentrations

Wine was prepared at the University of California at Davis from grapes sprayed with gibberellin, and tasted by a panel of experts. Preliminary results showed that wine from sprayed grapes scored just as high as wine from unsprayed.

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RECEIVED November 16, 1960.

Flowering and Fruiting of Strawberries in Relation to Gibberellins

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Gibberellins are known to alter the growth of plants. The influence of gibberellins, specifically the potassium salt of gibberellic acid, on the growth and fruiting of Sparkle strawberry plants has been investigated in the greenhouse and field during the past few years. These studies indicate the peak of the June harvest can be shifted into the early harvest period, if a 10-p.p.m. spray is applied three times at weekly intervals, starting in early September in the field. Greenhouse studies reveal earlier opening of flowers treated with gibberellins and a reduction in the number of short days required for formation of flower buds. Strawberries sold on the fresh market are worth more during the early part of the harvest period, and shifting the peak of the harvest into the early period returns greater profit to the grower.

Strawberry plants are an excellent tool in fruit research. They take up little valuable greenhouse space and in the field are suitable for small plot work, which is both economical and reliable. A fund of information was developed concerning the effect of factors, such as temperature and light, on the growth and fruiting of strawberry plants. The profound changes in growth and fruiting brought about by changes in the plant's environment are caused by changes in the biochemical systems within the plant—for example, the flowers of strawberry plants are initiated in the autumn under conditions of short days and cool temperatures.

Strawberry growers would like to control the fruiting of their plants so the crop can be marketed to advantage. This can be done in two basic ways: by increasing production per acre or so altering the harvest season that more of the crop is marketed at high prices. Interest in the use of growth-regulating chemicals on strawberries is motivated by these economic factors.

In 1957, work with gibberellins on strawberry plants was initiated at Rutgers University. The results of a small preliminary field study (*3*) indicated a possible

increase in the volume of fruit harvested early, but because of adverse weather conditions data concerning total yields could not be obtained. On the basis of these preliminary results, field studies were continued in the autumn of 1958. Uniform, 30-foot single row plots were selected in a field of Sparkle strawberries that had fruited in the spring and four studies were initiated. Each experiment was in a randomized block design with five replications.

Effect of Concentration

Potassium gibberellate (Gibrel, a solution of potassium gibberellate supplied by Merck & Co., Inc.) was applied three times (September 17, October 1, and October 15). These spray treatments consisted of 10, 20, 30, 40, 50, and 100 p.p.m. of potassium gibberellate plus an unsprayed check. These plots, as well as all others in the field studies, were kept under observation until the winter mulch was applied in December 1958, and observations were continued in the spring of 1959 when the mulch was removed.

Flowers appeared in the autumn of 1958 on plots treated with 40, 50, and 100 p.p.m. of potassium gibberellate and petiole elongation was noted on plants sprayed with 100 p.p.m. When the plants started to bloom in the spring, more flowers opened earlier on the plants sprayed with 10 and 20 p.p.m. of potassium gibberellate than on the unsprayed check plants. Plants in all plots started to bloom at about the same time. No other effects of the sprays were noted until harvest.

The yield data for the concentration study reported in Table I show that sprays at 10 and 20 p.p.m. of potassium gibberellate significantly increased early yields (first three harvests), compared to the unsprayed check. At the conclusion of the harvest season, all spray treatments except 10 p.p.m. had caused a significant reduction in yield when compared to the check. Thus, 10 p.p.m. of potassium gibberellate applied three times in the fall caused a highly significant increase in early yields of Sparkle strawberries but no significant change in total yields.

Table I. Effect of Rate of Potassium Gibberellate Application to Strawberry Plants

(Average accumulated yield in quarts per acre)

	Yield	
Concn., P.P.M.[a]	*First three harvests*	*Total, eight harvests*
0	2603	10,662
10	3758	9,519
20	3616	8,637
30	3515	8,252
40	2485	5,209
50	2390	5,241
100	832	1,673
L.S.D. 0.05	958	1,882
L.S.D. 0.01	1298	2,550

[a] Applied Sept. 17, Oct. 1, and Oct. 15.

Effect of Number of Gibberellate Applications

All spray applications were at the same concentration, 20 p.p.m. Three treatments consisted of single applications on September 17, October 1, and October 15. Two treatments received two spray applications, one on September 17 and October 1, and the other on October 1 and October 15. The sixth treat-

ment consisted of three spray applications on September 17, October 1, and October 15. An unsprayed check was included in this study.

Table II presents yield data from this study. Three applications of 20 p.p.m. of the material caused a highly significant increase in early yields with no significant reduction in total yields. The single application on October 15 and the two applications started October 1 significantly reduced total yields. Under conditions of this study it would appear that three applications of potassium gibberellate are needed to increase early yields.

Table II. Effect of Number of Potassium Gibberellate Spray Applications to Strawberry Plants

(Average accumulated yield in quarts per acre)

20 P.P.M. K Gibberellate Applied	*Yield*	
	First three harvests	*Total, nine harvests*
Sept. 17	2329	11,360
Oct. 1	2693	9,845
Oct. 15	2432	8,119
Sept. 17 and Oct. 1	2648	10,305
Oct. 1 and 15	2652	7,831
Sept. 17, Oct. 1, and Oct. 15	2981	9,053
Unsprayed	2363	10,036
L.S.D. 0.05	421	1,379
L.S.D. 0.01	571	1,868

Effect of Time of Initiation of Applications

A spray of 20 p.p.m. of potassium gibberellate was applied three times, at weekly intervals starting September 10, 17, 24, and October 1, 8, and 15. An unsprayed check was included, making a total of seven treatments. Yield data obtained in the spring of 1958 (Table III) indicate a highly significant increase in early yield with no significant reductions in total yield when the spray treatments were started on September 10 or 17. Neither early nor total yield was significantly affected when treatments were initiated on September 24. When treatment was delayed until October 1 or later, reduction in total yield was significant. The data from the study on numbers of applications (Table II) show that a single application at 20 p.p.m. made October 15 or two or three such applications initiated on or after October 1 cause a significant reduction in yield. This indicates that time of spraying can be very important in increasing early yields without significantly reducing total yields.

Table III. Effect of Time of Spray Application of Gibberellins to Strawberry Plants

(Average accumulated yields in quarts per acre)

Date of First Application[a]	*Yield*	
	First two harvests	*Total, seven harvests*
Sept. 10	3314	9,872
Sept. 17	3128	9,644
Sept. 24	2796	10,037
Oct. 1	3310	8,700
Oct. 8	2761	8,894
Oct. 15	1643	5,222
Unsprayed	2327	10,713
L.S.D. 0.05	586	1,465
L.S.D. 0.01	795	1,983

[a] Sprays applied at weekly intervals until three sprays of 20 p.p.m. of K gibberellate applied.

Effect of Potassium Gibberellate on Fruit Size

The treatments of 20 p.p.m. of potassium gibberellate applied three times (September 17, October 1, and October 5) and a nonsprayed check were combined in factorial design with treatments of 2-naphthoxyacetic acid (BNOA) at 50 p.p.m. sprayed on the plants in the spring during bloom. Two BNOA treatments were used: a single application on May 11, 1959, and two applications, on May 11 and May 18, 1959. In addition to harvest records obtained in the spring of 1959, average fruit size was determined by weighing 25 berries selected at random from each plot on each harvest date. Table IV indicates that spray applications of 20 p.p.m. of potassium gibberellate in the preceding autumn caused no significant reduction in total yield. Fruit size, as indicated by the average weight of 25 berries at the third harvest, was significantly reduced by applications of potassium gibberellate in the fall, but by the time of the seventh harvest, no significant difference in fruit size was noted. An increase in total fruit volume and a decrease in average fruit size in the early harvest period when potassium gibberellate is used mean that more berries are picked—ones which would ripen later in the season on nontreated plots. Data from plots in the fruit size study indicate that fruit harvested later in the season is smaller. The average reduction in fruit size observed in the early harvest period could be due to the presence of fruit which would usually ripen later in the season, but because of the treatment with potassium gibberellate, ripen early.

Table IV. Effect of Spray Applications of Potassium Gibberellate upon Strawberry Fruit Size and Yield

Treatments		*Accumulated Yields Quarts per Acre*		*Average Wt. of 25 Fruit, Grams*	
K gibberellate, .p.m.[a]	*2-Naphthoxyacetic acid, p.p.m.*[b]	*First three harvests*	*Total, seven harvests*	*At third harvest*	*At seventh harvest*
0	0	2711	10,298	240	123
0	50, 1 application	3199	11,031	201	123
0	50, 2 application	2765	10,991	210	123
20	0	4332	10,037	200	132
20	50, 1 application	3347	7,821	130	127
20	50, 2 applications	3740	8,339	175	128
	L.S.D. 0.05	726	1,873	38	23
	L.S.D. 0.01	990	2,254	51	32

[a] Sprays applied Sept. 17, Oct. 1, and Oct. 15, 1958.
[b] 1st application May 11, second application May 18, 1959.

Conclusions

These field studies indicate that sprays of potassium gibberellate in the autumn change the pattern of fruit production the following spring. It would appear that the flowers had all reached a similar stage of development at the bloom period on plots treated with 10 and 20 p.p.m. of the material. This is carried through to the harvest period, when more fruit is harvested early from the plots receiving these treatments.

At the time the field studies were in progress, greenhouse investigations were initiated. The Sparkle variety only was used. Greenhouse-grown, potted dormant strawberry plants were placed under a 16-hour photoperiod and sprayed with 0, 10, 20, 40, 80, and 160 p.p.m. of potassium gibberellate, applied first on October 13 and repeated on October 20 and 27, 1958. Each plot included four plants

treated alike and the plots were arranged in two randomized blocks. Greenhouse temperatures were maintained at 70° F.

The treated dormant plants produced longer petioles and appeared in a vegetative condition two weeks after treatment was initiated. These results are similar to those reported by Thompson and Guttridge (*4*) and Porlingas and Boynton (*2*), who comment on the vegetative stimulation caused by application of gibberellins to strawberry plants.

The aerial portions of the plants were harvested following the final spray application and extracted using the paper chromatographic method outlined by Vlitos and Meudt (*5*) for the determination of indoleacetic acid (IAA). To secure enough tissue for chromatographic analysis it was necessary to composite tissue from all plants receiving the same treatment. The IAA spots on the paper were eluted and quantitative determinations carried out by methods developed by Gordon and Paleg (*1*).

The results of the chromatographic analysis are reported in Table V. The presence of IAA in vegetative strawberry tissue was confirmed, but no simple relationship between free extractable IAA and applications of potassium gibberellate could be observed. The compositing of plant tissues prevented the statistical analysis of these data.

Table V. Effect of Potassium Gibberellate on Free Indoleacetic Acid Concentration in Aerial Portions of Sparkle Strawberry Plants

Concn. of Applied K Gibberellate, P.P.M.	*Indoleacetic Acid, μg./50 Grams Fresh Weight*
0	2.57
10	2.15
20	2.83
40	....[a]
80	2.16
160	2.22

[a] Extract accidentally lost.

In a second greenhouse study, stimulated by results of the field studies, the effect of gibberellins on rate of flower development was investigated. All plants were grown in a pretreatment photoperiod of 16 hours for several months to ensure that all flowers observed were formed under treatment conditions. Each plant was considered a plot and plants were selected for uniformity of size. The plants were placed on a greenhouse bench and shaded so that they received only 8 hours of light at the start of the study on October 28, 1959 (short-day conditions). One group of 36 plants was sprayed with 10 p.p.m. of potassium gibberellate immediately upon being placed under the short-day condition. This spray was applied once a week until three sprays had been accomplished. A similar group of 36 plants was left unsprayed as a check or second treatment. These two treatments represented sprayed and control during the flower initiation period.

A third group of 36 plants was subjected to 30 short days and then given the same spray treatment as the first group. This group represented treatment at a different stage of development, because after 30 short days flowers should have been initiated in the crowns. A check on floral initiation after 30 short days can be found in the control group for the first treatment. A fourth group of 36 plants was carried along as the control for the third treatment.

To gain information on the effect of potassium gibberellate on the number

of short days needed for flower initiation and development, groups of six plants, each plant a replicate, were withdrawn from each spray treatment and its respective control at 3-day intervals starting 15 days after spraying and continuing until six withdrawals had been made. These plants were placed under 16 hours of light each day (long-day conditions) and continued under these conditions until the study was terminated April 18, 1960. A record was kept of the number of opening of flowers produced by each plant. At the termination of the study all plants that had not produced flowers were dissected and examined for floral primordia. Greenhouse temperatures were maintained at 75° F. during the day and 65° F. at night. All data from this study were subjected to appropriate statistical examination.

Flowers were produced by the unsprayed plants that were exposed for only 30 short days. This confirms that flowers had been initiated in the crowns of plants when sprays were applied after 30 short days and that these plants represented a different population from those sprayed immediately on placement under short-day conditions. One clearly evident result was a sharp reduction in number of flowers produced. The plants treated with 10 p.p.m. of potassium gibberellate, regardless of time, produced about one half as many flowers as the control plants. This was not observed in the field studies at the 10-p.p.m. concentration, but 40 p.p.m. of potassium gibberellate used three times in the field reduced yields by about one half.

A statistical examination of the number of days between initiation of short-day treatments and the opening of the first flowers revealed a highly significant effect for gibberellin applications. When potassium gibberellate was applied to strawberry plants, it caused the plants to start to bloom earlier. In the field studies no differences were observed between treatments in time of flowering, but only in number of flowers that appeared early during the flowering period. To examine this point specifically, the time between initiation of short-day treatments and production of 50% of flowers by each plant was calculated. These data indicated that potassium gibberellate treatments significantly reduced the time required for the first 50% of the flowers to come into bloom. Thus, in the treated plants the peak of the normal blooming curve tends to move into the early season. This effect is similar to the changes in patterns of production in the field plots reported earlier.

Another aspect of this greenhouse experiment was the withdrawal of groups of plants from each spray treatment and their controls 15, 18, 21, 24, 29, and 30 days after treatment. The control or unsprayed treatment that received only 15 short days caused only two out of six plants to produce flowers. Five of the six plants sprayed with 10 p.p.m. of potassium gibberellate and receiving only 15 short days produced flowers. This difference was highly significant. It would appear that without the spray 15 short days were inadequate for good flower induction but spraying with gibberellins caused more plants to produce flowers. Eighteen short days caused five out of six plants to produce flowers in both sprayed and unsprayed plants. Another significant effect was due to the period the plants were exposed to short days. Plants exposed to periods of short days ranging between 15 and 30 days started to bloom earlier than those exposed to short days ranging between 45 and 60 days. Although short days are required for initiation of flowers, they appear to inhibit the emergence of the flowers from the crown.

Summary

Potassium gibberellate sprayed on strawberry plants in the field in the autumn at about the time flowers were initiated increased the quantity of fruit harvested during the early part of the picking season the following spring. Greenhouse studies indicate this is probably because more flowers come into bloom at the beginning of the bloom period. Fruit growers may be able to take advantage of this by placing on the market a greater volume of fresh fruit at a time when premium prices are offered.

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RECEIVED January 25, 1961.

Determination of Residual Tritium-Labeled Gibberellic Acid in Potatoes, Grapes, and Products Derived from Barley by Isotopic Dilution Techniques

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Isotopic dilution techniques were used to determine residual, tritium-labeled gibberellic acid in potatoes, grapes, and various products derived from barley. The seeds, the young plants, or the fruit were treated with labeled gibberellic acid and analyzed at the end of the growth period by extraction of the labeled residue in the presence of carrier gibberellic acid, isolation of a pure crystalline specimen, and subsequent assay by liquid scintillation counting.

To obtain federal registration for the use of gibberellic acid on various crops, the residual concentration at harvest time had to be determined. Zweig and Cosens (7) reported on the use of bioassay procedures in determining residual gibberellic acid in grapes and outlined the "total count method" for the determination of residual, tritium-labeled gibberellic acid.

Bioassay procedures for the determination of gibberellic acid have been developed (2, 5), but more recent chemical fluorometric assay methods are equally specific. However, both assay methods show a low response with samples containing less than 10 $\mu\mu$g. of the gibberellins. Consequently, in determining residual amounts within the part per billion (p.p.b.) range, relatively large samples must be extracted and extracts partially purified to satisfy the assay conditions. These operations are usually accompanied by some material losses or degradation, which impair quantitative interpretation of the results. Natural inhibitors can influence the results in the bioassay method (2), and fluorescent contaminants can interfere with the spectrophotometric analysis.

Because it is possible to label gibberellic acid with tritium, isotopic procedures are eminently suited for collecting the necessary data, circumventing some of the

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above difficulties. According to the principles of isotopic dilution analysis, an excess of unlabeled carrier material can be added during the extraction of a sample containing a labeled residue. As only a fractional recovery of the added material is required for the analysis, the sample size can be chosen within wide limits, and the resulting extracts can be subjected to vigorous purification steps to eliminate possible interference by degradation products or metabolites. Furthermore, by using materials with a specific activity of 400 μc. per mg. the detection limits can be lowered to 0.1 μμg.

On the other hand, the use of the total count method (7) largely forfeits the advantages which are inherent in the tracer method. Because the samples are extracted rather than assayed by combustion techniques, incomplete material recovery becomes a contributing factor. Furthermore, since the extracts are counted without preceding purification, the results cannot discriminate between residual gibberellic acid and possible degradation products. In spite of potentially increased sensitivity, the total count method offers no advantages over the bioassay or fluorometric procedures.

Isotopic Materials and Procedures

The preparation of tritium-labeled gibberellic acid and general isotopic methods have been described (*1*). Samples (10 to 100 grams) of the various plant tissues, or products, were extracted in a Waring Blendor with an acetone-water mixture (30% water) containing a known amount (100.0 to 200.0 mg.) of carrier gibberellic acid. The slurries were filtered, and the filtrates were concentrated under reduced pressure and extracted with ethyl acetate (pH 2.5). Volume equivalents of the concentrated and dried solutions were assayed directly by liquid scintillation counting, using internal standardization techniques. The remainders were chromatographed on acid-washed alumina, pure samples of the added carrier materials were recovered by crystallization and assayed, and the concentration of residual gibberellic acid was calculated as follows:

$$\mu\text{g. of residual gibberellic acid-H}^3 = \frac{\text{c.p.m. per mg. of isolated sample}}{\text{c.p.m. per } \mu\text{g. of standard sample}} \times C \text{ mg.}$$

where C represents the weight of carrier gibberellic acid originally added. Since the weight of residual gibberellic acid is small compared to the weight of the added carrier, use of this simplified equation is satisfactory.

Comparison of these data with the values obtained by direct isotopic assay of the extracts gave some indication as to the concentration of labeled metabolites. Since the isolation yields varied, these values may be regarded as estimates only. Furthermore, conversion of the gibberellic acid to some unknown metabolite might have been accompanied by a partial removal of the label.

Residue in Potatoes and Grapes

Potatoes. TREATMENT. Seed pieces of White Rose, Russet Burbank, and Pontiac potatoes were treated with an aqueous solution of 25 p.p.m. of tritium-labeled gibberellic acid (specific activity 108 μc. per mg.) for 5 minutes. The dried pieces were kept overnight at 68° F. (80 to 85% relative humidity) and then planted at the University Farm, Davis, Calif. Thirty days later, leaves and rhizomes of the White Rose variety were sampled to determine the translocation of gibberellic acid. New potatoes were harvested 60 days after planting. The

White Rose potatoes showed a pear-shaped gibberellin effect, as described by Rappaport and Lippert (*6*), whereas the other varieties were smaller than the controls.

RESULTS. The analytical results summarized in Table I show that during the early growth period gibberellin was translocated into the leaves and rhizomes. However, largely because of the mass increase, the residual amounts found in the new potatoes were very small.

