49. *The Biosynthesis of the Gibberellins. Part I. (−)-Kaurene as a Precursor of Gibberellic Acid.*

By B. E. Cross, R. H. B. Galt, and J. R. Hanson.

(−)-[17-^{14}C]Kaurene and ^{14}C-labelled gibberellin A_9 have been prepared, and their metabolism by cultures of *G. fujikuroi* has been investigated. (−)-Kaurene was found to be a precursor in the biosynthesis of gibberellic acid. Degradation of 7,18-dihydroxykaurenolide, derived from [2-^{14}C]-mevalonic lactone, has confirmed the α-orientation of the lactone ring in gibberellic acid.

Owing to the widespread and varied effects of the gibberellins on many aspects of higher-plant growth and development,[1] their biosynthesis is of great interest.

Four gibberellins have been isolated [2] from higher plants and gibberellin-like activity has been demonstrated in extracts from many higher plants [1c,3] and from representatives of some of the lower orders, *e.g.*, a seaweed (*Fucus vesiculosis* [4]) and a liverwort (*Marchantia polymorpha* L.[5]); however, the concentration of gibberellins in these sources is very low. The major source of the gibberellins is the fungus *Gibberella fujikuroi*, which is known to produce gibberellic acid (I; R = OH) in high yield [6] and five closely related gibberellins,[7] namely, A_1 (II; R = R′ = OH, R″ = CH_2), A_2 (II; R = OH, R′ = H, R″ = OH, Me), A_4 (II; R = OH, R′ = H, R″ = CH_2), A_7 (I; R = H), and A_9 (II; R = R′ = H, R″ = CH_2), in smaller amounts. It thus provides a convenient means of investigating the biosynthesis of the gibberellins.

[1] (*a*) Brian, Grove, and MacMillan, *Progr. Chem. Org. Nat. Prod.*, 1960, **18**, 350; (*b*) Phinney and West, *Ann. Rev. Plant Physiol.*, 1960, **11**, 411; (*c*) Phinney and West, "Encyclopedia of Plant Physiology," Springer-Verlag, Berlin, 1961, Vol. XIV, p. 1185.

[2] MacMillan, Seaton, and Suter, *Tetrahedron*, 1960, **11**, 60; 1962, **18**, 349; West and Phinney, *J. Amer. Chem. Soc.*, 1959, **81**, 2424; Kawarada and Sumiki, *Bull. Agric. Chem. Soc. Japan*, 1959, **23**, 343.

[3] Radley, M.Sc. Thesis, London University, 1960.

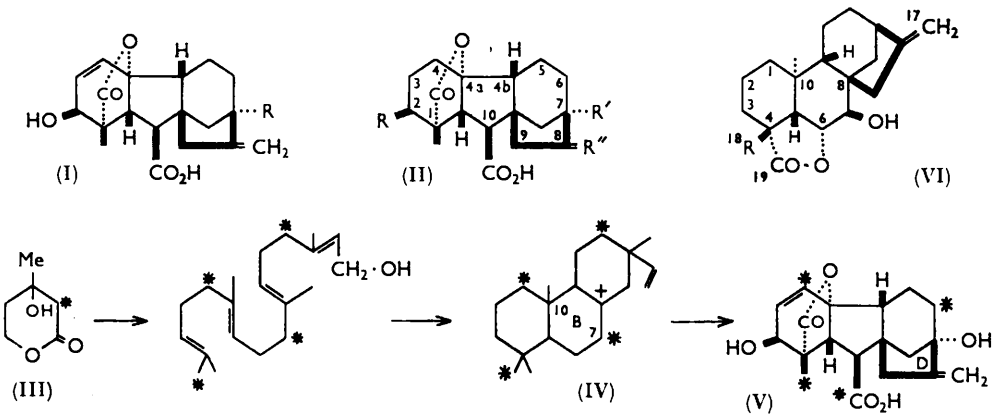
[4] Radley, *Nature*, 1961, **191**, 684.

