Spring 5-1-1970

Diterpene and Gibberellin Synthesis by Certain Fusarium Species and Bacteria

Chao-Ying Kuo
Indiana State University

DITERPENE AND GIBBERELLIN SYNTHESIS BY CERTAIN *FUSARIUM* SPECIES AND BACTERIA

A Master's Thesis

Presented to

the Faculty of the School of Graduate Studies

Indiana State University

Terre Haute, Indiana

In Partial Fulfillment

of the Requirements for the

Master of Arts Degree

---

by

Chao-Ying Kuo

May 1970

THESIS APPROVAL SHEET

The thesis of Chao-Ying Kuo, contribution of School of Graduate Studies, Indiana State University, Series I , Number 974, under the title, "Diterpene and Gibberellin Synthesis by Certain Fusarium Species and Bacteria," is approved as counting toward the completion of the Master of Arts Degree in the amount of six semester hours of graduate credit.

APPROVAL OF THESIS COMMITTEE:

Warren S. Silver
(Signature of Committee Member)

(Signature of Committee Member)

Frederick M. Rothwell
(Signature of Committee Member)

Mark O. Osler
(Signature of Committee Chairman) Jan. 26, 1970 (Date)

APPROVAL FOR SCHOOL OF GRADUATE STUDIES:

(Dean of Graduate Studies) 8-21-70 (Date)

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

BIOGRAPHICAL SKETCH

The author was born in Miaoli, Taiwan, Republic of China, April 27, 1940. He attended Provincial Miaoli High School in Miaoli-Town, Taiwan, and graduated from there in 1958. He attended Taiwan Provincial Normal University in Taipei, Taiwan, and received his B. S. degree in 1964. He then spent one year in ROTC and two years as a full time teaching assistant in his Alma University. He enrolled in the Indiana State University School of Graduate Studies in 1968 and held a teaching fellowship for the 1968-69 and 1969-70 years.

ACKNOWLEDGEMENT

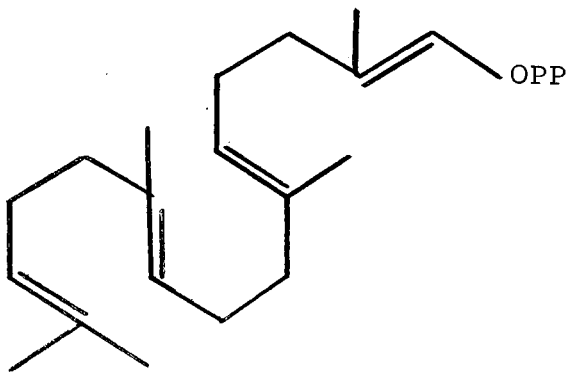
The author wishes to thank Dr. W. S. Silver, Dr. J. A. Gross and Dr. F. M. Rothwell for their advice and for serving as members of his committee. In addition he would like to express his gratitude to Dr. M. O. Oster for his guidance and understanding throughout this study and the preparation of this thesis.

INTRODUCTION

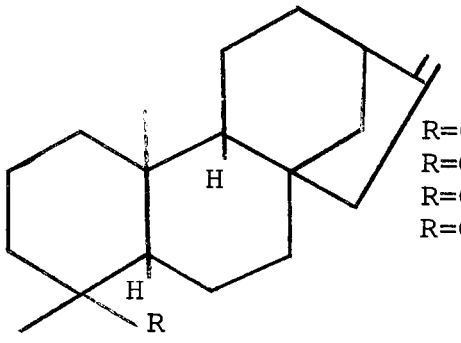
The gibberellins are a group of substances which serve as natural plant growth regulators. Their occurrence, biosynthesis, and physiological effects have been the subject of numerous reviews (Towe and Yamaki, 1957, 1959; Stodola, 1958; Brian, et al., 1960; Phinney and West, 1961; Paleg, 1965; Brian, 1966). They function, presumably, in conjunction with other plant hormones such as the auxins, cytokinins, and ethylene. Although a number of physiological effects produced by gibberellin treatment have been demonstrated and evidence has been obtained which supports the theory that the gibberellins serve to regulate RNA synthesis and, thus, protein synthesis, the exact mechanism by which they act remains to be established. Furthermore, the sites within plants at which the gibberellins are produced is incompletely described.