TABLE I. Residue Data on Potatoes and Grapes

Sample	*Days after Treatment*	*Extracted Activity Expressed as Gibberellic Acid, P.P.B.*[a]	*Gibberellic Acid Residue in Sample, P.P.B.*[c]
Potatoes			
White Rose, leaves	30	27	20
White Rose, rhizomes	30	64[b]	Not determined
New potatoes			
White Rose	60	2.1	1.7
Russet Burbank	60	0.7	0.6
Pontiac	60	2.5	1.1
Grapes			
Thompson Seedless treated in Indio			
Entire cluster	0	3400	4600
	34	88	58
Grapes only	34	75	38
	50	80	34
Thompson Seedless treated at Davis			
Grapes only	62	32	8

[a] Not corrected for extraction yield (50 to 80%).
[b] No carrier added.
[c] Values do not include any endogenous material present, since isotopic procedures determined concentration of residual labeled gibberellin only.

A comparison between the total amount of activity in the extracts and the activities actually associated with unchanged gibberellic acid shows that part of the material was metabolized. On the other hand, identification of unchanged gibberellic acid 60 days after application was unexpected, as gibberellic acid is known to be unstable in an aqueous medium.

Grapes. TREATMENT. Thompson seedless grapes were sprayed by hand atomizer to run off as described by Zweig and Cosens (*7*) with a 100 p.p.m. solution of tritium-labeled gibberellic acid. Samples were collected immediately after spraying, and after 34 and 50 days. At harvesting time (62 days), the treated fruits were significantly larger than the controls.

RESULTS. The analytical data summarized in Table I indicate that a large fraction of the applied gibberellin was recovered unchanged at the end of the growth period. Roughly one half of the activity was associated with materials other than gibberellic acid.

Zweig and Cosens (*7*) have reported the results of residue analysis of these same samples by bioassay and total count procedures; they are of the same order of magnitude. Residues in potatoes and grapes were determined jointly by Abbott Laboratories and the Pesticide Residue Research Laboratory, University of California.

Residue in Products Derived from Barley

Residue in Malt. TREATMENT. Samples of steeped barley (Kindred Variety, Northern-B grade, low protein 44.8% moisture) were treated with tritium-labeled

gibberellic acid solutions at various concentration levels. The samples were allowed to germinate under the standard conditions of the malting process. After drying, the sprouts were separated from the grains, and both parts were analyzed separately.

RESULTS. The analytical data are summarized in Table II. Absorption of the gibberellin from its solution is more nearly complete at the lower concentration range, and the applied material is relatively little degraded during germination and drying. The data clearly indicate translocation of gibberellin into the sprouts.

Work on products derived from barley was carried out jointly between Abbott Laboratories and the Research Laboratory, Rahr Malting Co.

TABLE II. Residues in Finished Malt

Gibberellic Acid Added per 454 G. Barley		*Gibberellic Acid Recovered*							
		Malt			*Sprouts*			*Total*	
Mg.	*P.p.m.*	*Mg.*	*P.p.m.*	*%*	*Mg.*	*P.p.m.*	*%*	*Mg.*	*%*
1.00	2.20	0.632	1.52	63.2	0.070	3.30	7.0	0.702	70.2
0.50	1.10	0.374	0.90	74.8	0.031	1.47	6.3	0.405	81.1
0.25	0.55	0.195	0.47	78.0	0.018	0.84	7.2	0.203	85.2

Residue in Distiller's Dried Grains with Solubles

Since malt is used as a source of liquefying and saccharifying enzymes in whiskey and grain spirits fermentations, and gibberellic acid increases the α- and β-amylase content of malt, it became desirable to determine gibberellin residues in a distiller's feed by-product.

Sample History. A malt sample which had been treated with gibberellic acid at a 1-p.p.m. level was submitted to Hiram Walker & Sons, Inc., for use in a pilot scale (0.16 bushel) bourbon fermentation. After fermentation, the beer was dealcoholized with steam and the grain residue was concentrated and dried in a tray dryer at 160° to 170° F.

Results. The results of this analysis are tabulated below. Contrary to natural growth processes, the distilling process (mashing, fermentation, distillation, and by-product feed recovery) destroyed considerable gibberellin, resulting in a material recovery of only 15%.

Materials used	Malt containing 0.88 p.p.m. gibberellin, %	8.24
	Carbohydrate sources (12% moisture), %	91.76
	Calculated over-all concentration of gibberellic acid in mixture, p.p.m.	0.072
Materials recovered	(Material recovery, solids, 34%)	
	Distillation residue, calculated concentration of gibberellic acid, p.p.m.	0.211
	Concentration found, p.p.m.	0.032

Gibberellic Acid in Brewer's Products

Sample History. All-malt microbrews containing 100 grams of malt which had been treated with 88 μg. of labeled gibberellic acid were brewed according to the technique reported by Kneen (3). Fermentation was carried out for 7 days at 12° C. and after fermentation, the beer was transferred to storage flasks, chill-proofed, and stored in the cold for 3 weeks. After storage, the beer was filtered, carbonated, and bottled.

Results. Aliquots of the beers, grain beds, and yeast and trub were assayed for labeled gibberellic acid as described above. The results of the residue analysis are summarized in Table III. Gibberellic acid is surprisingly stable in this system, considering that during preparation of the wort, the material is boiled for about an hour. The greatest percentage of the activity is recovered in the beer as gibberellic acid. A small amount remains on the grain bed, while the yeast and trub contain an almost insignificant amount of gibberellic acid. No effort was made to separate the yeast from the trub, to determine exactly how much gibberellic acid had accumulated in the yeast. It can be only an insignificant amount at best. Direct counts were made on the beers, extracts, and grain beds of the first two experiments to determine the total activity present; 84.5 and 75.2% of the total added activity were recovered in the first and second determinations, respectively. No attempt was made to calculate the recovery of total activity for the third determination, as all the grain bed extract was used in the chromatography of the sample.

TABLE III. Gibberellic Acid in Brewer's Products

Gibberellic Acid Added per 100 G. Malt		*Gibberellic Acid Recovered*										
		Beer			*Grain Bed*			*Yeast and Trub*			*Total*	
μg.	*P.p.m.*	*μg.*	*P.p.m.*	*%*	*μg.*	*P.p.m.*	*%*	*μg.*	*P.p.m.*	*%*	*μg.*	*%*
88	0.88	30.43	0.1268	34.6	19.65	0.459	22.2	0.0603	0.0374	0.068	50.14	56.9
88	0.88	24.84	0.092	28.3	6.05	0.203	6.88	0.040	0.0231	0.045	30.93	35.2
88	0.88	30.34	0.0812	34.4	8.66	0.198	9.34	0.0601	0.0387	0.068	39.06	44.4

Discussion

Although random labeling with tritium usually involves some element of uncertainty as to the identity of the labeled molecules with the original sample, this labeling method frequently offers the only practical means of preparing the necessary amounts of tagged materials for field studies. Carbon-14–labeled gibberellic acid has been prepared, but the yields have been too low to make its use practicable (*8*).

Prior to the use of tritium-labeled gibberellic acid in these studies, the identity of the labeled material was tested chemically and biochemically. On the basis of these tests, the labeled material can be regarded as unchanged gibberellic acid.

In many of the studies reported herein higher than recommended levels of gibberellic acid were used. As a result, concentrations of residual gibberellin at harvest time were higher than would be present under commercial circumstances. These higher levels were sought deliberately to permit check of isotopic data by biochemical assay procedures (*5*). Further, decomposition of the tissue-bound gibberellin was at a slower rate than had been anticipated. Treatment at lower levels might have been very difficult to follow.

A major factor which could not be evaluated in this study is the ratio between residual extraneous gibberellic acid and endogenous gibberellins. Because of the weight increase of the plants from the time of application of the gibberellic acid to harvest, these natural levels in many cases might be comparable with the artificial levels. This phase has since been studied (*4*).

Acknowledgment

The authors thank Marlin T. Leffler and Edward J. Matson of Abbott Laboratories and Michael R. Sfat of the Rahr Malting Co. for the coordination of this project, and J. M. Van Lanen of Hiram Walker & Sons, Inc., for the experimental sample of distiller's dry solids. They are particularly indebted to Gunter Zweig and Gladys R. Cosens of the Pesticide Residue Research Laboratory, University of California, Davis, for all planting data and samples for the grape and potatoes residue studies.

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RECEIVED September 15, 1960. Not a part of the symposium.

Translocation of C^{14}-Gibberellin in Red Kidney Bean, Normal Corn, and Dwarf Corn

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C^{14}-Gibberellin was synthesized by a culture of *Fusarium moniliforme* using C^{14}-1-acetate as the source of radioactivity. C^{14}-Gibberellin was applied to leaves of corn and beans as well as cotyledon nodes of bean plants. Translocation studies by autoradiography and growth response were followed under high and low humidity conditions for 1 to 216 hours. Evidence is presented of xylem and phloem transport of C^{14}-gibberellin in beans and possible phloem movement in corn. Growth response was correlated with the accumulation of radioactivity from C^{14}-gibberellin in the growing tissues at the stem tip of the bean plant. Foliar application of C^{14}-gibberellin in bean plants resulted in absorption and translocation only under high-humidity conditions. Significant movement of C^{14}-gibberellin into roots was observed only in bean plants but not in corn.

To elucidate the action of a plant growth regulator, it is necessary to relate its distribution in the plant to the site of action. To date, little is known about this relationship of gibberellic acid in plants. The first report on the movement of gibberellic acid was made by Watanabe and Scully (*10*, *11*), who observed the movement of C^{14}-gibberellic acid to be "nonpolar" in pinto bean plants, following internodal application. Nonpolar movement of gibberellic acid has also been observed by Kato (*6*) in pea stem sections using a spectrophotometric assay.

In the present studies the distribution of C^{14}-gibberellin by autoradiography was compared with internodal and foliar elongation in test plants. These studies included a time-rate series ranging from 1 to 216 hours after exogenous application. The synthesis of C^{14}-gibberellin used in these studies was based on previous work dealing with the biosynthesis of fungal gibberellic acid (*1*, *15*), implicating acetate, mevalonic, or senecioic acids as precursors.

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Materials and Methods

Synthesis, Isolation, and Characterization of C^{14}-Gibberellin. Cultural conditions for growing *Gibberella fujikuroi* (*Fusarium moniliforme* Sheld.), strain NRRL 2284, have been described (*15*).

To an experimental flask containing 75 ml. of medium were added aseptically 5 ml. of inoculum and 15 ml. of C^{14}-1-sodium acetate (specific activity 5.45 mc. per mmole). The flask was placed on a New Brunswick rotary shaker and agitated for 1 week.

At the end of the incubation period, the culture was filtered through Whatman No. 1 paper, and 1 gram of Darco G-60 charcoal was added to the filtrate. This suspension was agitated on the shaker for 1 hour and then filtered. The charcoal was air-dried and eluted once with 80% aqueous acetone. The eluate was concentrated to about 5 ml. in vacuo and was finally lyophylized.

After the dry powder had been dissolved in 1 ml. of 80% acetone, the solution was streaked on a sheet of Whatman 3-mm. paper (18 × 11 inches). Markers containing authentic gibberellic acid (Abbott Laboratories) were placed near both edges of the sheet on the origin. The paper was chromatographed by the ascending technique using Mitchell's solvent (*7*), *tert*-amyl alcohol–*n*-butyl alcohol–acetone–concentrated NH_4OH–water (25:25:25:10:15, v./v.).

Four major regions of radioactivity, detected with a Forro strip scanner and by autoradiography, had the following R_f values: 0.08, 0.41, 0.72, and 0.92. The region at the R_f of 0.41 corresponded to authentic gibberellic acid and was eluted with water. Acetone did not elute the radioactivity, since the chromatographic solvent contained NH_4OH, thus converting acids to the corresponding ammonium salts.

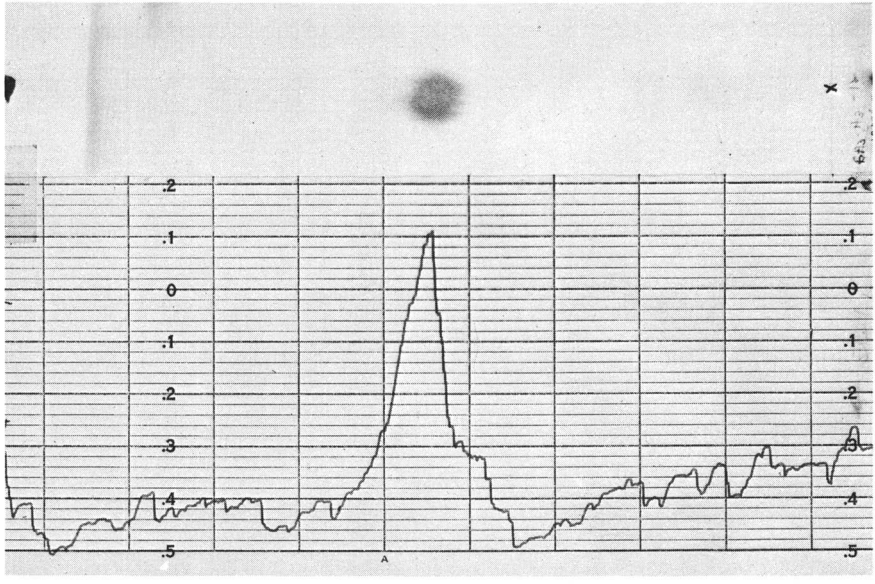


Figure 1. Chromatogram (upper) and corresponding radioactive scan of C^{14}-gibberellin (NH_4 salt)

The water eluate was lyophylized and analyzed quantitatively for gibberellic acid by the bioassay technique using d-1 dwarf corn (*14*). The C^{14}-gibberellin thus isolated was chromatographically identical with nonradioactive gibberellin A_3 (gibberellic acid) and was radiochemically homogeneous, as indicated by a single radioactive component (Figure 1). The sample had 62% of the biological activ-

ity of known gibberellic acid and a specific activity of 162,694 c.p.m. per mg. The total yield was 5.0 mg., equivalent to 0.47% radiochemical conversion of C^{14}-1-acetate to C^{14}-gibberellin.

The C^{14}-gibberellin was examined by infrared spectrophotometry, using the micropellet KBr technique and a Model 221 Perkin-Elmer infrared spectrophotometer. The spectrum of this material was not identical with that of the ammonium salt of authentic gibberellic acid (A_3), gibberellins A_1, A_2, and A_4, or *allo*gibberic acid. The 5.7 micron band (carboxyl group or lactone ring) was considerably weaker for the radioactive material, and an additional band at 6.15 microns was found (Figure 2). For these reasons the term "C^{14}-gibberellin" rather than "C^{14}-gibberellic acid" was chosen.

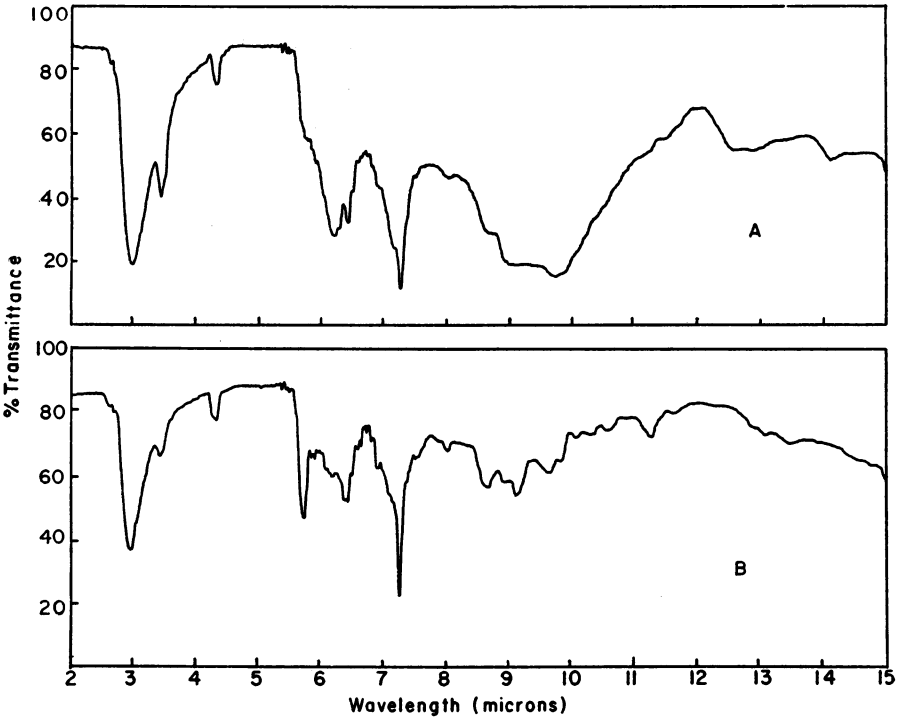


Figure 2. Infrared spectra
A. NH_4-C^{14}-gibberellin
B. NH_4-gibberellate

Culture Conditions of Plants. Red kidney beans were started in sand, transferred to Hoagland's solution in quart Mason jars, and grown under greenhouse conditions. At the time when the primary leaves were fully expanded, and the terminal bud was about 2.5 cm. long, the plants were treated as described later.

The dwarf mutant corn (d-1) plants were germinated in soil-vermiculite, transferred when they were nearly ready for bioassay treatment (*14*), and grown in a growth chamber at about 25° to 30° C. with continuous white fluorescent light of about 450 foot-candles. The roots of the corn were supported on filter paper wetted with Hoagland's solution and affixed to slanting glass plates, sup-

ported in a large metal tray containing about 1 inch of nutrient solution. The corn plants were treated when the first two leaves of the dwarf variety were still unfurled at a height of about 2 cm., and the normal corn had grown to a height of about 12 cm.

Treatment of Red Kidney Beans. In two preliminary experiments, 25μl. of a solution containing 100 μg. of C^{14}-gibberellin (equivalent to 62 μg. of A_3), dissolved in 80% acetone or 0.05% Tween 20 in water, was applied as a drop near the base of a primary leaf blade. The solution was confined to a small area by a Tygon ring $^1/_2$ inch in diameter, held in place with lanolin paste.

In a third experiment the C^{14}-gibberellin was applied at the cotyledon node. The solution was held in place by lanolin, which was covered with an aluminum-foil envelope.

In two subsequent experiments, following foliar and cotyledon applications, the bean plants were covered with polyethylene bags. To maintain a saturated atmosphere, water was applied daily, so that 1 inch of water covered the bench top on which the plants in Mason jars were growing.

In all experiments a sufficient number of plants was used for a time series of 1 hour to 9 days. All plants after harvesting were "freeze-dried" and autoradiographed (*12*).

Treatment of Corn. Ten microliters of an 80% acetone solution, containing 100 μg. of C^{14}-gibberellin, was applied with a micropipet near the middle of the upper surface of the first leaf, followed by 10 μl. of 0.1% Tween 20 and 50% ethyl alcohol in order to increase absorption of the radioactive material. The drop of solution was kept from running down by means of lanolin paste. Four normal and four dwarf corn plants were thus treated, while untreated plants were kept as controls. The same number of dwarf plants was treated with 0.05 μc. of C^{14}-5-aminotriazole in order to compare the pattern of translocation of gibberellin with that of a compound whose movement has been studied previously (*4*). The treated plants and controls were then placed in the growth chamber, and one or two specimens were harvested at the end of 1, 2, and 7 days, freeze-dried, and autoradiographed.