[5] Kato, Purves, and Phinney, *Nature*, 1962, **196**, 687.

[6] Curtis and Cross, *Chem. and Ind.*, 1954, 1066; B.P. 783,611.

[7] (*a*) Grove, *Quart. Rev.*, 1961, **16**, 56; (*b*) Cross, Galt, and Hanson, *Tetrahedron*, 1962, **18**, 451.

By the addition of [2-^{14}C]mevalonic lactone (III) to a fermentation of *G. fujikuroi* and isolation of the gibberellic acid produced, Birch and his collaborators [8] showed that gibberellic acid was derived from four molecules of mevalonic lactone. Degradation of the labelled gibberellic acid (V) showed that two of the four labelled atoms were accounted for by the 1-methyl and 10-carboxyl groups. It was concluded [8] that gibberellic acid was



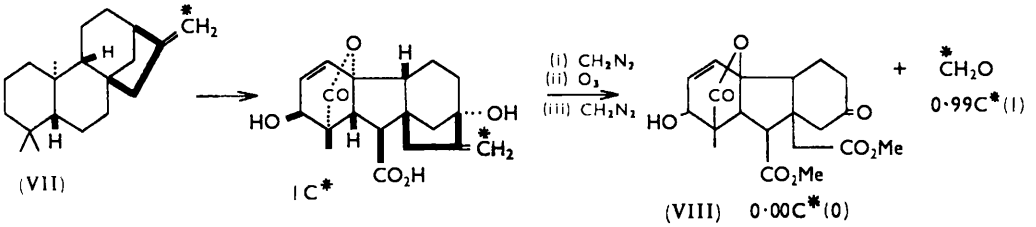
produced from a diterpenoid intermediate (IV; or its equivalent) by the reaction sequence (III) ⟶ (V) in which the angular 10-methyl group of (IV) is lost, ring D is formed, and contraction of ring B takes place with the extrusion of carbon-7 (diterpene numbering) to give a carboxyl group. The order and manner in which these reactions took place were unknown, as were the further transformations necessary to introduce the hydroxyl groups, the 3,4-double bond, and the γ-lactone ring of gibberellic acid. Our objectives were (*a*) to prove that gibberellic acid was derived from a normal diterpene skeleton, (*b*) to discover some of the intermediates between the pimaradiene carbonium ion (IV) and gibberellic acid, and (*c*) to determine at least part of the reaction sequence from (IV) to (V) and possibly the mechanism of some of the steps.

A careful examination of the compounds produced when *G. fujikuroi* (Saw.) Wr. strain ACC. 917 [9] was grown under a variety of conditions led to the isolation of many new metabolites,[10] the structures and absolute configuration of which have been determined.[10–12] Some of these were obviously of interest to the biosynthesis of the gibberellins. Thus 7-hydroxykaurenolide [11] (VI; R = Me) carried a lactone-carbonyl group at position 4 and was oxygenated at positions 6 and 7 as might be expected in an intermediate before contraction of ring B. 7,18-Dihydroxykaurenolide [12] (VI; R = CH_2·OH) looked less promising as a precursor since C-18, required as a methyl group in the gibberellins, is at the oxidation level of a hydroxymethyl group. These kaurenolides, which are known [11,12] to have the carbon skeleton of (—)-kaurene, are probably derived (see below) from the latter by microbiological oxidation; their role in the biosynthesis of the gibberellins is under investigation. (—)-Kaurene, which was also found [10] in *G. fujikuroi* fermentations and shown [10a,11,13] to have the absolute configuration (VII), is derivable from the intermediate carbonium ion (IV) by the Wenkert rearrangement [14] and might therefore be a precursor