The structural formula of gibberellic acid shown in Fig. 1 was proposed by Cross et al. (1959; Storle and Newman, 1959), based on the structures of its degradation products. Up to date, there are more than twenty-five gibberellins that have been structurally identified (Paleg, 1965; Cavell, et al. 1967; MacMillan and Yakahashi, 1968; Harrison, et al., 1968). Of these, GA_1, GA_3, GA_4, GA_7, GA_9, GA_{12}, GA_{13}, GA_{16}, and GA_{24} are produced in cultures of

a. GERANYLGERANYL PYROPHOSPHATE



b. KAURENE COMPOUNDS



R=CH_3----KAURENE
R=CH_2OH--KAURENOL
R=CHO----KAURENAL
R=COOH---KAURENOIC ACID

c. GIBBERELLIC ACID (GA_3)

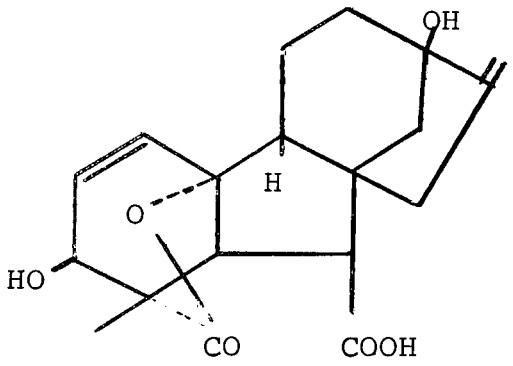


Figure 1. Structural Formulae.

Fusarium moniliforme (or Gibberella fujikuroi) (Takahashi et al., 1955, 1957; Stowe and Yamaki, 1957; Cross et al., 1962; Paleg, 1965; Galt, 1968; Harrison et al., 1968).

One of the most pronounced effects of gibberellic acid, or gibberellins, is a rapid elongation of the shoots and internodes of plants. The elongation apparently is the result of an increase in both number and length of the cells depending on the nature of the plant material investigated. The physiological actions of gibberellins differ from those of auxins in several respects. The most striking difference is that light plays no direct role in the expression of effects produced by the gibberellins in contrast to the effects produced by auxins. Gibberellins apparently do not show polar movement within the plant which is characteristic of auxins.

In several species of plants, genetic dwarf mutant strains have been described which respond specifically to the application of gibberellins. One such dwarf mutant of Zea mays L. described by Phinney (1956) has been used extensively for a biological assay for gibberellins as described by Neely (1959). The biochemical-genetic relationships of the maize dwarf mutants have been shown to be single gene defects in which the defects are individual blocks in the biosynthetic pathway for gibberellin synthesis (Phinney and West, 1957, 1961). The specific responses to

gibberellins by dwarf plants are not duplicated with auxin.

An outline of the biosynthetic pathway for the synthesis of gibberellins is shown in Fig. 2 which represents many experimental findings obtained during the last decade. Cross et al. (1956) first suggested the probable biogenetic relationship between gibberellic acid and diterpenes. Birch et al. (1958) and Birch and Smith (1959) provided experimental support for this suggestion with the demonstration of the number and positions of ^{14}C-atoms in gibberelliac acid produced from 2-^{14}C-mevalonate or 1-^{14}C-acetate by cultures of F. moniliforme and proposed a general route from acetate or mevalonate to gibberellin A_3 via diterpenoid intermediates. This was understood to involve the following steps; a) the phosphorylation and condensation of mevalonic acid to geranylgeranyl pyrophosphate, b) the cyclization of geranylgeranyl pyrophosphate to kaurene (a diterpene hydrocarbon) and c) the oxidation of kaurene to gibberellins.

Cross et al. (1962 and 1963) isolated (-)-kaurene as one of several neutral diterpenoid compounds present in culture filtrates of F. moniliforme. Phinney et al. (1964) demonstrated that kaurene has gibberellin-like activity in the dwarf-5 mutant assay of maize. Direct evidence that geranylgeranyl pyrophosphate is the substrate for kaurene formation in higher plants was obtained by Dennis et al.