Results

Absorption of NH_4–C^{14}-Gibberellin. Absorption of NH_4–C^{14}-gibberellin by red kidney beans depended on the site of application and the humidity conditions under which the plants were grown. Foliar application under normal greenhouse conditions resulted in an extremely low uptake of C^{14}-gibberellin from 80% acetone or 0.05% Tween 20 solutions. This was demonstrated by autoradiographs showing an intense black spot at the area of application and almost no distribution of radioactivity throughout the plant. Even 4 days after the original application of 100 μg. of C^{14}-gibberellin no enhanced elongation of the internode over the control was observed, indicating that no appreciable amount of gibberellin was taken up by the bean plants. Under high humidity conditions, using equal amounts of C^{14}-gibberellin, a higher proportion of the applied materials entered the plant, as is shown in the autoradiogram (Figure 3,*C*) and the visible growth response in the internode. A slight chemo-pseudoautoradiograph was observed in the control plant (Figure 3, *A*). Application at the cotyledon node under high humidity and normal greenhouse conditions resulted in greater absorption than that from foliar applications. Even so, by far the larger amount of applied radioactivity remained at the point of application (Figure 4).

The autoradiographs of C^{14}-gibberellin-treated corn plants (Figure 5) revealed limited absorption as shown by the light distribution pattern in the plant outside the treated spots. However, enough of the radioactive material was absorbed by the corn to give a significant gibberellin-response in dwarf corn (d-1) (8). In comparison, 0.05 μc. of C^{14}-5-aminotriazole in corn plants showed a much higher absorption of the applied material by the plants (Figure 6).

Transport of C^{14}-Gibberellin in Red Kidney Bean Plants. When NH_4–C^{14}-gibberellin was applied at the cotyledon node of red kidney bean plants, elongation of the first internode above the primary leaf was significant 8 hours after application. Table I gives the growth increment (millimeters per hour) due to applied NH_4–C^{14}-gibberellin, contrasted with controls. The greatest response was observed between 8 and 72 hours after treatment, but no further response in the first internode was seen after this time. However, the internodes above the first continued to respond even after this period, indicating that gibberellin-response in the plant had not yet terminated at the 72-hour treatment time.

Table I. Growth Increment Due to C^{14}-Gibberellin on First Internodal Elongation under Greenhouse Conditions

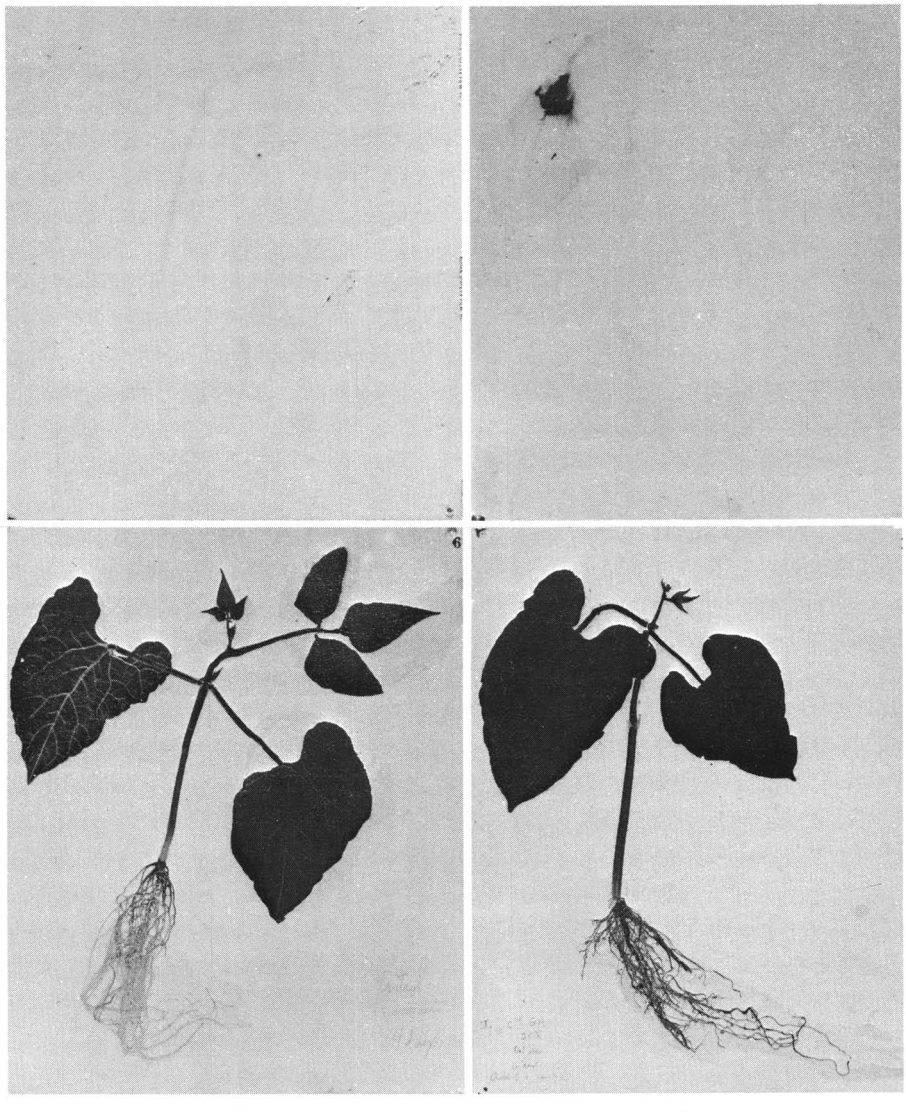
	Hours after Treatment				
	3	*8*	*24*	*72*	*144*
Treatment			*Mm./Hour*[a]		
C^{14}-gibberellin (100 μg.)	0	0.4	0.5	0.3	0
Control	0	0.1	0.2	0.1	0

[a] Average of 3 plants.

Elongation of the internode due to C^{14}-gibberellin was compared with the distribution of radioactivity. Figure 4 depicts autoradiographs of some of the plants used for the growth-response studies summarized in Table I. After 8, 24, and 72 hours treatment, radioactivity accumulated in the growing tissues at the stem tip, most pronounced in the 24-hour sample where movement proceeded all the way to the root tips. General distribution of radioactivity throughout the plant was observed as early as 3 hours after treatment, but after 1 hour all parts, except the roots, contained some radioactivity. Therefore, as shown in Table I and Figure 4, the growth response due to C^{14}-gibberellin and the distribution pattern of radioactivity may be interrelated. Some evidence that this translocated radioactivity is due to intact gibberellin was obtained by a preliminary experiment. Twenty-four hours after application of 500 μg. of nonradioactive authentic gibberellic acid at the cotyledon node of a bean plant, an acetone extract of the trifoliate leaves was made. This extract was assayed by (d-1) dwarf corn test and had an activity equivalent to 1.0 μg. of gibberellic acid.

Figure 4 also shows the decline of radioactivity in the growing regions at the stem tip 72 hours after treatment. Beyond this, radioactivity appears to decrease also in the primary leaves, suggesting a redistribution of radioactivity to the growing leaves. Future experiments will determine whether the radioactivity which has been redistributed is due to intact C^{14}-gibberellin.

Movement of C^{14}-gibberellin 96 hours after foliar application under high humidity proceeded into all parts of the plant except the opposite primary leaf (Figure 3,*C*). This observation suggests phloem movement of C^{14}-gibberellin. Additional evidence favoring this hypothesis is the accumulation of radioactivity in the growing regions at the stem tip, following cotyledon node application (Figure 4).

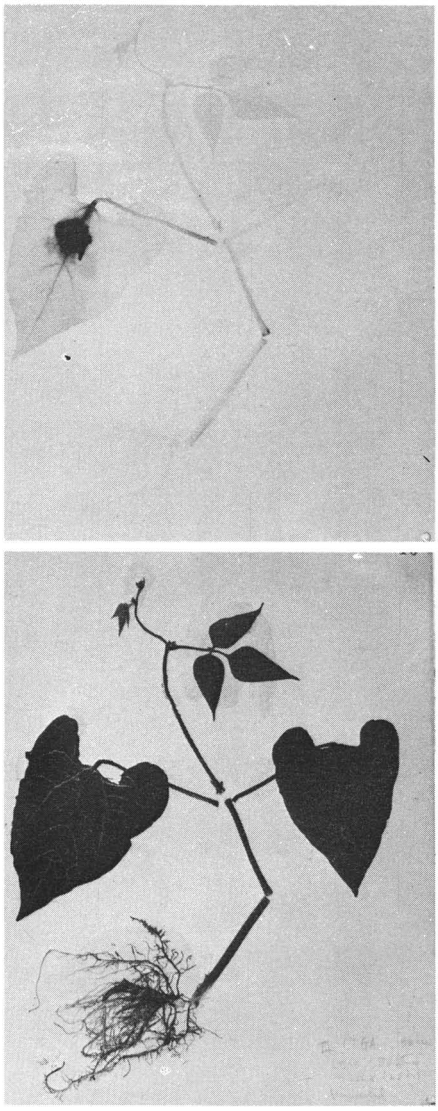


A B

Figure 3. Foliar application of C^{14}-gibberellin on red kidney bean plants 96 hours after treatment

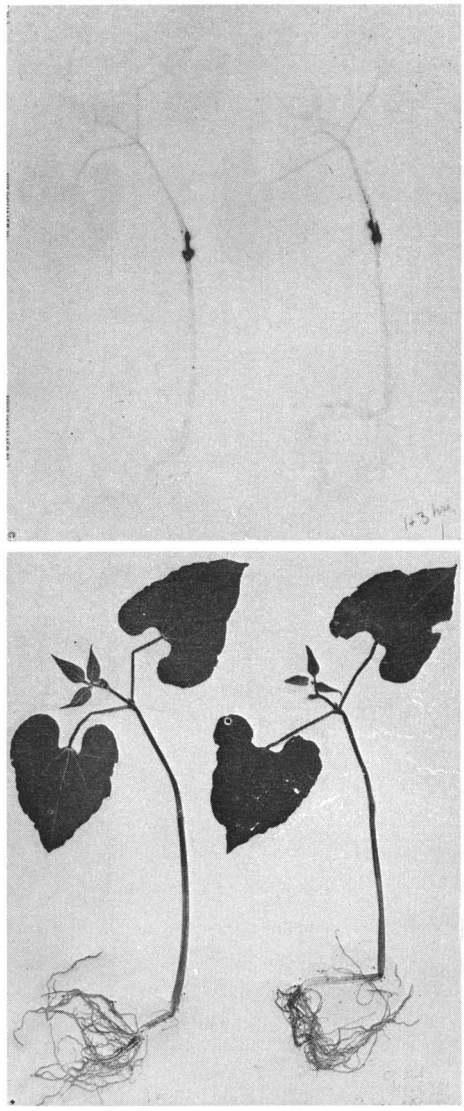
Upper. Autoradiograms
Lower. Lyophylized plants
A. Control; no C^{14}-gibberellin applied
B. Greenhouse conditions; C^{14}-gibberellin
C. High-humidity conditions; C^{14}-gibberellin

Figure 3,*C* shows an additional movement of radioactivity following foliar application of C^{14}-gibberellin. This was a wedge-shaped movement from the site of application towards the leaf tip and has been ascribed by Strugger to transpirational movement in the cell walls (*9*). This movement has also been observed with the more mobile compounds, like maleic hydrazide, monuron, simazine, and aminotriazole (*5*).



C

Figure 3 (Continued)



1 3

Figure 4

Transport of C^{14}-Gibberellin in Corn Plants. Corn plants which had received 0.05 μc. of C^{14}-gibberellin showed a light distribution to all developing leaves after 1 to 2 days treatment. There was a tendency for radioactivity to accumulate in the growing regions (Figure 5), but no evidence of movement into the roots was found. Although maximum distribution of radioactivity occurred 24 to 48 hours after initial application of C^{14}-gibberellin, faint radioactivity in the roots was observed 7 days after treatment, but only after the plants had been exposed to x-ray film for 10 weeks instead of the usual 2 weeks. Judged by the autoradiographs, only a trace of the applied C^{14}-gibberellin was absorbed and translocated at all treatment times studied.

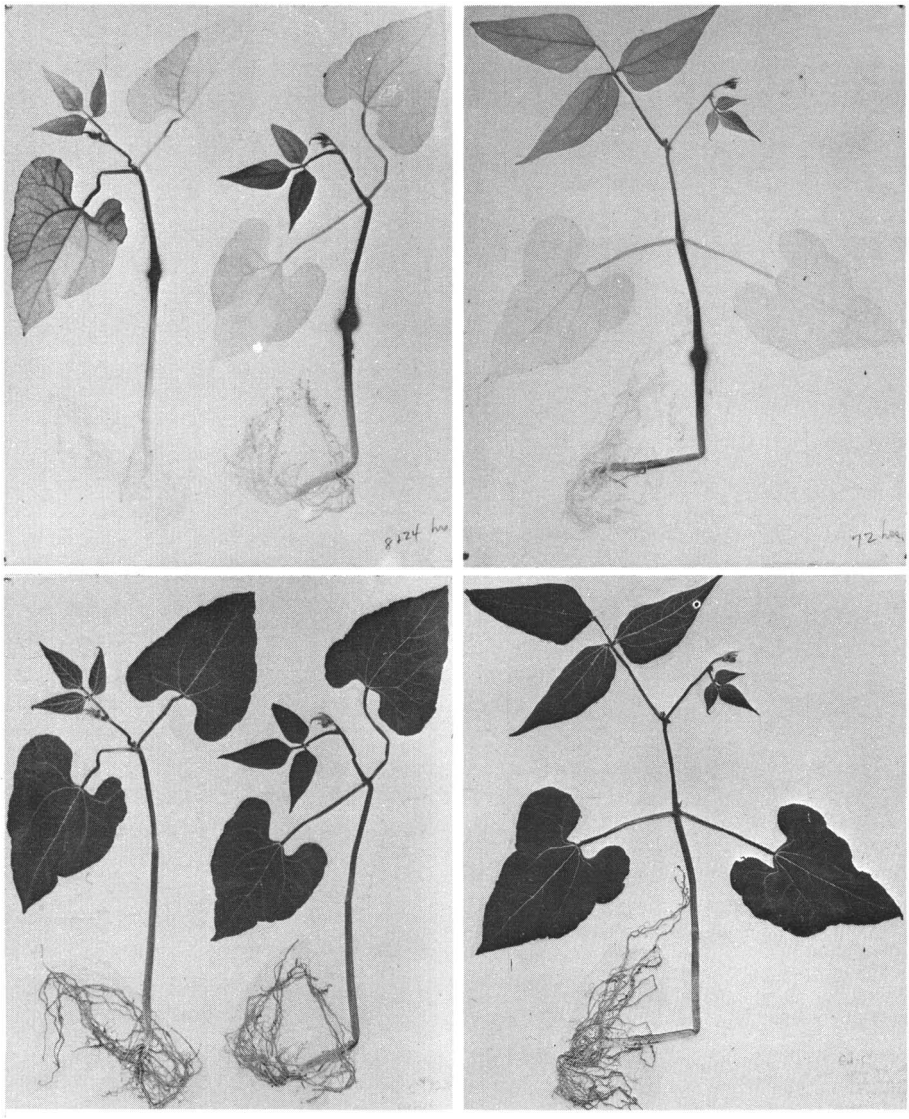
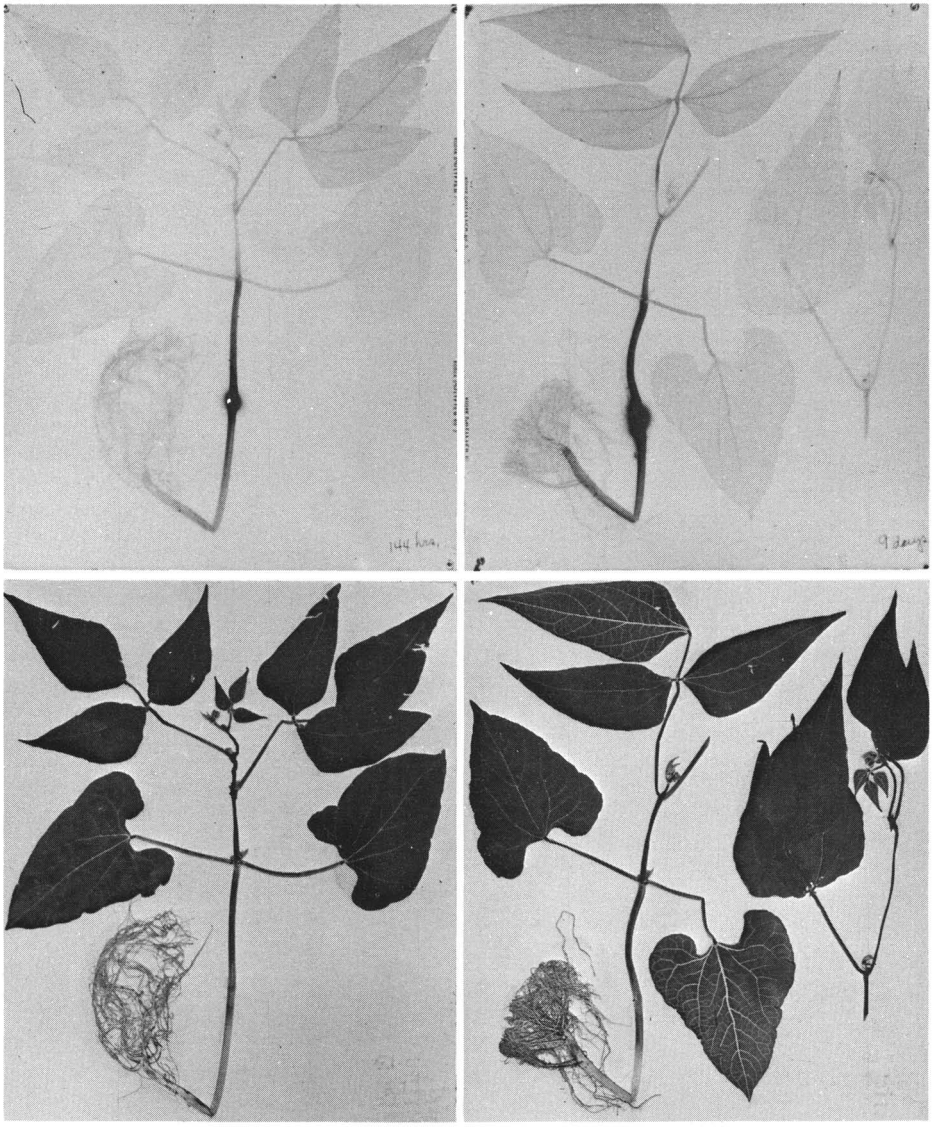


Figure 4 (Continued). Cotyledon node application of C^{14}-gibberellin on red kidney bean plants under greenhouse conditions

Numbers refer to treatment time in hours
Upper. Autoradiograms
Lower. Lyophylized plants

The amount of radioactivity in the roots was too small to make extraction and bioassay possible, but further attempts are being made to investigate the nature of the radioactive material. No essential differences were observed in the distribution pattern of C^{14}-gibberellin in normal and dwarf corn plants.

The movements of C^{14}-gibberellin was compared with that of C^{14}-5-aminotriazole in corn plants. The translocation of aminotriazole has been studied (*4*) and shown to occur readily from mature green leaves to the growing regions, in-



144 216

Figure 4 (Continued)

cluding root tips. The same general distribution of C^{14}-5-aminotriazole has now been demonstrated in dwarf corn (Figure 6). The movement of C^{14}-aminotriazole into the roots clearly shows that no blockage of translocation exists. Therefore, the difference in distribution of gibberellin and aminotriazole must be ascribed to specific translocation characteristics of these two compounds.

As in the studies with bean plants, it was indicated that a correlation existed between the presence of radioactivity due to C^{14}-gibberellin in the growing regions of the leaves of dwarf corn and the observed growth response.