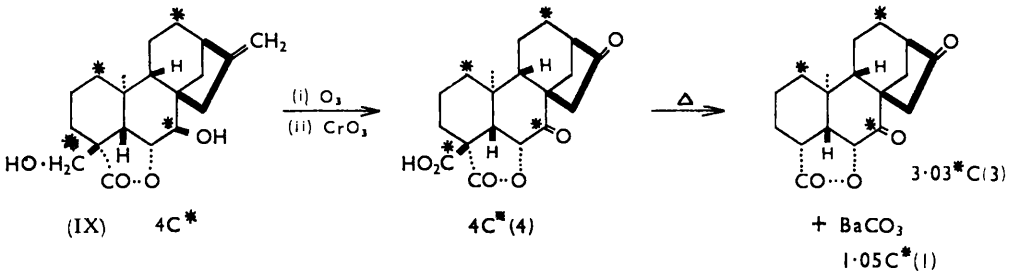
[8] Birch, Rickards, and Smith, *Proc. Chem. Soc.*, 1959, 192; Birch, Rickards, Smith, Harris, and Whalley, *Tetrahedron*, 1959, **7**, 241.
[9] Borrow, Brian, Chester, Curtis, Hemming, Henehan, Jefferys, Lloyd, Nixon, Norris, and Radley, *J. Sci. Food Agric.*, 1955, **6**, 340.
[10] (*a*) Cross, Galt, Hanson, and Klyne, *Tetrahedron Letters*, 1962, 145; (*b*) Cross, Galt, Hanson, Curtis, Grove, and Morrison, *J.*, 1963, 2937.
[11] Cross, Galt, and Hanson, *J.*, 1963, 2944.
[12] Cross, Galt, and Hanson, *J.*, 1963, 3783.
[13] Vorbrueggen and Djerassi, *J. Amer. Chem. Soc.*, 1962, **84**, 2990.
[14] Wenkert, *Chem. and Ind.*, 1955, 282.

in the biosynthesis of gibberellic acid if formation of ring D takes place before contraction of ring B.

(—)-[17-^{14}C]Kaurene (VII) was prepared by reacting kaurene norketone with the Wittig reagent $^{14}CH_2{=}PPh_3$ (derived from $^{14}CH_3I$ and triphenylphosphine).[15] The labelled



(—)-kaurene was added to a fermentation of *G. fujikuroi* which was harvested after 72 hours and the products were isolated as previously described.[10b] The gibberellic acid was



shown to be labelled (incorporation 5·7%) by crystallisation of both the acid and its methyl ester to constant radioactivity.* Ozonolysis [16] of the latter and isolation of the formaldehyde as its dimethone showed that 99% of the activity resided in the terminal methylene group. The seco-ester (VIII) was inactive. This result shows that (—)-kaurene acts as a precursor of gibberellic acid and that rearrangement of (IV) to form a tetracyclic intermediate takes place before oxidative attack and contraction of ring B. However, (—)-kaurene may not be an obligatory precursor of gibberellic acid and there may be alternative pathways from (IV) to the gibberellins.

7-Hydroxy- and 7,18-dihydroxy-kaurenolide (VI; R = Me and $CH_2{\cdot}OH$, respectively), isolated from the fermentation, were also labelled (incorporation 0·05 and 0·44%, respectively).