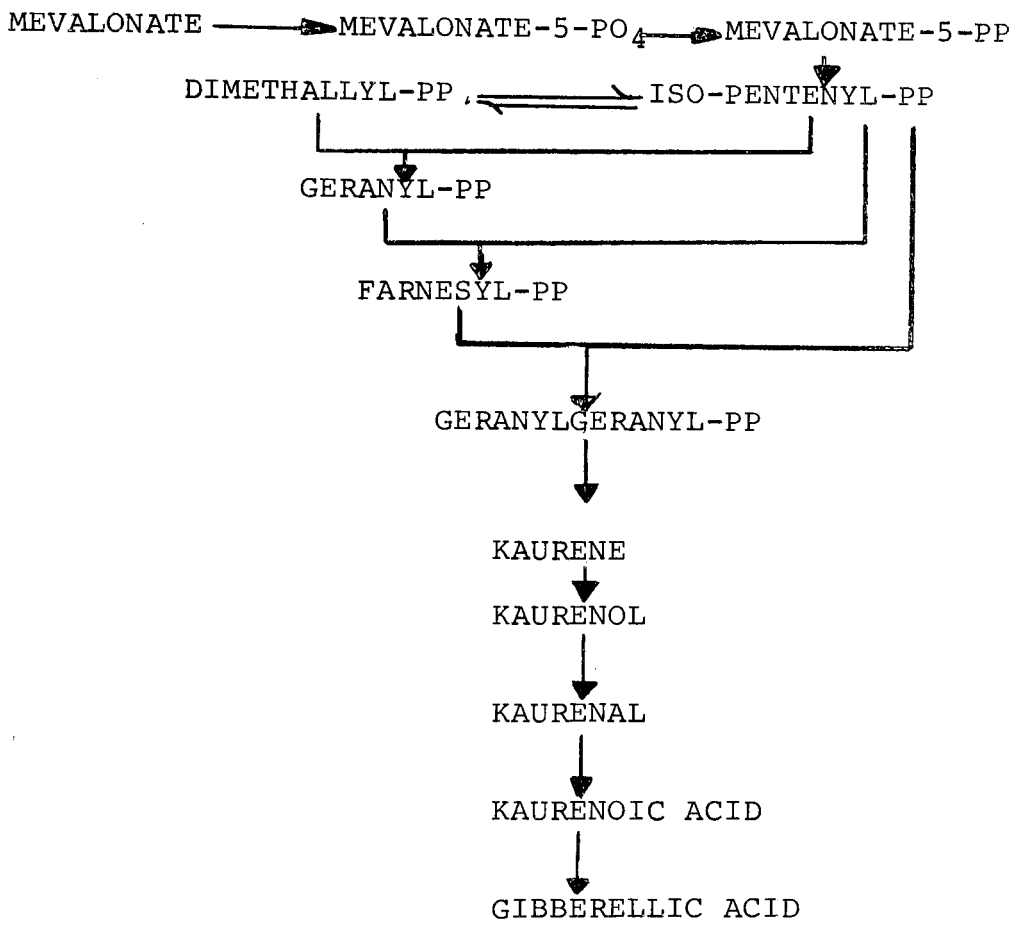


Figure 2. Outline of the proposed pathway for the biosynthesis of gibberellins.

(1967) who incubated chemically prepared ^{14}C-substrate with extracts from immature seeds of Echinocystis macrocarpa Greene and isolated ^{14}C-kaurene as the major product. Later, ^{14}C-geranylgeranyl pyrophosphate prepared enzymatically was shown to be the precursor for kaurene (Oster and West, 1968). Cross, Galt and Hanson (1964) found that (-)-kaurene-17^{14}C was transformed by G. fujikuroi cultures into GA_3 without alternation of the position of the label. Phinney et al. (1966) demonstrated the conversion of kaurenoic acid into gibberellins in cultures of G. fujikuroi. Katasumi et al. (1964) showed that kaurenoic acid has gibberellin-like activity in dwarf-5 and anther-1 mutants of maize. The formation of the diterpenes (-)-kaurene, (-)-kaurene-19-ol from 2-^{14}C-mevalonate in cell-free homogenates of the endosperm of E. macrocarpa was described by Graebe et al. (1965). They also demonstrated that (-)-kaurene-19-ol serves as a precursor for the synthesis of gibberellic acid. Dennis and West (1967) showed that the oxidation of kaurene in endosperm occurs in the sequence kaurene to kaurenol to kaurenal to kaurenoic acid to more polar acid substances from kaurenoic acid, and proposed an hypothesis of gibberellin biosynthesis in E. macrocarpa endosperm.