Discussion

It is necessary to review the chemical and biological properties of the C^{14}-gibberellin used in these studies. The C^{14}-gibberellin had 62% of the biological activity of authentic gibberellic acid (A_3). Furthermore, the C^{14}-gibberellin was radiochemically homogeneous and identical with gibberellic acid as determined by co-chromatography. However, the partial disappearance of the 5.7-micron band in the infrared suggested that the C^{14}-gibberellin was not gibberellic acid but might resemble *allo*gibberic acid. The lack of response to *allo*gibberic acid in dwarf pea (2) and dwarf corn from our own observations, in fact, precluded the possibility that C^{14}-gibberellin was *allo*gibberic acid. Furthermore, C^{14}-gibberellin gave an infrared spectrum which was different from that of *allo*gibberic acid, as well as gibberellins A_1, A_2, A_3, and A_4. It is concluded that the C^{14}-gibberellin may be a mixture of gibberellins or a new fungal gibberellin produced under our cultural conditions.

The translocation studies indicate that C^{14}-gibberellin can move in the bean plant by xylem as well as phloem. The evidence for xylem transport is the general distribution of radioactivity in the mature green leaves following cotyledon node application 8 hours after treatment. The autoradiographs showed this generalized movement 1 and 3 hours after treatment. Phloem movement, on the other hand, seemed to occur simultaneously with xylem movement, as shown by the accumulation of radioactivity in the growing regions at the stem and root tips. Additional evidence for phloem movement is that C^{14}-gibberellin deposited on a primary bean leaf moved into stem, hypocotyl, and root but not into the opposite primary leaf. If xylem transport had also been involved, movement into the opposite leaf would have occurred. However, apoplastic movement was observed within the treated leaf, as shown by the wedge-shaped pattern of movement of radioactivity towards the leaf tip (Figure 3, *C*). In the corn plant the transport of C^{14}-gibberellin and C^{14}-aminotriazole may be attributed to phloem rather than xylem. As shown in Figures 5 and 6, movement occurred only to the developing leaves and the tip of the treated leaf. If xylem and apoplastic as well as phloem movement had occurred, one would have expected an accumulation of radioactivity near the tips of the developing leaves (3), and this did not occur.

Acknowledgment

The authors are grateful to A. S. Crafts for encouraging them to carry out this research. The advice and assistance of J. E. DeVay, Beth Hertlein, and R. William Breidenbach are gratefully acknowledged.

Infrared spectra of gibberellic acid, gibberellins A_1, A_2, A_3, and A_4, and *allo*-gibberic acid were kindly furnished by Abbott Laboratories.

Seeds of the dwarf mutant corn (d-1) were obtained from B. O. Phinney, University of California, Los Angeles.

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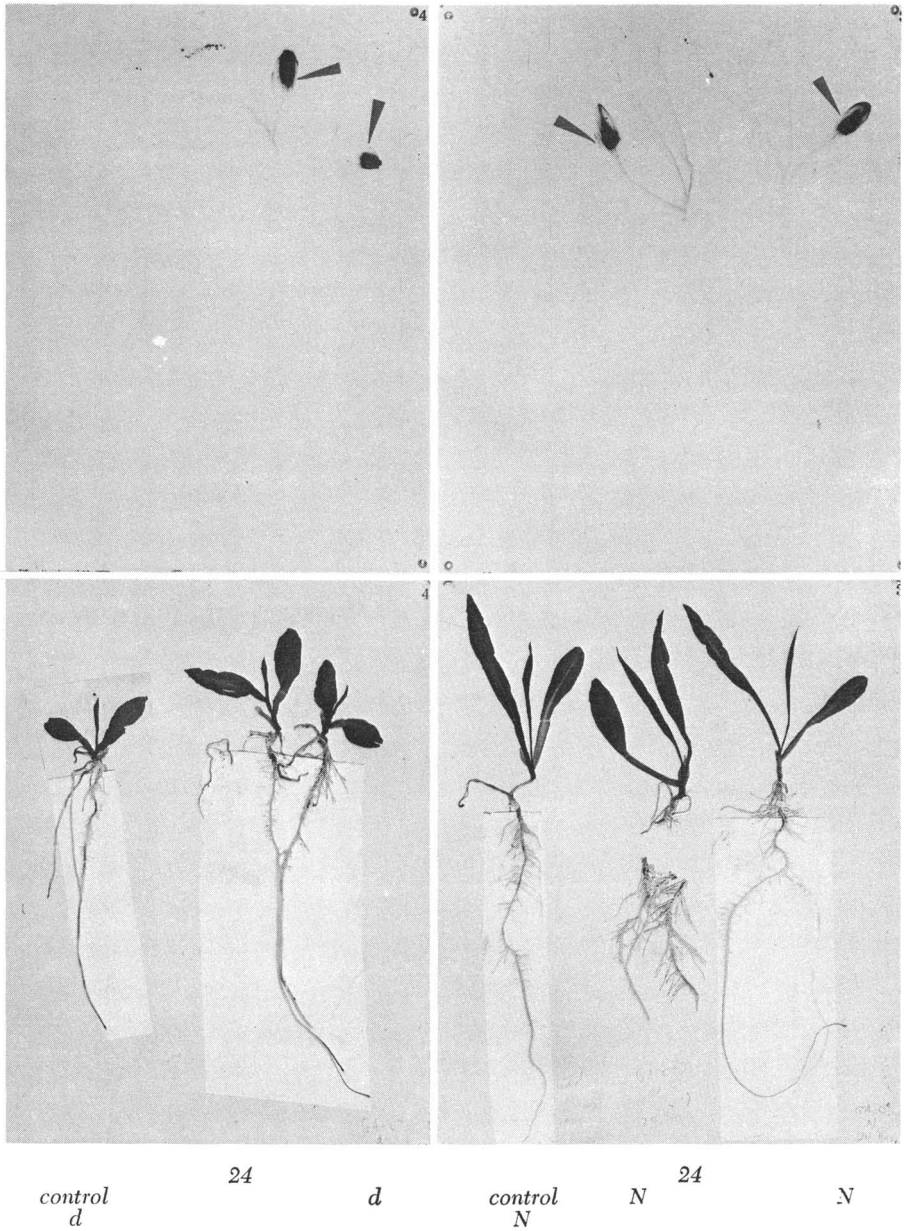

Figure 5. Foliar application of C^{14}-gibberellin on normal (N) and dwarf (d) corn

Numbers refer to treatment time in hours
Upper. Autoradiograms, 2 weeks' exposure, except 168 hours: 10 weeks' exposure
Lower. Lyophylized plants

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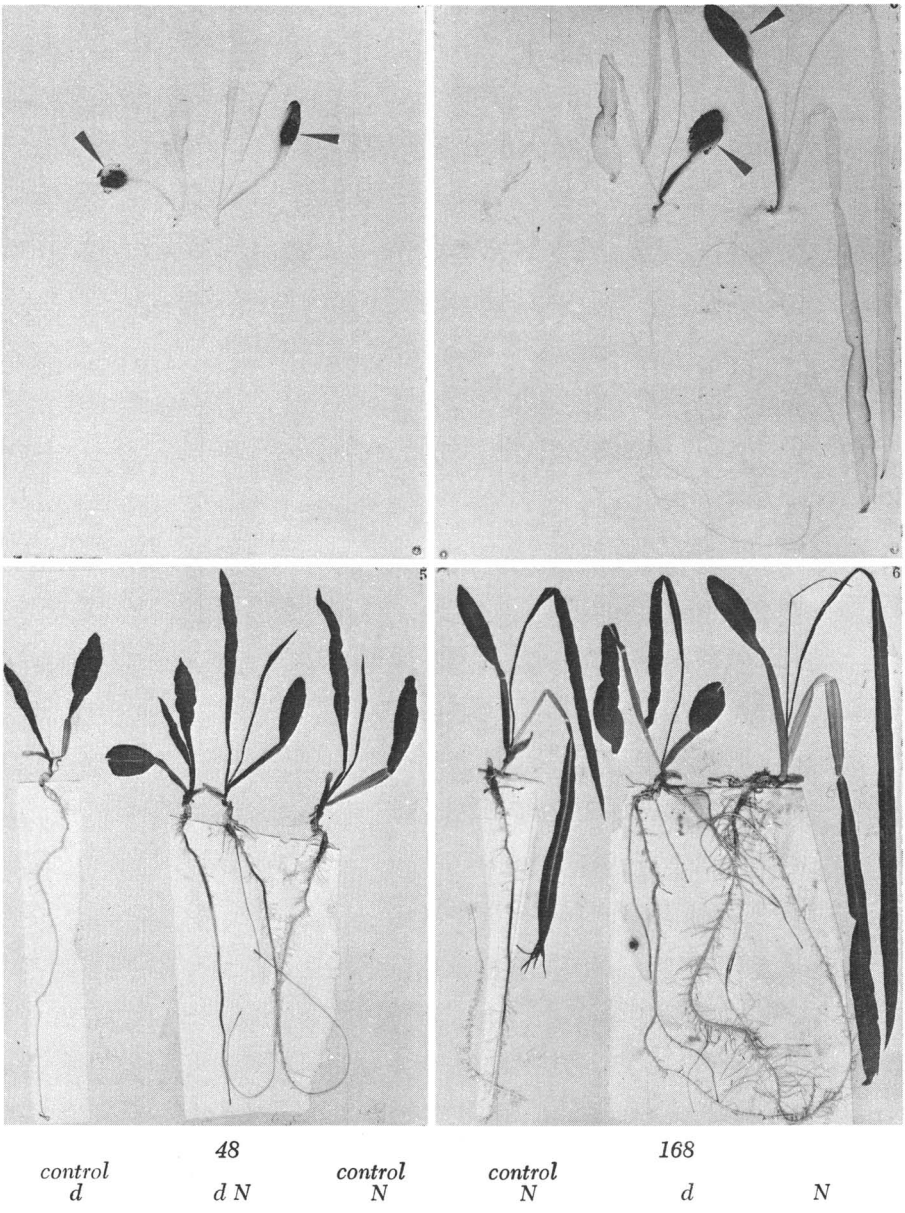

Figure 5 (Continued)

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RECEIVED October 13, 1960. Research partially supported by a grant-in-aid, Abbott Laboratories, North Chicago, Ill.

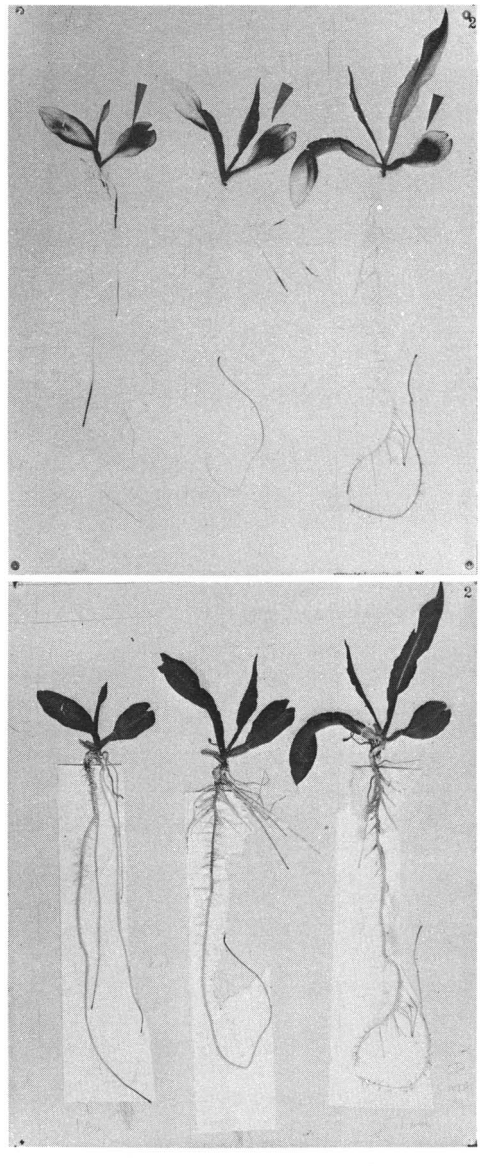

Figure 6. Foliar application of C^{14}-5-aminotriazole on dwarf corn

Numbers refer to hours after treatment, arrows point to site of application
Upper. Autoradiograms
Lower. Lyophylized plants

Enhancement of Gibberellin-Induced Phenomena

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Adjusting the solution containing gibberellin to pH 3.2 with citrate-phosphate buffer promoted much higher percentage germination of lettuce and *Lepidium* than did unbuffered water solution. When added to low concentrations of applied gibberellin, hydrangenol (4',8-dihydroxy-3,4-dihydroisocoumarin), isolated from the leaves of Engel's White hydrangeas,, enhanced the stem extension observed with mutant dwarf maize, peas, beans, chrysanthemums, and hydrangeas. Hydrangenol exhibited little or no growth-regulation activity when applied alone or in combination with high concentrations of gibberellin. Other chemicals affecting the growth-promoting activity of gibberellin are cinnamic acid, *p*-coumaric acid, and β-hydroxyethylhydrazine.

The rate and amount of growth of certain plants can be modified by applications of aqueous solutions of gibberellic acid. Often high concentrations of gibberellin are necessary for effectiveness, or the limited tissue area for uptake greatly reduces the usefulness of applied gibberellin. This report deals with factors influencing the uptake and activity of gibberellin applied to seeds and plants.

Effect of Surfactants on Gibberellin

Tween 20 (nonionic, polyoxylethylene sorbitan monolaurate) was reported as the only surfactant among several tested which increased the effectiveness of the gibberellin solution over that of a solution of gibberellin in water alone when applied to dwarf maize (*15*) and bean (*5*). No consistent response from adding Tween 20 to the gibberellin solution has been observed by the authors. Tween 20 aided the spraying and pipetting operations, but apparently was not associated with enhancement of chrysanthemum and hydrangea growth. This area of research needs further study.

Effect of Chemical Structure of Gibberellins and Derivatives on Plant Growth

The gibberellins were defined by Phinney and West (*16*) as substances possessing the same carbon skeleton as gibberellin A_3, gibberellic acid, or one very

Figure 1. Elongation of second leaf sheath of d-1 maize following application of various gibberellins (0.1 μg.) to base of first leaf sheath

Left to right. Untreated; gibberellic acid A_3; mixture of gibberellins A_1 and A_3; potassium gibberellate; zinc gibberellate; methyl ester of gibberellic acid; and allogibberic acid

closely related to it, and biologically active in stimulating cell division, cell elongation, or both in plants. Various derivatives of gibberellic acid have been made to determine if its activity could be enhanced.

Salt. When compared on a molar basis, the mineral ion salts (ammonium, calcium, rubidium, copper, silver, lead, manganese, cobalt, potassium, and sodium, and cyclohexylamine salt) were as effective as the free gibberellic acid in promoting stem elongation (*10*, *14*). As shown in Figure 1, the potassium and zinc salts of A_3 were as active as the acid in promoting the growth of d-1 dwarf maize.

Acyl. The acetyl, butyryl, benzoyl, and diacetyl derivatives of gibberellin were reported by Moffatt and Radley (*14*) to be as active as the acid when applied to the roots or leaves of pea.

Ester. Esterification of the hydroxyl group of gibberellin A_3 did not reduce biological activity, and none was more active than the acid (*14*). Straight aliphatic esters prepared by esterifying the carboxyl group were relatively inactive when assayed on seed (*10*) and growing plants (*4*). As an example, the methyl ester of gibberellic acid was inactive on cucumber seedlings (*10*), on d-1 maize (Figure 1), and on the induction of parthenocarpic fruit set of tomato (*4*), but was slightly active when applied to dormant lettuce seed (*17*) and to pea seedlings grown in water solutions containing the ester (*14*). The observed activity may have resulted from the presence of ethanol (*19*), or from the slow hydrolysis of the ester into the acid. The complete lack of activity shown by acyl esters may have been due to their low solubility.

Although the *n*-butyl ester had little effect on the growth of cucumber seedlings, butyl Cellosolve ester was almost as effective as the acid (*10*). Since the Cellosolve esters are relatively insoluble in water, hydrolysis may have occurred.

Acids. All the previous comparisons were made with gibberellin A_3. Other gibberellins have been isolated, six from the metabolic products of the fungus *Gibberella fujikuroi* (Saw) Wr. (A_1, A_2, A_3, A_4, A_7, and A_9) and four from the immature seed of *Phaseolus multiflorus* Lem. (A_1, A_5, A_6, and A_8) (*8*, *13*). The biological activity of the first four gibberellins has been characterized (*4*, *6*, *10*, *11*, *20*). On most plant processes, gibberellin A_3 was the most active, whether it was assayed on d-1 mutant maize (Figure 2), Black Valentine snap bean (*4*), lettuce, or hydrangea (Figure 2). The remaining gibberellins were less effective in the following order: $A_1 \geqslant A_4 > A_2$. Stem extension of cuttings of Fred Shoesmith chrysanthemums treated with the four gibberellins was similarly affected.

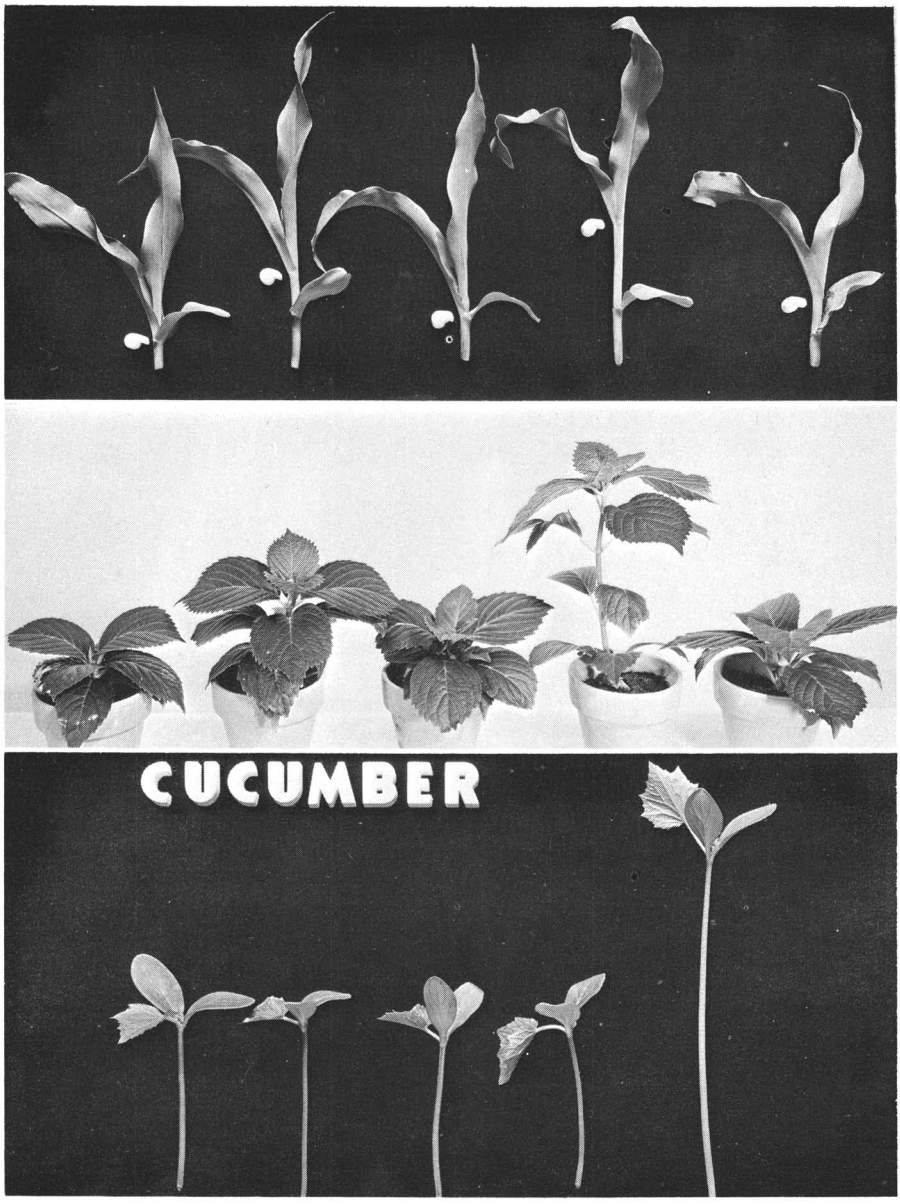

Figure 2. Elongation following application of gibberellins A_1, A_2, A_3, and A_4
1 μg. per plant
Top. To d-1 maize
Middle. 3 times 5 days apart to Engel's White hydrangeas
Bottom. One time to Marketer cucumber

Roots on cuttings treated with A_4 were developed as well as or better than those on untreated cuttings. The other gibberellins tended to retard root development. The only exception to the order as measured by stem extension was with cucurbits (*10–12*, *20*), where gibberellin A_4 was always the most active, followed by A_3, A_1, and A_2

Enhancement of Gibberellin Effects in Seed Germination

Gibberellin A_4 was 50 to 100 times more effective than the other gibberellins in promoting the germination of lettuce and tobacco seed (*11*) in darkness. Although gibberellin A_4 was the most active on the germination of lettuce seed, A_3 was used for extended tests because larger amounts were available.