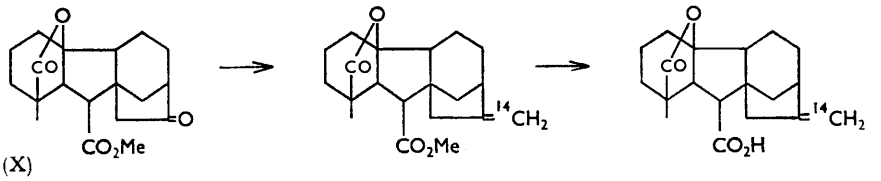
Addition of [2-^{14}C]mevalonic lactone to a *G. fujikuroi* fermentation and isolation of the kaurenolides [10b] afforded, as expected, labelled 7-hydroxy- and 7,18-dihydroxy-kaurenolide. Degradation of the latter (IX) by established reactions [12] gave the results shown. Thus of the four radioactive carbon atoms, one was accounted for by C-18 which is known to be β-oriented.[11,12] This result illustrates the great value of the biosynthetic approach to structural and stereochemical problems in natural products. Since (—)-kaurene is a precursor of both 7,18-dihydroxykaurenolide (IX) and gibberellic acid, and when the latter is derived from [2-^{14}C]mevalonic lactone the 1-methyl group is labelled,[8] it followed that this group must be β-oriented. This showed unequivocally that the lactone ring in gibberellic acid was α-oriented [17,18] at a time when its orientation was in dispute.[19] Subsequently the complete stereochemistry of gibberellic acid was delineated by *X*-ray

* For a preliminary announcement of this result see Cross, Galt, and Hanson.[11]

[15] Cf. Bell, Ireland, and Partyka, *J. Org. Chem.*, 1962, **27**, 3741.
[16] Cross, *J.*, 1960, 3022.
[17] Cross, Grove, McCloskey, Mulholland, and Klyne, *Chem. and Ind.*, 1959, 1345.
[18] Stork and Newman, *J. Amer. Chem. Soc.*, 1959, **81**, 5518.
[19] Edwards, Nicolson, ApSimon, and Whalley, *Chem. and Ind.*, 1960, 624.

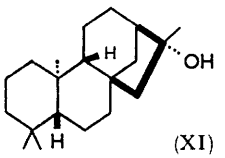
crystallography;[20] the stereochemistry at all centres except 4b was also deduced from nuclear magnetic resonance and other data.[21]

Inspection of the structure of gibberellin A_9 [7b] (II; R = R′ = H, R″ = CH_2), which is the simplest known gibberellin, suggested that it might be an intermediate at a late stage

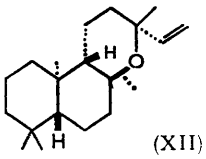


(X)

in the transformation of (—)-kaurene to gibberellic acid. In this connection it should be noted that addition of gibberellin A_4 (II; R = OH, R′ = H, R″ = CH_2) to a fermentation has been claimed to increase the yield of gibberellic acid,[22] from which it might be inferred that the former is a precursor of the latter. Gibberellin A_9 labelled with ^{14}C in the terminal methylene group was prepared from the norketone (X) [7b] by the reaction sequence shown,



(XI)



(XII)

in 28% overall yield. It was added to a fermentation and the products were isolated in the usual way. Chromatography of the acids furnished crude gibberellic acid which, although initially radioactive, rapidly lost its activity on repeated crystallisation. The nature of this radioactive impurity, which does not correspond to any of the known gibberellins, is being investigated by thin-layer radioautography. The same technique applied to the other acid fractions has shown the presence of unincorporated starting material and several as yet unidentified transformation products. Hence, gibberellin A_9 does not appear to be on the main biosynthetic pathway from (—)-kaurene to gibberellic acid.

The isolation [10b] from *G. fujikuroi* fermentations of (—)-kaurene, (—)-kauranol (XI), and (—)-13-epimanoyl oxide (XII), previously only known as rather rare compounds derived from Australasian trees and shrubs, provides an important link between the biosynthetic processes of higher plants and fungi. If (—)-kaurene is an obligatory precursor of the gibberellins which are widely distributed in the plant kingdom it is possible that (—)-kaurene will be found to be a common constituent of plants.

Experimental

Melting points were determined on a Kofler block and were corrected.

The following chromatographic materials were used: silica gel M.F.C. (Hopkin & Williams), Celite 545 (Johns-Mandeville), and alumina (Woelm neutral alumina, grade II).

Assays of radioactivity were carried out by essentially standard methods [23,24] except that the cross-sectional area of samples was reduced to 0·33 cm.^{2}.