Toole and Cathey (*19*) compared two seed kinds— Grand Rapids lettuce and *Lepidium virginicum* L.—in germination tests with gibberellin A_3. The germination of Grand Rapids lettuce increased gradually with increasing concentrations of gibberellin; the optimum concentration was $10^{-3}M$. In contrast, *Lepidium* required a higher and very restricted range of gibberellin concentration ($2.5 \times 10^{-3}M$) for promotion of germination. Lower concentrations had no apparent effect, whereas higher concentrations caused the seedlings literally to "pop out" of the seed coat with no development of the radicle.

Potassium gibberellate was inactive on *Lepidium* at all concentrations tested from 10^{-7} to $10^{-1}M$. Gibberellic acid always bleached the seed coat because of the acidity. The pH of the solution ($2.5 \times 10^{-3}M$) of the potassium gibberellate was 6.0, whereas that of the acid was 3.2. When the potassium salt was dissolved in a citrate ($0.005M$) and phosphate ($0.01M$) buffer at pH 3.5, it promoted seed germination as well as did the acid. Solutions buffered at a higher pH were ineffective in promoting germination. The buffer alone did not affect the germination of *Lepidium* seed held in darkness but greatly increased the sensitivity of the seed to light.

Solutions of varying concentrations of gibberellin weakly buffered at pH 3.2 were much more effective at a lower and over a broader range in promoting the germination of lettuce and *Lepidium* seeds than were those that had inbibed unbuffered gibberellin solutions (Figure 3).

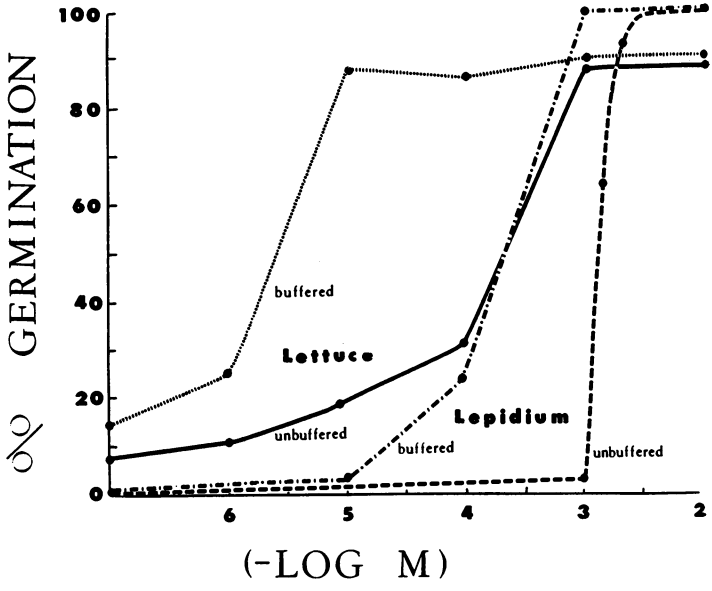

Figure 3. Germination of lettuce and Lepidium seeds held in darkness at 25° and 20° C., respectively, after moistening with various concentrations of gibberellin A_3, dissolved in unbuffered or dilute citrate phosphate buffer

The increased germination was not the direct effect of pH alone or of the one particular buffer. Phthalate-HCl, glycine-HCl, and sodium aconitate buffers at pH 3.2 had no effect in the dark but did increase the sensitivity of seed to light. Germination in darkness occurred only when the acid or a buffered solution of gibberellin was used to moisten the substrate.

These results suggested that the undissociated, rather than the total, gibberellin concentration determined the germination of *Lepidium*. The pK_a of gibberellin A_3 was 3.8 as reported by Cross (*7*). The pH's of the buffered or active solutions were below that value. Gibberellin did not degrade to allogibberic acid in the acid solution, since this substance was inactive in tests with seed reported by Halevy and Cathey (*10*) or with growing plants reported by Wittwer and Bukovac (*20*) (Figure 1).

Enhancement of Gibberellin Effects in Stem Elongation

Stowe (*18*) reported that certain fatty acid esters exerted a synergistic effect on gibberellin and indoleacetic acid rather than being growth regulators in themselves. The experiments were conducted with stem sections of pea. No growth factor or combination of known factors restored more than half of the growth produced on intact plants. In the present studies, various substances promoted the activity of applied gibberellin (*1*). The first chemical studied, hydrangenol (4',8-dihydroxy-3,4-dihydroisocoumarin), was isolated as the aglycone and the glucoside from the leaves of Engel's White hydrangeas. A treating solution was prepared by dissolving the aglycone of hydrangenol in hot water and adjusting the pH to 7 with sodium hydroxide. Hydrangenol applied alone had little or no effect on the elongation of the second leaf sheath of d-1 maize.

When 10 μl. of hydrangenol and 10 μl. of gibberellin, in appropriate concentrations, were placed separately at the base of the first sheath of d-1 maize, elongation of the second leaf sheath increased with increased concentrations of

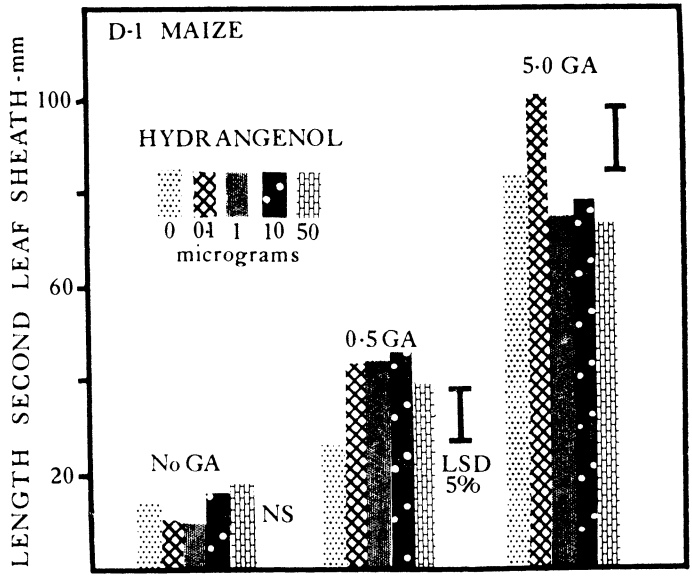

Figure 4. Mean length of fully expanded second leaf sheath of d-1 mutant maize treated with gibberellin and hydrangenol

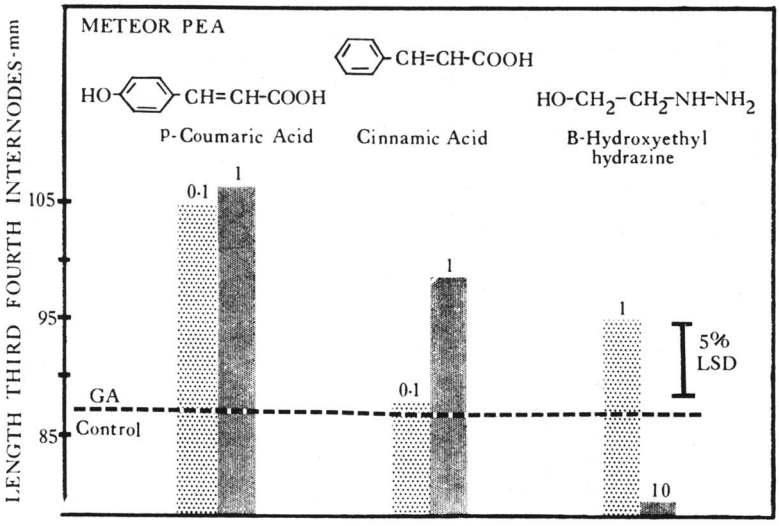

Figure 5. Mean length of fully expanded third and fourth internodes of Meteor pea treated with 0.1 μg. of gibberellin and 0.1 and 1 μg. of p-coumaric acid and cinnamic acid and 1 and 10 μg. of β-hydroxyethylhydrazine

gibberellin applied to the plants (Figure 4). The response to 0.5 μg. of gibberellin was increased significantly by addition of hydrangenol. Elongation of the leaf sheath treated with 5 μg. of gibberellin was increased by the application of 0.1 μg. of hydrangenol, but higher concentrations of hydrangenol tended to reduce the growth promotion of gibberellin. The range in concentration of gibberellin in which hydrangenol was active was very narrow.

In the previous test the solutions were applied to the same area of the first leaf sheath. To determine if the activity depended upon application of the chemicals to the same area, hydrangenol and gibberellin were applied from a mutual solution to the same area and from separate solutions to separate areas of the first leaf sheath. The total concentration of the surfactant applied to the plants was always constant. Hydrangenol enhanced the growth-promoting activity of gibberellin to the same degree regardless of method of application.

Similar results have been observed with the stem elongation of Black Valentine bean, Meteor pea, hydrangea, and chrysanthemum. Hydrangenol did not increase the observed effectiveness of gibberellin in replacing the cold requirement of hydrangeas nor in hastening the flowering of chrysanthemums.

Hydrangenol is not the only chemical that acts in this manner. *p*-Coumaric acid, cinnamic acid, and β-hydroxyethylhydrazine (Figure 5) increased the activity of gibberellin applied to Meteor pea.

Solutions of gibberellins A_1, A_2, A_3, and A_4 were applied to plants of Meteor pea; growth response was maximum with A_3, followed in order by A_1, A_4, and A_2. The addition of 0.1 μg. of hydrangenol per plant significantly increased the growth promotion of gibberellins A_1 and A_3. The activities of gibberellins A_2 and A_4 were unaffected by application of hydrangenol. These results indicate that hydrangenol promoted primarily the activity of applied gibberellin but did not affect the endogenous gibberellin.

Discussion

The stem extension results suggest that hydrangenol is similar in action to the third factor postulated by Brian (2) and Brian and Hemming (3) and further expanded by Galston and Warburg (9) but interpreted by them as a sparing action of auxin. This possibility should be considered. If this mechanism be accepted, hydrangenol should enhance the activity of gibberellin naturally occurring in the test plants. Also, larger amounts of hydrangenol should enhance the activity of higher levels of applied gibberellin unless auxin became limiting. Neither action was observed. When additional auxin was supplied, the response to a combination of hydrangenol and gibberellin was decreased rather than increased.

Since hydrangenol affected gibberellin when applied to separate leaf areas, the response must be associated with metabolism, rather than with penetration. Our experiments have been inconclusive in regard to the combination of buffered solutions of gibberellin and hydrangenol. Buffered solutions apparently affect the uptake, whereas hydrangenol affects the action of gibberellin within the plants. Chemical modifications of the acid usually rendered the compounds less active than the original substance. Our work is continuing; it is not possible at present to suggest any simple explanation of the mechanism by which hydrangenol and several other compounds enhance the growth-promoting activity of applied gibberellin.

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RECEIVED December 19, 1960.

Enhancement of Gibberellin and Auxin Action by Alkyl Lipides

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Pea stem sections are much less responsive to gibberellin than are intact plants. The section response is greatly enhanced by low concentrations (1 to 50 mg. per liter) of a large number of lipides. The specificity of this response has been surveyed and the common characteristic of the active lipides has been shown to be possession of a long hydrocarbon chain of the fatty acid ester or isoprenoid type. The lipides do not increase pea section growth by themselves, but enhance the growth stimulation of auxin and gibberellin. The role of this effect in natural plant growth regulation is under investigation. Preliminary evidence indicates that there are similarities between this lipide stimulation of plant hormone action and the activation by lipides of components of the cytochrome system.

A puzzling lack of effect of the gibberellins is their failure to increase the growth of plant stems much, when these are separated from the rest of the plant. A stem left on the intact plant will respond greatly to a gibberellin application which is nearly without effect on a similar stem that has been excised in the manner usually employed for growth hormone bioassays. In the belief that study of this difference might provide a clue to the nature of gibberellin's action, we initiated studies 3 years ago of etiolated pea seedlings and of 10-mm. stem sections cut from their third internodes at 7 days of age.

These studies indicated that a 10-mm. zone marked on the intact plant elongated 132% in 24 hours (2). When the same zone was cut out and floated in a Petri dish with buffered solutions containing several growth-promoting substances—gibberellic acid, indoleacetic acid, cobalt ion, and sucrose—even at optimum concentrations, the zone did not attain a length of more than 84% over the original 10 mm. Even more strikingly, the zone on the intact plant when treated with gibberellic acid elongated 201%. Hence plant stem sections grown under the long-employed standard bioassay conditions give less than half of the

response of the intact plant to treatment with gibberellic acid. These figures were obtained with dwarf pea sections, which we had anticipated would be more sensitive to gibberellin; in fact, the gibberellin response of standard Alaska pea sections was comparable.

Effect of Plant Extracts and Detergents

In the belief that the failure of the stem section to grow meant that some essential cofactor must normally be supplied to the stem section by the rest of the plant, we began investigating the effect of plant extracts. A mixture of glycerides isolated from the pea nearly doubled the growth response of the section to auxin and gibberellin, even when applied at the extremely low concentration of 10 mg. per liter (3). Even so, the growth attained by the section is still less than that of the intact plant, and a cofactor other than those discussed in this paper may be involved.

Much to our surprise, this effect of the isolated fatty materials is not a specific one. We accidentally discovered that some of the Tween detergents are also active (3). Other detergents showed this property only when they contained a long hydrocarbon chain and an ester group. Pure methyl esters of known fatty acids also enhanced the growth promotion of auxin and gibberellin on pea stem sections. Clearly then, the growth enhancement is not limited to a single substance or even a small group of substances.

Specificity of Lipides

In an effort to delineate the limits of specificity of these lipide substances, a large number of long-chain alkyl compounds have been surveyed (2). No compound of chain length less than C_{12} has shown a growth-promoting effect, but C_{12} and all longer straight-chain fatty acid methyl esters, saturated and unsaturated, are effective, as well as all the triglycerides we had available. No free fatty acid, however, had any influence on the sections except toxicity at much higher concentrations. Some monoglycerides and alcohols had moderate promotive action; others were ineffective.

Further studies have extended this promotion of auxin and gibberellin action to other fat-soluble substances, in particular some isoprenoid vitamins and related compounds (1). For instance, vitamin E, vitamin K_1, and phytol are quite comparable in their growth-promoting ability to the fatty acid ester, methyl linoleate. But some fat-soluble vitamins are not active—for instance, vitamin A, β-carotene, and vitamin D_2.

This striking effect of a large number of alkyl esters and alcohols poses the difficult question of trying to explain what they are doing. No well understood metabolic pathway seems to be involved, and permeability or surface action effects seem to be excluded (2).

The most promising leads have come from studying the hormonal requirements of the system. By themselves, we can find no indication whatsoever of any growth promotion by any of the alkyl lipides. When gibberellic acid is also applied, response remains small. If indoleacetic acid, or another auxin, is added, the sections show the classic bioassay response—and this is further stimulated by the active lipides. In the presence of both auxin and gibberellic acid, however, the sections show their greatest capacity to elongate still further in the presence of the alkyl lipides (1). Remarkably, the lipides are most effective at concentrations com-

parable to those of these hormones—as low as 3 μM in some cases, but 10 to 30 μM is the usual optimum range.

In the pea stem section then, it is evident that both an auxin and a lipide are required for maximal effectiveness of gibberellic acid in promoting cell elongation, and that all are effective at hormonal levels. However, unless an energy source such as sucrose is present, very little effect of the lipide will be noted. This introduces the possibility that the lipides synergize with the hormones by directing metabolic energy to the growth mechanism. Support for this idea is provided by the observation, detailed elsewhere (*1*), that the specificity of this stem section system is closely paralleled by cytochrome c reductase reactivation in iso-octane–extracted animal mitochondria. The cytochrome c reductase in these particles is restored also by fatty acid esters, vitamin E, vitamin K_1, phytol, natural lipide extracts, triglycerides, and certain other compounds, but not by free fatty acids, carotene, or vitamin A. As far as we are aware, no other biological system has such close correspondence to our pea stem sections. In further support of this notion is the fact that the respiration of the pea stem sections is increased in the presence of the active lipides. We believe, therefore, that cytochrome activation best expresses the results obtained. A close linkage of plant hormone action with the cytochrome system was shown long ago by several investigators.

Our present efforts are being devoted to studying pea lipides *in situ*, in the hope of determining whether hormone activation by lipide substances is a natural method of growth regulation. Gas chromatography has already confirmed that our original active pea extract is a mixture of conventional fatty acid esters.

Peas are a low-fat plant, and it is likely, therefore, that our results should not be freely extended to all other auxin- and gibberellin-induced systems. In fact, the equally classic oat coleoptile section hormone assay does not seem to respond to the fatty substances. Even the pea section fails to respond, unless the seedling has received some red light during the early part of its development.

It has long been known that detergents increase the effectiveness of auxin herbicides. This may now be interpreted as perhaps not due entirely to the wetting properties of the detergents; and it would be of interest to compare fatty acid ester detergents with those not containing fatty acid groups in herbicide trials.

Vlitos and Crosby's results with fatty acid alcohols in certain respects resemble ours (*4*). Thus it seems likely that alkyl lipides play some fundamental role in hormone action and that their importance to gibberellin activity will gradually be extended.

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RECEIVED November 10, 1960. Investigation supported by a Public Health Service research grant RG-6921 from the National Institutes of Health, U. S. Public Health Service.

Structural Relationships among Chemicals Which Act Like Antigibberellins

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Certain organic compounds, varying widely in structure, cause plants to grow with short and thick stems or of appearance opposite to that obtained with gibberellin. Gibberellin can reverse the action of most of these compounds. The tall growth of plants obtained with gibberellin is likewise prevented by simultaneous application of these chemicals with gibberellin. The biological growth response may be described by the concept of substances with antigibberellin action. However, there is yet no proof that these compounds are gibberellin antagonists in a biochemical or physiological sense and the compounds are not structurally related to gibberellin. The most active are derivatives of (2-chloroethyl) trimethylammonium chloride. This structure is varied without losing biological activity by substituting other small, nucleophilic and nonionizable groups for the beta-chloro substituent of the ethyl group. Maleic hydrazide, AMO 1618, and phosfon D also promote growth of short plants. There is no apparent chemical relationship between any two groups of these compounds which are correlated with biological activity.

Gibberellin causes plants to grow tall and elongated, with light green leaves. It stimulates seed germination and other growth phenomena. Compounds which alter plant growth toward shorter height and darker green leaves and in general inhibit growth phenomena, such as seed germination, are often thought of as inhibitors. Such compounds are actually growth regulators and this concept is particularly true if the chemicals, when properly applied, are effective without decreasing the weight of the plant. Application together of gibberellin (GA) and chemicals which promote shorter growth may result in plants which appear

more normal in height than those obtained after treatment by either chemical applied singly. Thus the concept exists that chemicals which cause plants to grow short are antigibberellins in a biological sense.

In plant research we are interested in the discovery of compounds which cause plants to grow as a bush or rosette form, and in biochemical studies we are concerned as to how such compounds might function and alter growth. Such research on antigibberellin-like compounds would aid in understanding the mode of action of gibberellin. Then, too, there are numerous commercial applications for chemicals which would hold back the growth in height of plants and induce shorter plants, more resistant to damage from nature and machinery.

From the literature a list of widely varying chemical structures can be selected which cause plants to grow with shorter and sometimes thicker stems or the opposite type of growth in appearance from that obtained with gibberellin. Gibberellin in low concentration can reverse in part the action of most of these compounds. Four compounds have been selected as representatives of this class of plant growth substances. These particular compounds are not extremely inhibitory to growth at the low concentrations where growth alteration can be observed. Also, plant growth obtained from simultaneous application of each of these four substances with gibberellin has been studied.

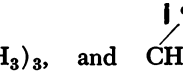
(2-Chloroethyl)trimethylammonium Chloride and Related Structures

Application of these chemicals to plants results in shorter and stockier growth without change in total weight (*15*, *17*, *20*, *21*). This compound, $CH_2Cl–CH_2–N^+(CH_3)_3 \cdot Cl^-$, is an analog of choline, in that the hydroxy group in choline has been replaced with a chlorine substituent. Consequently, its trivial name is chlorocholine chloride, abbreviated CCC. About 90 compounds of this class have been tested upon plant growth and as a group of plant growth chemicals they are being referred to as derivatives of (2-chloroethyl) trimethylammonium chloride.

Growth alteration after treatment with the (2-chloroethyl)trimethylammonium chloride derivatives has been described for wheat (*17*), tomato (*20*), chrysanthemum (*9*), poinsettia (*9*), soybeans (*21*), dwarf corn (*21*), lettuce (*21*), and peas (*21*). This class of compound has been considered as a new growth substance rather than an antigibberellin. In general, the growth produced by CCC appeared the opposite from that induced by gibberellin and a mutually antagonistic effect was obtained from simultaneous application of this compound and gibberellin (*17*, *20*, *21*). Gibberellin produced tall plants with thin stems and light green leaves. The most characteristic growth alterations produced by CCC were shorter plants with thick stems and dark green leaves. Gibberellin accelerated seed germination and stimulated in many plant species the effects of long photoperiod and exposure to high temperatures. Conversely the (2-chloroethyl)trimethylammonium chloride derivatives inhibited seed germination and tended to duplicate the effect of short photoperiod and exposure to low temperatures (*21*). In the growth of tomato ovaries the synergistic effect from application of (2-chloroethyl)trimethylammonium chloride, IAA, and gibberellin was greater than that from the application of any two of these compounds together (*21*).

The various derivatives of (2-chloroethyl)trimethylammonium chloride have been considered as analogs of choline in which the alcohol group has been designated as X. The general formula of this cation would be $CH_2X–CH_2–N^+(CH_3)_3$.

The enzyme choline esterase has been shown to have two binding points on its protein surface for these substances—one site for the quaternary ammonium group and one for X. This enzyme catalyzes the hydrolysis of an ester at the X position. From a consideration of the structure of the (2-chloroethyl)trimethylammonium chloride derivatives which were active as plant growth substances, a similar protein-binding site in the plant has been postulated. This site would have a point of attachment for both the ammonium cation and the X constituent of the molecule. This postulated site in the plant is thus similar, but not identical, to cholinesterase, which is an enzyme not known to occur in plants. There is no direct proof for this hypothetical site in the plant.

Of the derivatives of (2-chloroethyl)trimethylammonium chloride which have been synthesized and tested as plant growth substances for the reduction of the height of plants, numerous substituents at the X site produced derivatives with biological effect (*15*). The most active derivatives were the chloride or bromide salts of $CH_2Cl-CH_2-N^+(CH_3)_3$, $CH_2Br-CH_2-N^+(CH_3)_3$, $CH_2=CH-CH_2-N^+(CH_3)_3$, $CH_2=CCl-CH_2-N^+(CH_3)_3$, and  $N^+(CH_3)_3$. Other active derivatives were $CH_3-O-CH_2-CH_2-N^+(CH_3)_3$, $CH_3-S-CH_2-CH_2-N^+(CH_3)_3$, $CN-CH_2-N^+-CH_3)_3$, $CH\equiv C-CH_2-N^+(CH_2)_3$, $CH_3-CH_2-CH_2-N^+(CH_3)_3$, and $SH-CH_2-CH_2-N^+(CH_3)_3$. The trimethyl quaternary ammonium cation was shown to be necessary for activity because any substitution for even one methyl group produced nearly inactive compounds. For optimal activity, the carbon chain which contained the substituent, X, at the end should be two carbons in length. In the active compounds, substituents at X were of small size, nucleophilic and nonionizable.

The sensitivity of many plants to all the active derivatives of (2-chloroethyl)-trimethylammonium chloride has not been systematically surveyed. Among the plants so far tested which were affected by 10^{-3} to 10^{-6} *M* concentrations of these chemicals when applied as soil drenches are some grains, all tomatoes, and many cucurbits. Another group of plants which were markedly reduced in height in greenhouse tests by 10^{-2} to 10^{-3} *M* solutions included some chrysanthemums, poinsettias, azaleas, beans, peas, some grasses, and woody plants such as apple and peach trees. Other plants were affected by these compounds only when 10^{-2} *M* or higher concentrations or repeated applications were used.

Many compounds have been tested simultaneously with (2-chloroethyl)trimethylammonium chloride on Thatcher wheat, to ascertain, if possible, whether these chemicals were affecting a particular metabolic process. Other cholinesterase inhibitors such as eserine, diisopropyl fluorophosphate, and nitrogen mustard, neither negated the effect from (2-chloroethyl)trimethylammonium chloride nor altered the growth of the plant themselves. Many other substances were also without effect on the action of (2-chloroethyl)trimethylammonium chloride. A very slight reversal of the alteration by (2-chloroethyl)trimethylammonium chloride was obtained by 10^{-2} and 10^{-3} *M* choline, betaine, and adenine. Only gibberellin completely and rapidly reversed the shorter growth pattern of a plant which had been treated with (2-chloroethyl)trimethylammonium chloride.

The action of gibberellin and (2-chloroethyl)trimethylammonium chloride in altering the growth of Thatcher wheat was mutually antagonistic (*15*). The same seems to be true for the effect of (2-chloroethyl)trimethylammonium chloride and gibberellin when applied together on tomato (*20*), chrysanthemum (*9*), dwarf corn (*21*), and germinating lettuce seed (*21*). The elongation from excess gib-

berellin always dominated over the opposite effect from excess (2-chloroethyl)-trimethylammonium chloride. By judicious selection of the right molarities of each type of compound, a final plant growth could be obtained which in appearance was more normal than that from either chemical by itself. Also the two growth substances can be applied at different times for different effects. Chrysanthemums, which generally grow too tall in floricultural practice, have been grown until flower bud initiation as a bushy plant due to treatment with (2-chloroethyl)-trimethylammonium chloride. During flowering the plants were treated with gibberellin to promote big flowers with long stems. Since growth of the rest of the plant was completed by that time, the base of the plant remained short and strong.

Additional data in Table I are from measurements of the length of Thatcher wheat plants after treatment with gibberellin A_1 (GA_1) and gibberellin A_3 (GA_3) in combination with CCC. GA_3 by itself was effective on this wheat at 10 p.p.m., whereas GA_1 at 30 p.p.m. was needed for an elongation of growth. The 50% stimulation in height by GA_3 at 30 p.p.m. was more than reversed by 5×10^{-4} *M* CCC or 79 p.p.m. Another combination showed that as little as 1 p.p.m. of GA_3 could reverse part of the short growth pattern from the action of CCC. These results were obtained on measurement of height; there was no change in wet or dry weight in any of the treatments.

Table I. Height of Thatcher Wheat Seedings after Treatment with Both CCC and Gibberellin[a]

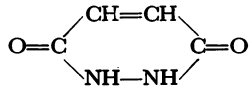
Molarity of CCC	Gibberellin A_1, P.P.M. 0 %	1 %	3 %	10 %	30 %
0	100	..	98	94	121
5×10^{-4}	35	34	33	49	51
5×10^{-3}	23	24	25	28	34
	Gibberellin A_3, P.P.M.				
0	100	..	101	121	150
5×10^{-4}	35	46	49	56	76
5×10^{-3}	23	30	35	64	127

[a] Plants were grown and treated with CCC as in bioassay procedure (*15*). Two days after CCC treatment, plants were sprayed until wet with gibberellin solutions. Two weeks after CCC treatment lengths between bases of first and second leaf blades were measured and expressed as per cent of length for untreated controls. Results are average of several experiments.

Maleic Hydrazide (MH)

Of these compounds which act as if they were antigibberellins, so far only MH (1,2-dihydropyridazine-3,6-dione) has been extensively used industrially as a growth inhibitor. MH suppresses apical dominance and bolting in vegetables (*11, 14*), and it has been considered as an antiauxin. The ultimate effects of MH and gibberellin on plant growth and development are opposite. Thus MH is a gibberellin antagonist in that it will block the growth-promoting action of gibberellin, whereas its own inhibition of growth is negated by gibberellin (*1, 2*). Application of MH to growing plants generally results in dwarf plants with short internodes and dark green leaves. MH breaks apical dominance, causing plants which normally grow by a single main axis to assume a bushy habit. In considering the mechanism of action, however, MH should probably not be referred to as an antigibberellin. MH primarily inhibits cell division, while gibberellin affects cell expansion as well (*4, 13*). Thus only in final growth appearance is MH a gibberellin antagonist.

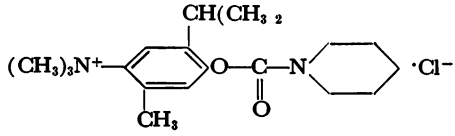
In some respects, the actions of (2-chloroethyl)trimethylammonium chloride derivatives and MH in altering the growth of plants appear similar. There is no apparent similarity in the chemical structure of maleic hydrazide,



with that of CCC which would suggest why both may be gibberellin antagonists. In actuality the two compounds probably affect plants by different mechanisms. At high concentrations MH is very toxic to plants; CCC even at 10^{-2} *M* is not toxic, and growth inhibition, as measured by total weight, may not be severe even though the treated plants may have extremely short internodes. CCC derivatives do not affect apical dominance to the degree exerted by MH, but rather CCC affects primarily the internodal distance. Very low concentrations of ferric sulfate and boric acid will greatly reduce maleic hydrazide inhibition of *Avena* coleoptile sections (*4*), but neither ferric sulfate nor boric acid affects the action of CCC on wheat. In MH-treated plants there is an altered metabolism as manifested by an increase in free sugars. Neither gibberellin nor CCC significantly altered sugar metabolism as studied by $C^{14}O_2$ fixation and measurement of the formation of labeled sugars (*5*, *16*).

AMO 1618

A comparison of the structure of AMO 1618, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-pipiridinecarboxylate methyl chloride, suggests no relationship to



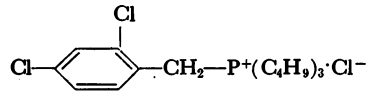
gibberellin. Yet experimentally this compound and certain closely related structures cause plants to grow short and sturdy with intensely green foliar coloration (*7*, *10*, *19*). Compounds of this class are also antagonistic to the growth-accelerating effect of gibberellin (*3*, *9*). In these respects AMO 1618 and CCC have similar plant growth-altering properties. Both compounds possess a quaternary nitrogen as a trimethylammonium chloride, but the rest of the molecule is completely different. Significant alteration of the aromatic ring of AMO 1618 produced inactive derivatives, while increase in the size of the 2-chloroethyl side chain of CCC decreased activity. In fact, benzyltrimethylammonium chloride was inactive in the assays which were used on CCC. It seems that the active structures of AMO 1618 and CCC are structurally unrelated and they would therefore affect plant growth at different sites.

Both AMO 1618 and CCC derivatives are effective on chrysanthemums and cucurbits. For chrysanthemums AMO 1618 is slightly more active on a molar basis than CCC; for cucurbits AMO 1618 is less active and more toxic. On tomatoes, wheat, and other plants so far tested AMO 1618 is inactive or toxic, whereas CCC is active. CCC has the distinct advantages of being more active on a wider variety of plants, much less toxic to the plants, and easier to synthesize.

Phosfon D

Few published data are available concerning phosfon D and its effects on plants (*12, 18, 21*), but it is included in this report because its effect on the growth of chrysanthemums is similar to that of AMO 1618 and CCC (*8*). Phosfon D is effective on chrysanthemums at 10^{-4} and 10^{-3} *M* by causing a decrease in height of the plant up to 75%. The effect is reversed by gibberellin (*8*). Snap beans, cucurbits, and tomatoes, which are dwarfed or shortened by the proper level of phosfon D treatment, are often injured when only slightly higher concentrations of the compound are applied (*12, 21*).

Phosfon D, tributyl-2,4-dichlorobenzylphosphonium chloride,



and related active derivatives are phosphonium cations in comparison to the ammonium cations of AMO 1618 and CCC. Both CCC and phosfon D contain a nucleophilic group, Cl, about the same distance from this cationic site. Yet the available data suggest that phosfon D and CCC are not affecting the plant at the same site. The corresponding tributyl or triethyl derivatives of CCC—(2-chloroethyl)tributylammonium chloride or (2-chloroethyl)triethylammonia chloride—are completely inactive. Phosfon D is inactive on wheat and many other plants which are affected by CCC. Phosfon D has no influence upon the effectiveness of CCC on wheat, which suggests that the two compounds are not capable of affecting the same site.

Concept of Antigibberellins

A consideration of the data on treatment of plants with the four groups of chemicals discussed above and in combinations with gibberellin suggests that these compounds might be antigibberellins. Investigators in this field have been justifiably hesitant to use the term antigibberellin without more exacting evidence. In all the investigations to date the only measurement has been one of effect on final growth in height and weight. The results could be caused by the additive effect of two opposite types of growth alteration.

The remarkable oppositeness in growth which is obtained by application of such substances as (2-chloroethyl)trimethylammonium chloride as compared to gibberellin does not prove that these compounds have an opposite effect on the same reaction in the complexity of growth or even upon the same stage of growth. To a biochemist the term antigibberellin suggests compounds which compete with gibberellin for one or more specific reaction centers in the growing plant. Antigibberellins should therefore be chemical analogs of gibberellin possessing the ability to block the gibberellin activity. No such structures and corresponding biological activity have so far been reported. In fact, the structures of (2-chloroethyl)trimethylammonium chloride, maleic hydrazide, AMO 1618, and phosfon D are so different from gibberellin that it is difficult to see how they would affect a system which gibberellin could also attack. Thus none of these chemicals is an antigibberellin in a chemical sense and none has been proved to be an antigibberellin in a biochemical or physiological sense. They are antigibberellins only with respect to the observed plant growth obtained from their in vivo use. In

actuality they may be altering growth through biochemical processes entirely different from those influenced by gibberellins.

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RECEIVED November 29, 1960. Article 2719, Michigan Agricultural Experiment Station.

Natural Inhibitors of Gibberellin-Induced Growth

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Extracts from the fruit or seed of several plant species will suppress the growth induced by gibberellic acid. The inhibitory extract from immature fruit of *Ceratonia siliqua* L. has been partially purified and four inhibitory substances are now known to be present. One is an inorganic component which is inhibitory after ashing. Three organic inhibitors—A, B, and C—are responsible for most of the inhibitory activity of the extract. B and C are adsorbed by activated charcoal and can be separated in this way from organic inhibitor A, which is not adsorbed. C can be extracted with ethyl ether from aqueous solution at pH 5, while B is not removed under these conditions.

Gibberellin-like substances have been found in many plant species (*5*). However, a number of plants give no evidence of the presence of such substances. One possible explanation for some of these apparently negative results is the simultaneous occurrence of inhibitors which mask the presence of gibberellin-like substances. To test this idea extracts from the fruit or seed of eight plant species which gave no evidence of the presence of gibberellin-like substances were tested for their ability to inhibit the growth induced by gibberellic acid on a dwarf mutant of *Zea mays*. Five of the extracts proved inhibitory, among them an extract from immature seed of *Ceratonia siliqua* L., the carob tree. This extract inhibited the growth stimulation caused by gibberellins A_1, A_2, A_3, and A_5 and the gibberellin-like substances in *Aesculus* and *Lupinus* extracts. The inhibition was demonstrated on Black Valentine beans, Little Marvel peas, and the dwarf-1, dwarf-2, dwarf-3, and dwarf-5 mutants of maize. The above findings have been reported (*1*, *2*).

Because of the apparently large amount of inhibitory activity exhibited by the extract and the availability of material for extraction, *Ceratonia* was selected for further study. Evidence has been obtained of at least four inhibitory substances during the purification of this extract.

Bioassay

Seedlings of two species of plants were used for bioassay to detect inhibition: the dwarf-1 mutant of maize, *Zea mays* L., and the Little Marvel cultivar of peas,

Pisum sativum L., which has a dwarf habit of growth. One set of plants received no treatment, one set was treated with a mixture of gibberellic acid solution and an equal volume of water, and other sets were treated with a mixture of gibberellic acid solution and an equal volume of solution to be tested for inhibitor. Each set consisted of ten plants on which the treatments were replicated. The gibberellic acid solution contained 0.05% Tween 20 (sorbitan, polyoxyethylene monolaurate).

A variation of the maize gibberellin bioassay developed by Neely (3) and Neely and Phinney (4) was used. Seedlings were treated 6 to 7 days after the seed was planted, when the first leaf was just unfolding to form a cup-shaped enclosure. The test solution was dropped onto the surface of the unfolding first leaf from a 0.5-ml. hypodermic syringe. Each maize seedling treated with gibberellic acid received 0.5 μg. of the acid in a total solution of 0.1 ml. Five to 7 days after the time of treatment measurements were taken of the first leaf sheaths from the coleoptilar node to the point of insertion of the ligule.

The bioassay with dwarf peas is similar. Six to 7 days after the seed was planted the seedlings were about 4 cm. high. The first leaf infolded the growing point at the apex of the plant. The test solution was introduced onto the apical region with a 0.1-ml. pipet. Each treated plant received 0.05 μg. of gibberellic acid in a total volume of 0.01 ml. Five to 7 days after the time of treatment measurements were obtained for the shoot length from the point of seed attachment to the epicotyl.