Thin-layer chromatography was carried out on layers (0·275 μ thick) of silica gel (G E. Merck, A.-G., Darmstadt), prepared by Stahl's method.[25] Ilford *X*-ray film (Industrial G) and Ilford PQX-1 high contrast developer were used for radioautography.

Known compounds and degradation products were identified by their infrared spectra.

[20] McCapra, Scott, Sim, and Young, *Proc. Chem. Soc.*, 1962, 185.
[21] Aldridge, Grove, Speake, Tidd, and Klyne, *J.*, 1963, 143.
[22] Schmidt, *Flora*, 1961, **151**, 455.
[23] Birch, Massy-Westropp, and Smith, *J.*, 1958, 360, and subsequent papers.
[24] Popjak, *Biochem. J.*, 1950, **46**, 560.
[25] Stahl, *Chem.-Ztg.*, 1958, **82**, 323.

Light petroleum refers to the fraction of b. p. 60—80°.

(−)-[17-^{14}C]*Kaurene*.—[^{14}C]Methyl iodide (0·5 mc) was diluted to 0·5 g. with unlabelled methyl iodide, dissolved in benzene, and added to a slight excess of triphenylphosphine in benzene. After 2 days the [^{14}C]triphenylmethylphosphonium iodide (1·35 g.) was filtered off, dried, and powdered; 1·2 g. (1·4 mol.) were suspended in freshly distilled dry ether (30 ml.) under nitrogen. Butyl-lithium in pentane (15% w/v; 2·1 mol.) was added slowly to the stirred suspension until the solution was almost homogeneous and the characteristic orange colour appeared. (When only the theoretical amount of butyl-lithium was used, the reaction yield was very low.) After 2 min. kaurene norketone (0·58 g., 1 mol.) in the minimum amount of ether was added rapidly and the mixture was stirred for 5 hr. at room temperature. Water and more ether were added and the organic layer was washed with water. The gummy product was chromatographed on alumina. Elution with light petroleum gave crude (−)-[17-^{14}C]-kaurene (VII) (0·24 g.) which was crystallised twice from methanol, giving needles (0·17 g., 41 μc) (Found: r.m.a. × 10^{-4}, 1422). Elution with 15% of ether in light petroleum afforded starting material (0·25 g.).

[^{14}C]*Gibberellic Acid, 7-Hydroxy*[^{14}C]*kaurenolide, and 7,18-Dihydroxy*[^{14}C]*kaurenolide from* (−)-[^{14}C]*Kaurene*.—*Gibberella fujikuroi* was grown on a Dextrosol–ammonium nitrate medium (4 l.) in stirred, aerated culture until the inorganic nitrogen was exhausted. Then (−)-[17-^{14}C]-kaurene (0·17 g.; 41 μc) in ethanol (50 ml.) was added and the fermentation was run for a further 72 hr. The acidic and neutral metabolites were isolated and separated in the usual way.[10b] The [^{14}C]gibberellic acid (0·77 g.; 2·38 μc, 5·7%) (Found: r.m.a. × 10^{-4}, 22·4) was methylated and the product chromatographed on alumina. Elution with 50—70% of ethyl acetete in light petroleum gave methyl [^{14}C]gibberellate (Found: r.m.a. × 10^{-4}, 21·2).

The neutral fraction was chromatographed on alumina. Elution with light petroleum gave a colourless gum (0·097 g.) which on rechromatography afforded [17-^{14}C]kaurene (61 mg.; 3·6 μc, 8·8% of initial activity). The fraction eluted with 15% of ethyl acetate in light petroleum was a gum (0·058 g.) containing 7-hydroxykaurenolide. Dilution with unlabelled material (0·02 g.) and rechromatography afforded 7-hydroxy[^{14}C]kaurenolide, m. p. 187—188° (0·025 g.; 0·02 μc, 0·05%). Elution with 35% of ethyl acetate in light petroleum gave crude 7,18-dihydroxy[^{14}C]kaurenolide (0·129 g.) which on crystallisation afforded pure material, m. p. 208—212° (0·06 g.; 0·18 μc, 0·44%) (Found: r.m.a. × 10^{-4}, 21·5).

Ozonolysis of Methyl [^{14}C]*Gibberellate*.—(1) Ozonised oxygen (16 mg. of O_3 per min.) was passed through methyl [^{14}C]gibberellate (0·1 g.; r.m.a. × 10^{-4}, 21·2) in acetic acid (20 ml.) at room temperature for 2 min.[16] Water (20 ml.) was added, and the solution was shaken for 1 hr. and steam-distilled. The distillate (50 ml.) was added to a saturated aqueous solution of dimedone (50 ml.). After 24 hr. formaldehyde dimethone (12 mg.), m. p. 189—191° (0·016 g.; 20% yield) [Found: r.m.a. × 10^{-4}, 20·9 (1C, 21·2)], was collected.

(2) The same stream of ozonised oxygen was passed through methyl [^{14}C]gibberellate (0·18 g.; r.m.a. × 10^{-4}, 21·2) in ethyl acetate at −70° for 1·5 min.[16] The ethyl acetate was removed *in vacuo* at room temperature and the resultant foam treated with water for 48 hr. The product was extracted with ethyl acetate and separated into acid and neutral fractions with sodium hydrogen carbonate solution. The acidic gum was methylated with diazomethane and chromatographed on alumina. Elution with 60% of ethyl acetate in light petroleum gave the methyl ester (VIII) [16] (0·02 g.) (Found: r.m.a. × 10^{-4}, 0).

7-*Hydroxy*[^{14}C]*kaurenolide and* 7,18-*Dihydroxy*[^{14}C]*kaurenolide from* [2-^{14}C]*Mevalonic Lactone*.—*G. fujikuroi* was cultured as above. After 402 and 474 hr. 0·125 mc portions of (±)-[2-^{14}C]-mevalonic lactone were added in aqueous solution. The fermentation was harvested after 542 hr. and the culture filtrate extracted with chloroform. The extract was washed with sodium hydrogen carbonate solution and with water and dried. The recovered gum (690 mg.) was chromatographed on Celite–silica gel (2 : 1, 24 × 2 cm.). Elution with 10% of ethyl acetate in light petroleum gave 7-hydroxykaurenolide (29 mg.) which crystallised from ethyl acetate–light petroleum in needles (14 mg.), m. p. 188°, which were diluted with pure unlabelled material (52 mg.). Crystallisation from ethyl acetate–light petroleum gave 7-hydroxykaurenolide (Found: r.m.a. × 10^{-4}, 112). The fractions eluted with 20—25% of ethyl acetate in light petroleum afforded 7,18-dihydroxykaurenolide (214 mg.) which crystallised from ethyl acetate–light petroleum in needles (175 mg.), m. p. 214°. It was diluted with an equal weight of pure unlabelled material and after crystallisation gave 7,18-dihydroxykaurenolide (3·16 μc) (Found: r.m.a × 10^{-3}, 647).

Degradation of 7,18-*Dihydroxy*[^{14}C]*kaurenolide*.—(i) Ozonolysis as previously described [12] gave the norketone which after dilution with inactive norketone and crystallisation had m. p. 261—264° (Found: r.m.a. $\times 10^{-3}$, 209).

(ii) The norketone (100 mg.) in acetone (5 ml.) was treated [12] with the 8N-chromium trioxide reagent [26] (0·25 ml.) for 45 min. The excess of oxidant was discharged with methanol, water (10 ml.) was added, and the mixture was heated on a water-bath for 2 hr. in a current of nitrogen. Passage of the effluent gases through barium hydroxide solution gave barium carbonate [Found: r.m.a. $\times 10^{-3}$, 54·6 (1C, 52·2)]. Extraction of the reaction mixture with ethyl acetate gave 6α-hydroxy-7,16-dioxo-17,18-bisnorkauran-19-oic acid lactone [12] (65 mg.), m. p. 270—273° [Found: r.m.a. $\times 10^{-3}$, 158·7 (3C, 156·8)].