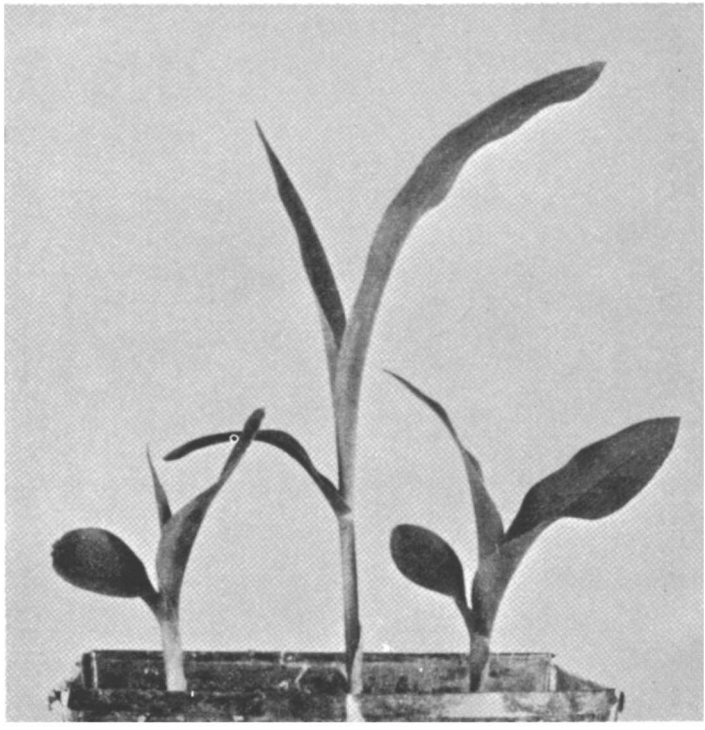

Figure 1. Response of dwarf-1 maize to gibberellic acid and a mixture of gibberellic acid and Ceratonia extract

Left to right. Nontreated control, treated with 0.5 μg. of gibberellic acid, and treated with mixture of 0.5 μg. of gibberellic acid and extract from 0.1 gram of Ceratonia *fruit*

Measurements were taken to the nearest millimeter for both maize and pea seedlings. An average and a standard error were determined for each set of plants. Net responses were obtained by subtracting the average of the nontreated control plants from the average of the treated plants. The average per cent reduction of the gibberellin-induced growth was calculated from the average net response of the plants receiving a mixture of gibberellic acid and solution to be tested for inhibitory activity and the average net response of the plants receiving gibberellic acid alone.

In no case was any injury to the assay plant evident after treatment with *Ceratonia* extract. Even at high concentrations there was no apparent leaf or stem damage, such as is frequently observed after application of toxic substances.

Extraction

The original inhibitory extract from *Ceratonia* was obtained from immature seed. It was later found that extracts from the green pod alone were also inhibitory, and all the studies reported here were carried out with extracts from whole fruit which had reached full length but were still green.

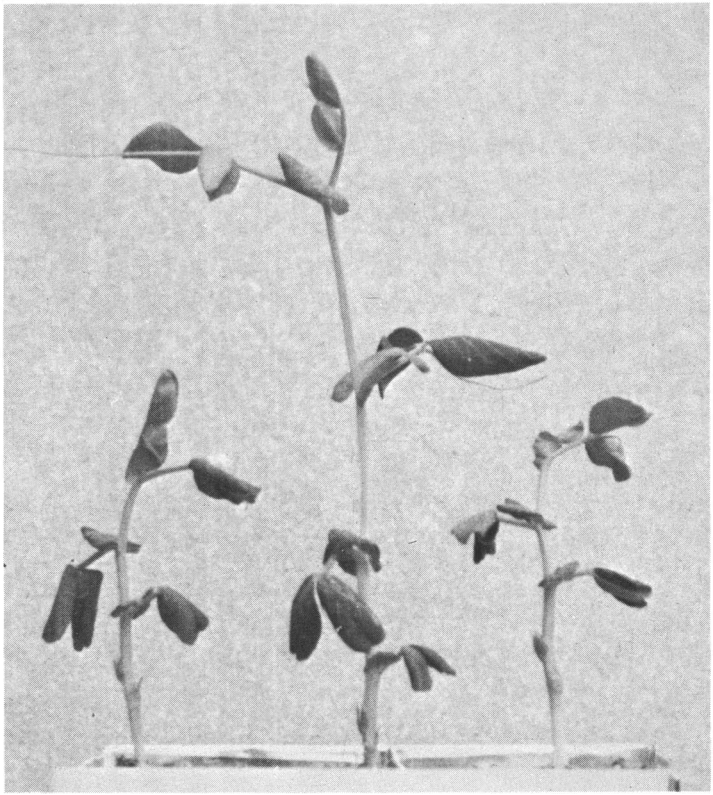

Figure 2. Response of Little Marvel pea seedings to gibberellic acid and a mixture of gibberellic acid and Ceratonia extract

Left to right. Nontreated control, treated with 0.05 μg. of gibberellic acid, and treated with mixture of 0.05 μg. of gibberellic acid and extract from 0.1 gram of Ceratonia *fruit.*

Four hundred grams of fruit were cut in half lengthwise and broken into pieces from 3 to 6 cm. long. This material was covered with 1 liter of acetone and water (1 to 1 by volume) and allowed to stand for 3 days at 1° C. The diffusate was then decanted and concentrated under vacuum to a volume of 200 ml. In this way the acetone was removed and the extracted material left in an aqueous medium. Each milliliter of aqueous residue contained the extractable material from 2 grams of fruit. In all experiments an extract of this concentration was used unless otherwise indicated.

Properties of Inhibitory Extract

The extract from *Ceratonia* completely or almost completely suppressed the growth which usually occurs after application of gibberellic acid. This inhibitory action on maize and peas is illustrated in Figures 1 and 2.

The relationship between response and dosage was determined by varying the concentration of inhibitory extract while keeping the gibberellic acid concentration constant. A series of tenfold dilutions of the extract was made with water to give five concentrations, the lowest 10,000 times more dilute than the highest. The dwarf-1 mutant of maize was used for bioassay. The per cent reduction in leaf-sheath length is plotted against concentration of the extract in Figure 3. The two highest concentrations show over 80% inhibition, the two lowest concentrations less than 10% inhibition. The extract from 10 mg. of *Ceratonia* fruit is capable of inhibiting over 80% of the growth induced by 0.5 μg. of gibberellic acid; the inhibitory material is very active and/or present in very high concentrations.

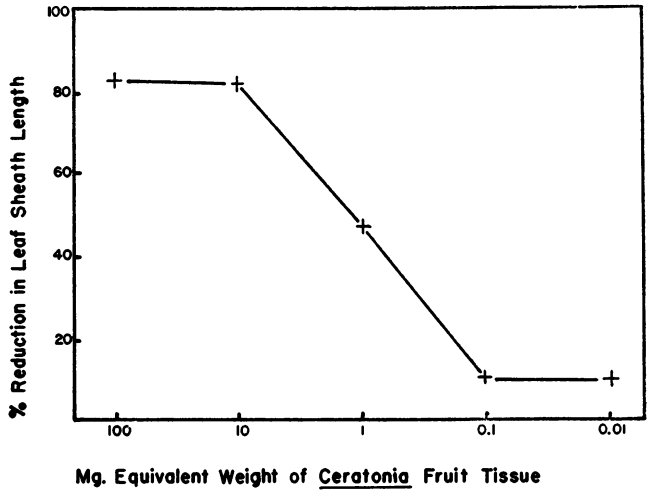

Figure 3. Inhibition of gibberellic acid–induced growth by Ceratonia extract

To determine whether the inhibitor was organic, a 2-ml. aliquot of extract was ashed and the ash suspended in 2 ml. of water. A suspension of 100 times greater concentration was also obtained by ashing an aliquot of 100 ml. and suspending the ash in 1 ml. of water. Both suspensions were mixed with gibberellic acid and assayed on dwarf-1 seedlings. A nonashed aliquot of extract was tested directly by mixing with gibberellic acid and assaying. The data in Table I indicate that prior to ashing the extract was highly inhibitory, the ashed extract was not inhibitory, and the concentrated ashed extract was inhibitory. These results indicate that

Ceratonia extract contains an inorganic substance which is inhibitory after ashing, and that most of the inhibition is due to a substance(s) which is destroyed by ashing, and is presumably an organic compound.

Table I. Effect of Ashing on Inhibitory Properties of *Ceratonia* Extract

	Length of First Leaf Sheath,[a] Mm.	*% Inhibition*
Nontreated control	23.0 ± 0.7	
Gibberellic acid control	51.0 ± 2.1	
Extract and gibberellic acid	27.7 ± 1.7	83
Nontreated control	23.3 ± 0.9	
Gibberellic acid control	54.3 ± 2.5	
Ash and gibberellic acid	47.5 ± 1.3	22
Nontreated control	23.1 ± 0.8	
Gibberellic acid control	44.6 ± 2.7	
Concentrated ash and gibberellic acid	33.2 ± 1.0	53

[a] Av. and standard error of 10 plants.

At least some inhibitory material was extractable into ethyl ether, which gives further evidence of the presence of an organic inhibitor.

Purification

It was thought that adsorption onto charcoal might prove a useful step in purification of the organic inhibitor(s). Accordingly, an extract was mixed with activated charcoal, the mixture was filtered, and the charcoal was washed with water and eluted with acetone. Both filtrate and eluate proved to be inhibitory. Either the amount of charcoal used was insufficient to adsorb all of the inhibitor, or two types of inhibitors were present, only one of which was adsorbed by charcoal.

To determine which possibility was correct, an extract from 200 grams of *Ceratonia* fruit was concentrated to 200 ml. and mixed with 20 grams of a mixture of 1 part of charcoal to 2 parts of Celite. The mixture was then filtered. A 1-ml. aliquot of the filtrate was removed for testing and the remainder mixed with an additional 20 grams of charcoal and Celite. This mixture was filtered and the steps were repeated until the extract had been mixed with four separate portions of charcoal and Celite. Each batch of charcoal and Celite was washed separately with 500 ml. of water and then eluted with acetone. Each eluate was evaporated to dryness and the residue dissolved in 20 ml. of water.

The inhibitory properties of the four filtrates and the four eluates were determined by bioassay with the dwarf-1 maize mutant (Table II). Only the first eluate was inhibitory, the next three showing little if any reduction in leaf-sheath length. In contrast, all filtrates were inhibitory. Since the initial treatment with charcoal and Celite removed only some inhibitory material while subsequent treatments left the remaining inhibitory material in the supernatant, there must be at least two inhibitory materials in the extract, only one of which is adsorbed by charcoal.

Both the filtrate after charcoal-Celite adsorption and the eluate from the charcoal-Celite mixture were ashed and the ash was concentrated 100 times. Bioassay revealed that the inorganic inhibitor was not adsorbed by the charcoal-Celite mixture but remained in the filtrate.

Thus, the filtrate, after charcoal adsorption, contains a mixture of at least two inhibitors: an inorganic component which is inhibitory after ashing and an organic component (organic inhibitor A) which is responsible for most of the total inhibitory activity and is destroyed by ashing.

Table II. Effect of Repeated Charcoal Adsorptions on *Ceratonia* Extract

Eluate 1. Eluate from first charcoal adsorption, etc.
Filtrate 1. Filtrate after first charcoal adsorption, etc.

	Length of First Leaf Sheath,[a] *Mm.*	*% Inhibition*
Nontreated control	25.5 ± 1.8	
Gibberellic acid control	50.3 ± 2.5	
Eluate 1 and gibberellic acid	34.5 ± 2.5	60
Eluate 2 and gibberellic acid	45.0 ± 3.3	21
Eluate 3 and gibberellic acid	49.7 ± 1.8	2
Eluate 4 and gibberellic acid	48.6 ± 2.8	7
Nontreated control	24.4 ± 0.5	
Gibberellic acid control	48.9 ± 1.8	
Filtrate 1 and gibberellic acid	29.0 ± 1.9	81
Filtrate 2 and gibberellic acid	34.5 ± 3.3	59
Filtrate 3 and gibberellic acid	38.4 ± 2.1	43
Filtrate 4 and gibberellic acid	36.3 ± 2.5	51

[a] Av. and standard error of 10 plants.

Purification of the inhibitory substance which adsorbs onto charcoal was continued by partitioning into ethyl ether. An amount of extract derived from 286 grams of *Ceratonia* fruit was mixed with charcoal and Celite. The mixture was filtered; the charcoal-Celite was washed with water and eluted with acetone. The eluate was concentrated to remove the acetone and the residue was suspended in 20 ml. of water (pH approximately 5). This solution was partitioned 12 times with 40 ml. of ethyl ether each time. An aliquot of the final aqueous fraction was assayed for inhibitory activity.

The ether fractions of the first and second partitions were pooled, those of the next two were pooled, and this procedure was continued until the 12 fractions were in six groups. Each of the pooled ether fractions was dried overnight with anhydrous sodium sulfate. The ether solution was decanted and evaporated to dryness. The residue from each ether extract was dissolved in 5 ml. of water and an aliquot assayed with Little Marvel pea seedlings.

Table III. Effect of Repeated Extraction with Ethyl Ether

	Shoot Length,[a] *Mm.*	*% Inhibition*
Nontreated control	54.7 ± 1.7	
Gibberellic acid control	151.9 ± 8.0	
Pairs of ether partitions (mixed with gibberellic acid)		
First	68.8 ± 5.4	67
Second	103.9 ± 7.1	50
Third	108.6 ± 6.4	45
Fourth	120.4 ± 5.0	32
Fifth	138.3 ± 6.0	14
Sixth	146.1 ± 8.1	6
Water phase and gibberellic acid	78.2 ± 3.6	76

[a] Av. and standard error of 10 plants.

The results shown in Table III reveal strong inhibition in the first pair of ethyl ether partitions and in the final water phase. The inhibition was progressively less through the remaining ether partitions, with none in the final pair of ether fractions. This evidence indicates the presence of two inhibitory substances. Organic inhibitor B is not extractable with ether at pH 5; organic inhibitor C is extractable with ether at pH 5. Both are adsorbed by activated charcoal.

An experiment was conducted to measure the distribution of the three organic inhibitors into ethyl ether at pH 2, 7, and 12. Organic inhibitors A and B tended to distribute into ether only at the acidic pH, which is suggestive of a carboxylic acid. The results with organic inhibitor C are somewhat different, although the

same trend is evident. At pH 12 almost all of the inhibitory property was lost. What little remained was in the water phase only. At pH 7 both water and ether phases were inhibitory. At pH 2 the inhibitor was present in the ether phase and little if any appeared in the water phase. Again with this inhibitor, solubility in ether is favored by an acidic pH. These partition characteristics again might be expected of a weak acid.

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RECEIVED October 14, 1960. Research supported in part by grant 67-543 from the Cancer Research Committee of the University of California.

Spectrophotometric Method for Determination of Gibberellic Acid

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A rapid quantitative method for the determination of gibberellic acid is based on the conversion of gibberellic acid to gibberellenic acid, followed by measurement of the absorption of the latter compound at 254 mμ. The method has been shown to be specific for gibberellic acid and tolerant of the presence of other known impurities and decomposition products. It has been successfully applied to a wide range of fermentation samples.

Fermentation broth samples may contain, in addition to gibberellic acid, varying amounts of closely related but inactive compounds such as gibberellenic and isogibberellic acids, together with other acidic products of the fermentation. The resolution of these from the active constituent can prove extremely difficult and methods hitherto used for the determination of gibberellic acid in broths have not been completely satisfactory: Physical techniques employing infrared spectrophotometry (*4*), polarography (*2*), or fluorimetry (*3*) all lack specificity, while biological methods (*1*) are time-consuming and require greenhouse facilities, not generally available to the analytical laboratory, for their successful application. The present work was undertaken in an attempt to resolve these difficulties.

Experimental

Solutions of gibberellic acid in dilute mineral acid decompose slowly at room temperature, giving first gibberellenic acid and, on prolonged standing, allogibberic and gibberic acids. Gibberellenic acid absorbs strongly in the ultraviolet and has an extinction coefficient ($E_{1\,cm.}^{1\%}$) of 613 at 254 mμ, whereas the other three acids are only very weakly absorbent at this wave length.

It was thought that acceleration of this reaction might form the basis of a selective method of gibberellic acid determination, and the effects of acid and elevated temperature were studied using various strengths of hydrochloric, phosphoric, perchloric, acetic, formic, trichloroacetic, and methanesulfonic acids over a temperature range of 20° to 50° C.

Hydrochloric acid proved to be by far the most effective, and decomposition

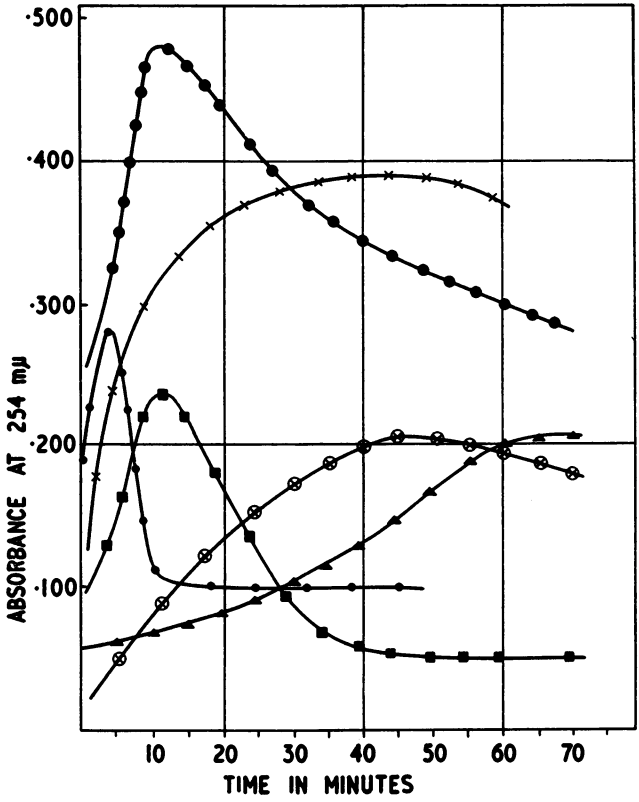

Figure 1. Decomposition of gibberellic acid by hydrochloric acid

	Concd. HCl, Ml./100 Ml.	° C.
●	40	20
×	30	20
▲	10	20
⊗	10	37
•	30	55
■	10	55

curves covering the conditions employed are illustrated in Figure 1. At 20° C. a reagent consisting of 3 volumes of concentrated hydrochloric acid and 7 volumes of water showed considerable promise, giving a sharp rise in gibberellenic acid concentration over the first 30 minutes, followed by a relatively flat portion where the rates of formation and decay of gibberellenic acid are almost equal. Addition of alcohol to the medium retards the rate of decomposition of gibberellenic acid, as shown by the increase in maximum absorption from a constant weight of gibberellic acid (Figure 2). A 10% (v./v.) alcoholic concentration in the solution gave the most satisfactory curve and all future work was carried out with this amount of alcohol present.

A calibration graph was prepared from known aliquots of pure gibberellic acid and a linear relationship established over a concentration range of 0 to 4 mg. per 100 ml. with a reaction time of 75 minutes and a temperature of 20° C. The

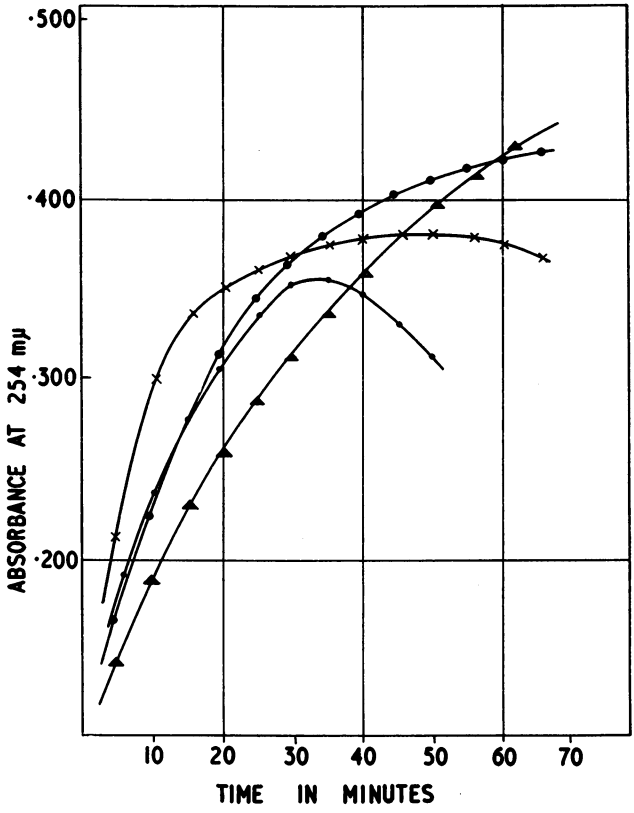

Figure 2. Effect of alcohol concentration on rate of decomposition of gibberellic acid by hydrochloric acid

• 2% v./v. × 5% v./v. ● 10% v./v. ▲ 20% v./v.

results of four replicate calibration graphs, given in Table I, illustrate the high degree of reproducibility.