Preparation of [^{14}C]*Gibberellin* A_9.—[^{14}C]Methyl iodide (~1 mc) was diluted with unlabelled halide and treated with triphenylphosphine in benzene solution to give [^{14}C]triphenylmethylphosphonium iodide (2·4 g., 3 mol.). A stirred suspension of the salt in pure tetrahydrofuran (100 ml.) was treated with butyl-lithium under nitrogen as above. Gibberellin A_9 methyl ester norketone [7b] (0·65 g., 1 mol.) in tetrahydrofuran (20 ml.) was added rapidly and the mixture refluxed for 5 hr. Water was added, the solution was extracted with ethyl acetate, and the product chromatographed on alumina. Elution with 15% of ethyl acetate in light petroleum gave a gum (0·33 g.). The next fraction, eluted with 20% of ethyl acetate in light petroleum, contained gummy crystals (0·2 g.) of gibberellin A_9 methyl ester. Without further purification, these fractions were combined in methanol (16 ml.), 2N-sodium hydroxide (60 ml.) was added, and the solution refluxed for 7·5 hr. Extraction with ethyl acetate removed unhydrolysed neutral material. The aqueous layer was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The gummy product solidified after being heated on the steam-bath for 5 min. and was crystallised from acetone–light petroleum, giving 4aα-hydroxy-1β-methyl-8-[^{14}C]methylenegibbane-1α,10β-dicarboxylic acid 1→4a-lactone (0·17 g., 28% overall yield; 59 μc), m. p. 207—210° (Found: r.m.a. $\times 10^{-4}$, 2376).

Addition of [^{14}C]*Gibberellin* A_9 *to a* Gibberella fujikuroi *Fermentation*.—[^{14}C]Gibberellin A_9 from the preceding experiment (0·17 g.; 59 μc) in ethanol was added to a fermentation of *G. fujikuroi* as for the experiment with (−)-[17-^{14}C]kaurene. The metabolites were isolated and separated into acid (0·938 g.) and neutral (0·115 g.) fractions in the usual way [10b] and the former was chromatographed on Celite : silica gel (2 : 1; 90 g.). Elution with 65%, 70%, 75%, and 80% of ethyl acetate in light petroleum gave crude gibberellic acid (0·446 g.) which was initially radioactive. However, when a portion was repeatedly crystallised the activity rapidly disappeared. Methylation of the remainder gave methyl gibberellate which was crystallised four times from acetone–light petroleum, a sample of each stage of purification being retained. Thin-layer chromatography of these four samples of methyl gibberellate (benzene–acetic acid–water, 8 : 3 : 5 [27]) separated methyl gibberellate (R_F 0·11), gibberellin A_1 methyl ester (R_F 0·16), and an unidentified compound (R_F 0·55) which was only present in the first two " less pure " samples. Radioautography revealed that the fast-running spot was the only radioactive impurity in the gibberellic acid. Exposure of the purest sample of methyl gibberellate to *X*-ray paper for 12 days failed to show any radioactivity.

Elution with 35%, 40%, 45%, 50%, and 55% of ethyl acetate in light petroleum gave radioactive acidic gums (10, 26, 53, 57, and 49 mg., respectively), the composition of which is being investigated.

We are indebted to Mr. G. L. F. Norris who did the fermentations.

Imperial Chemical Industries Limited, Akers Research Laboratories,
The Frythe, Welwyn, Herts. [*Received, June 14th,* 1963.]

[26] Curtis, Heilbron, Jones, and Woods, *J.*, 1953, 457.
[27] MacMillan and Suter, *Nature*, 1963, **197**, 790.