Table I. Reproducibility of Points on Calibration Graph

	Absorbance at 254 Mμ			
Gibberellic Acid, Mg.	1	2	3	4
1.0	0.219	0.216	0.214	0.216
2.0	0.431	0.435	0.430	0.428
3.0	0.644	0.648	0.658	0.640
4.0	0.863	0.868	0.860	0.858

Influence of Impurities and Decomposition Products. GIBBERELLENIC ACID. Gibberellenic acid decomposes slowly in the presence of strong concentrations of mineral acids and therefore, if this acid is initially present in the sample, the net absorbance due to gibberellic acid decomposition will be low by that fraction of the initial gibberellenic acid that has decomposed during the 75-minute reaction period of the method described below. The amount by which the net absorbance due to gibberellic acid decomposition is low can be calculated from the initial gibberellenic acid content and a simple correction applied. In practice it is rarely

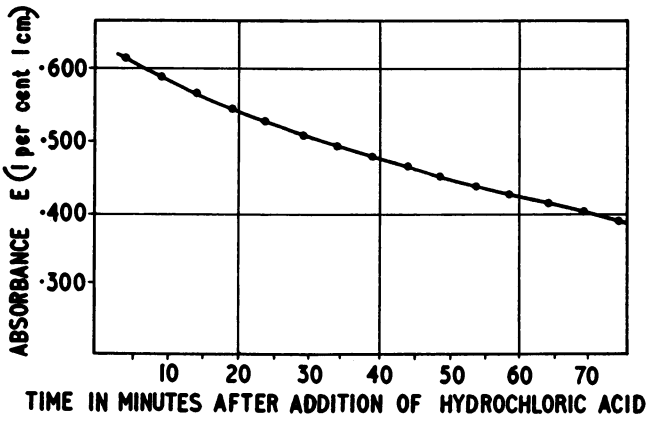

Figure 3. Rate of decomposition of gibberellenic acid by hydrochloric acid

necessary to correct for gibberellenic acid, as it is present in significant amounts only in stored solutions or artificially decomposed samples.

The rate of decomposition of gibberellenic acid has been measured under the conditions employed for gibberellic acid determination. The graph relating fall in absorbance with time is reproduced in Figure 3.

ISOGIBBERELLIC ACID (gibb-4-ene 1 → 3 lactone). This compound exhibits a small absorption at 254 mμ, but as it is unchanged by the conditions employed for the decomposition of gibberellic acid, no complication is introduced by its presence.

ALLOGIBBERIC AND GIBBERIC ACIDS. Both these compounds exhibit weak absorption in the ultraviolet region with maxima at 265 mμ. No change in absorption takes place on treatment with hydrochloric acid and hence no interference is caused by either compound.

SUMIKI'S ACID (5-hydroxymethylfuran-2-carboxylic acid). This acid is often present to a greater or lesser extent in fermenter broth and is occasionally found in solid isolates in significant amount. It absorbs strongly in the ultraviolet, exhibiting a maximum at 260 mμ in acid solution ($E_{1\,cm.}^{1\%}$ 950). The absorption is unchanged under the conditions employed for the gibberellic acid determination and hence this compound causes no direct interference. The close similarity of the spectrum of Sumiki's acid to that of gibberellenic acid, however, precludes the estimation of the latter compound by direct ultraviolet measurement for correction of the gibberellic acid content. Where these three acids are present in admixture, it is necessary first to estimate the Sumiki's acid and to subtract the contribution of this acid from the absorption curve of the sample before the gibberellenic acid content can be calculated. Sumiki's acid can be estimated by taking advantage of the difference in absorption spectra under acid and neutral-alkaline conditions displayed by this compound (Figure 4). The absorbance of the sample solution is measured under both neutral and acid conditions and the Sumiki's acid content calculated from the change in absorbance at 268 mμ.

Application to Broth Filtrates

Samples of gravity-filtered broth are invariably turbid and, without treatment, totally unsuitable for spectrophotometric work. The form of treatment depends

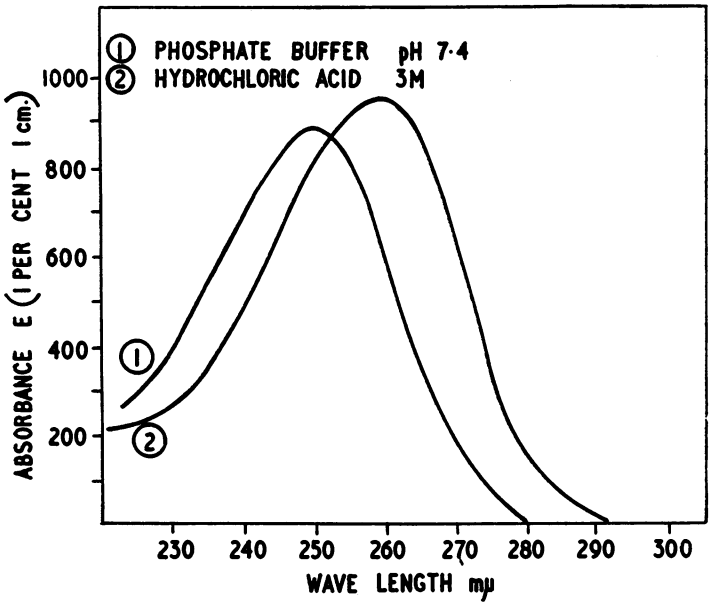

Figure 4. Spectra of Sumiki's acid under acid and alkaline conditions

largely on the composition of the culture medium used. Treatment with zinc acetate–potassium ferrocyanide has been found satisfactory for clarifying a wide range of media; on no occasion has it failed to give an optically clear solution. In a few instances, where complex media had been used, the absorbance of the filtrate was very high and prior separation of the gibberellic acid by extraction into ethyl acetate, followed by re-extraction into phosphate buffer, was preferred. Stronger hydrochloric acid was used to overcome the effect of the phosphate buffer and the reaction time was extended from 75 to 80 minutes.

A series of broth samples taken at intervals during the course of fermentations using a variety of media has been examined after zinc acetate–potassium ferrocyanide clarification and hydrochloric acid treatment. Full spectra of blank and sample were measured in each case between 230 and 300 mμ. On the assumption that the increase in absorbance at 254 mμ was due solely to gibberellenic acid, the whole spectral curve was, with the aid of a reference curve for pure gibberellenic acid, corrected for the presence of this compound. The degree of coincidence obtained when the corrected curve was compared with that of the blank indicated that no other ultraviolet-absorbing substance was produced in significant amount. A typical set of spectra is illustrated in Figure 5 .

Method

On the basis of the foregoing experimental work, the following method was developed for the determination of gibberellic acid in solid samples and broth filtrates.

Reagents. 1. Dilute Hydrochloric Acid, 30%. Dilute 300 ml. of concentrated hydrochloric acid (specific gravity 1.18) to 1 liter in a volumetric flask with water, cool to 20° C., and adjust the volume to 1 liter with water if necessary.

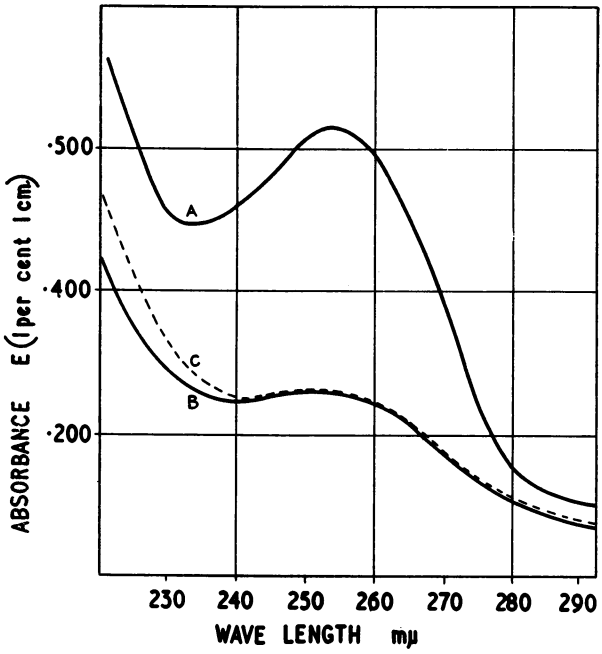

Figure 5. Typical spectrum of sample before and after acid treatment

A. After acid treatment
B. Before acid treatment
C. Curve *A* corrected for gibberellenic acid

2. Dilute Hydrochloric Acid, 35%. Dilute 350 ml. of concentrated hydrochloric acid (specific gravity 1.18) to 1 liter with water in a volumetric flask, cool to 20° C., and adjust the volume to 1 liter with water if necessary.

3. Dilute Hydrochloric Acid, 5%. Dilute 100 ml. of concentrated hydrochloric acid (specific gravity 1.18) to 2 liters with water.

4. Standard Gibberellic Acid Solution. Dissolve 0.040 gram of pure gibberellic acid in absolute alcohol and dilute to 100 ml. in a volumetric flask with absolute alcohol. 1 ml. = 0.400 mg.

5. Zinc Acetate Solution. Dissolve 21.9 grams of crystalline zinc acetate [$Zn(CH_3COO)_2.2H_2O$] in about 80 ml. of water, add 1.0 ml. of glacial acetic acid, and dilute to 100 ml. with water.

6. Potassium Ferrocyanide Solution. Dissolve 10.6 grams of analytical reagent grade potassium ferrocyanide in 100 ml. of water.

7. Phosphate Buffer, pH 7.4. Dissolve 47.7 grams of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) and 4.53 grams of potassium dihydrogen phosphate (KH_2PO_4) in about 250 ml. of warm water and dilute to 2.5 liters with water.

Preparation of Calibration Graph. Transfer aliquots of 2, 4, 6, 8, and 10 ml. of standard gibberellic acid solution to each of five 100-ml. volumetric flasks and add sufficient absolute alcohol to bring the volume in each flask to 10.0 ml. In a sixth flask (to provide a reagent blank) place 10 ml. of absolute alcohol. Commencing with the flask containing the smallest aliquot, dilute to volume with dilute hydrochloric acid (30%). Mix the contents and place the flask in a water bath at 20° ± 1° C. At 3-minute intervals repeat the procedure successively with the remaining flasks. Allow each flask to stand for 75 minutes in the water bath and then immediately measure the absorbance of the contents at 254 mμ in 1-cm. silica cells on a suitable spectrophotometer against water in the blank cell. Subtract the reagent blank from each reading and construct a calibration graph from the figures so obtained.

Treatment of Samples. SOLID SAMPLES OF GIBBERELLIC ACID. Dissolve an accurately weighed quantity of between 50 and 60 mg. of sample in absolute alcohol and dilute to 100 ml. with absolute alcohol in a volumetric flask (Solution A). Transfer 5.0-ml. aliquots of Solution A to each of two 100-ml. volumetric flasks and add 5.0 ml. of absolute alcohol. Dilute the contents of the first (sample) flask to 100 ml. with dilute hydrochloric acid (30%), allow to stand in a water bath at 20° ± 1° C. for exactly 75 minutes, and measure the absorbance at 254 mμ against water as previously described. To the second (blank) flask add 35 ml. of dilute hydrochloric acid (5%), dilute to volume with water, mix, and immediately measure the absorbance at 254 mμ against water. Subtract the blank reading from the sample reading and obtain the gibberellic acid content of the sample by reference to the calibration graph.

Where gibberellenic acid is present in significant amount, either alone or accompanied by Sumiki's acid, the following procedure has been found satisfactory for the correction of blank reading to compensate the interference introduced as a result of the presence of the former compound.

Transfer 25.0-ml. aliquots of Solution A to each of two 100-ml. graduated flasks. Dilute the contents of the first flask to 100 ml. with water: Mix the solution and measure the absorbance at 242, 254, 266, and 268 mμ in 1-cm. silica cells against a reference solution consisting of 1 part of absolute alcohol and 3 parts of water. To the second flask add 35 ml. of 5% hydrochloric acid (reagent 5) and dilute to 100 ml. with water. Measure the absorbance of the solution at 268 mμ.

Let x = the absorbance of the neutral aqueous alcoholic solution at 268 mμ.
Let y = the absorbance of the acid aqueous alcoholic solution at 268μ.

$$\text{Then \% Sumiki's acid} = (y - x) \times \frac{100}{25} \times \frac{1}{\text{wt. taken}} \times \frac{100}{443}$$

where 443 is the $E^{1\%}_{1\text{cm.}}$ difference for Sumiki's acid in neutral and acid solution.

Correct the absorbances measured at 242, 254, and 266 mμ for the Sumiki's acid present as follows:

$$\text{Corrected reading at 242 m}\mu = \text{observed reading} - \left(\frac{\text{\% Sumiki's acid}}{100} \times 510 \times \frac{\text{wt. taken}}{4}\right)$$

$$\text{Corrected reading at 254 m}\mu = \text{observed reading} - \left(\frac{\text{\% Sumiki's acid}}{100} \times 893 \times \frac{\text{wt. taken}}{4}\right)$$

$$\text{Corrected reading at 266 m}\mu = \text{observed reading} - \left(\frac{\text{\% Sumiki's acid}}{100} \times 841 \times \frac{\text{wt. taken}}{4}\right)$$

where 510, 893, and 841 are the $E^{1\%}_{1\text{cm.}}$ values for pure Sumiki's acid.

$$\text{Then \% gibberellenic acid} = \text{corrected reading at 254 m}\mu - \frac{\left(\frac{\text{corrected reading at 242 m}\mu + \text{corrected reading at 266 m}\mu}{2}\right) \times 400}{156 \times \text{wt. taken}}$$

where 156 is $E^{1\%}_{1\text{cm.}}$ 254 mμ $- \frac{E^{1\%}_{1\text{cm.}}\ 242\ \text{m}\mu + E^{1\%}_{1\text{cm.}}\ 266\ \text{m}\mu}{2}$

for pure gibberellenic acid.

Then: Corrected blank = observed blank − (gibberellenic acid content × $\frac{\text{wt. of sample}}{2{,}000} \times 226$)

where 226 represents the fall in terms of $E^{1\%}_{1cm.}$ of a solution of gibberellenic acid over the 75-minute standing period.

BROTH SAMPLES. Clarification with Zinc Acetate–Potassium Ferrocyanide. Transfer an aliquot of filtered broth expected to contain between 5 and 15 mg. of gibberellic acid to a 50-ml. volumetric flask, add 10.0 ml. of absolute alcohol, and dilute to about 40 ml. with water. Add 2.0 ml. of zinc acetate solution, followed, after 2 minutes, by 2.0 ml. of potassium ferrocyanide solution. Adjust the contents of the flask to 50 ml. with water, mix, allow the flask to stand at room temperature for 5 minutes, and filter the contents through a Whatman No. 52 filter paper. Transfer 10.0-ml. aliquots of the filtrate to each of two 100-ml. volumetric flasks and add 8.0 ml. of absolute alcohol to each. Complete the determination as described above commencing with the addition of dilute hydrochloric acid (30%) to the first (sample) flask.

Extraction with Ethyl Acetate. Transfer an aliquot of filtered broth expected to contain between 2 and 6 mg. of gibberellic acid to a 100-ml. separating funnel, and add sufficient water to bring the volume to about 10 ml. Adjust the pH of the solution by spotting out on Universal indicator paper to between pH 1 and pH 2 by the careful addition of 0.1*N* hydrochloric acid. (About 2 ml. is usually required.) Add 20 ml. of analytical reagent grade ethyl acetate and shake vigorously for 1 minute. Allow the layers to separate and quantitatively transfer the lower aqueous layer to a second separator. Repeat the extraction with a further 20-ml. portion of ethyl acetate, reject the aqueous layer, and combine the ethyl acetate layers in the first separating funnel. Re-extract the gibberellic acid from the ethyl acetate layer with successive portions of 20, 15, and 10 ml. of phosphate buffer, shaking each extraction for 1 minute, and combine the buffer extracts in a 50-ml. volumetric flask. Adjust the contents of this flask to 50 ml. with phosphate buffer. Transfer 20.0-ml. aliquots of the phosphate buffer extract to each of two 100-ml. volumetric flasks and add 10.0 ml. of absolute alcohol to each. Adjust the contents of the first (sample) flask to 100 ml. with dilute hydrochloric acid (35%) and measure the absorbance at 254 mμ in 1-cm. silica cells after standing at 20° ±1°C. for 80 minutes. To the second flask, add 35 ml. of dilute hydrochloric acid (5%), make to volume with water, and immediately measure the absorbance at 254 mμ. Subtract the blank reading from that of the sample and obtain the gibberellic acid content by reference to a calibration graph prepared from standard quantities of gibberellic acid dissolved in 20 ml. of phosphate buffer, employing dilute hydrochloric acid (35%) and the extended reaction time of 80 minutes.

Results

A series of solid samples to which known quantities of the principal impurities and decomposition products had been added was analyzed by the proposed method (Table II).

Table II. Recovery in Presence of Impurities and Decomposition Products

Gibberellic Acid Present, Mg.	*Allogibberic Acid, Mg.*	*Gibberic Acid, Mg.*	*Isogibberellic Acid, Mg.*	*Gibberellenic Acid, Mg.*	*Sumiki's Acid, Mg.*	*Gibberellic Acid Recovered*	
						Mg.	*% w./w.*
2.80	—	—	—	—	—	2.83	101
2.80	2.48	—	—	—	—	2.83	101
2.80	—	2.12	—	—	—	2.83	101
2.80	—	—	0.65	—	—	2.83	101
4.80	—	—	—	0.80	—	4.72	98.4
2.80	—	—	—	—	0.56	2.80	100
2.80	—	—	—	0.14	0.56	2.80	100
2.80	—	—	—	—	—	2.80	100

A series of culture media was prepared, containing known amounts of gibberellic acid, and assayed by the two methods described (Table III).

Table III. Recovery from Fortified Fermentation Broth

Gibberellic Acid		*Gibberellic Acid Recovered*			
		Ferrocyanide Precipitation Method		*Extraction Method*	
Sample	*Added, mg.*	*Mg.*	*% w./w.*	*Mg.*	*% w./w.*
1	3.50	3.40	97	3.40	97
2	4.90	4.85	99	4.90	100
3	6.20	6.30	101.5	6.30	101.5
4	8.05	8.32	103.5	8.10	100.5
5	8.27	8.10	98	8.20	99
6	8.80	8.50	96.5	8.85	100.5
7	9.60	9.20	96.0	9.40	98
8	10.10	9.90	98	9.80	97

Statistical Evaluation of Method

Statistically designed experiments were carried out to assess the reproducibility of the method as applied to both solid isolates and broth samples.

In the first series of experiments, six samples of solid, chosen to cover a wide fluctuation of gibberellic quality, were assayed by three operators in sextuplicate on six occasions during 3 days. The results indicated a standard error of 1% over the whole series, slightly better on the purer samples and slightly worse on material of poor quality.

A similar series of experiments, carried out using two broth samples typifying extremes of medium complexity and fermentation conditions, gave standard errors of 2 and 3%, respectively.

Acknowledgment

The authors express their thanks to F. Colledge and G. M. Wells for assistance with some of the experimental work and J. F. Grove for providing pure samples of some of the impurities.

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RECEIVED August 22, 1960.