Evidence has been obtained which suggests that several other microorganisms possess activities for the

synthesis of gibberellins (Katzenelson and Cole, 1965). Vacura (1961) and Burlingham (1964) detected gibberellin-like activity in old culture medium of Azotobacter chroococcum. Three gibberellin-like substances were separated on paper chromatography from culture media of A. chroococcum by Brown and Burlingham (1968). None were identified. Geranylgeranyl pyrophosphate, the precursor of kaurene, is synthesized in extracts of Micrococcus lysodeikticus (Kandutsh et al., 1964). Silver et al. (1963) demonstrated that the physiological response of the plant leaves of Psychotria bacteriophila to the presence of bacterial leaf nodules could be partially simulated by applying gibberellic acid to leaves devoid of nodules. This observation has suggested that the nodular bacterium, a Klebsiella species, possibly may be able to synthesize gibberellins or intermediates of gibberellin biosynthesis.

The present investigation was undertaken to gain further information about the activities in certain microorganisms for diterpene and gibberellin synthesis. Such information is of value for understanding the interaction of microorganisms with higher plants. In addition, these studies may reveal new biological tools for the study of gibberellin biosynthesis.

Several strains of the fungus Fusarium were assayed for their activities. Previous studies have demonstrated

that isolates of F. moniliforme from infected rice carry out gibberellin synthesis, but other isolates do not. It was of interest to determine what parts of the pathway to gibberellin compounds are generally present in species and strains of the genus Fusarium. Such finding might provide further insight into the evolution of the capacity for gibberellin synthesis.

The present studies deal with the measurement of activities in cell-free extracts of fungal and bacterial cells with respect to (a) diterpene synthesis from mevalonate and (b) cyclization of geranylgeranyl pyrophosphate to diterpene hydrocarbons. Activities of cells to produce gibberellins during culture growth and activities of washed cell suspensions to incorporate mevalonate into neutral compounds and gibberellin precursors have also been measured. The known gibberellin-producing strain, F. moniliforme Lily strain, was used in these studies as the positive control.

MATERIALS AND METHODS

Chemicals.

2-^{14}C-Mevalonate (DBEB salt) was purchased from New England Nuclear Corp. ^{14}C-Geranylgeranyl pyrophosphate and reference standard compounds, farnesol, kaurene, kaurenol, and gibberellic acid (GA_3), were obtained from the laboratory of Dr. C. A. West, Biochemistry Division, University of California, Los Angeles.

Silica gel F-254 (Merck) pre-coated thin layer plates were purchased from Brinkmann Instruments Inc. PRE MIX "M" (PPO 91 %, Dimethyl POPOP 9 %) was purchased from Packard Instrument Company, Inc.

Organisms.

F. moniliforme Lily strain M-119 obtained from the laboratory of Dr. C. A. West, Biochemistry Division, University of California, Los Angeles, was used as the control strain for this study.

The following strains representing five species of Fusarium were obtained through the courtesy of Mr. Rodney Caldwell of the laboratory of Dr. John Tuite, Department of Botany and Plant Pathology, Purdue University: F. moniliforme strains 1, 2, 6, and 8; F. tricinctum strain 10. F. oxysporum strains 2 and 8, F. roseum strains 22 and 46; F. solani strains 2 and 3. Two species of soil bacteria,

Azotobacter vinelandii and Arthrobacter globiformis, were obtained from Dr. W. S. Silver, Department of Life Sciences, Indiana State University.

Media.

Fungal stock cultures were grown and maintained on Potato-Dextrose Agar slants (PDA) prepared as follows: 200 g of peeled, sliced potatoes were boiled for 5 minutes in 500 ml water. The resultant infusion was filtered through cheesecloth. The filtrate was restored to a volume of 500 ml. To this infusion were added 100 ml of a solution containing dextrose (20 g), $CaCO_3$ (0.2 g), $M_gSO_4.7H_20$ (0.2 g) and 400 ml of a solution containing 15 g of agar.

Azotobacter vinelandii was grown in Burk's nitrogen-free medium as described by Wilson and Knight (1952). The medium contained sucrose (3.5 g), KH_2PO_4(0.41 g), K_2HPO_4 (0.52 g), $CaCl_2$(0.02 g) $MgCl_2.6H_20$(0.17 g), $Na_2MoO_4.2H_20$ (0.00025 g), $FeSO_4.7H_2O$(0.005 g) and Na_2SO_4(0.15 g) in 1 l. of distilled water.

Arthrobacter globiformis was grown in a Yeast extract medium as described by Lochhead and Burton (1955). This medium contained glucose (1.0 g), K_2HPO_4(1.0 g), KNO_3 (0.5 g), $MgSO_4.7H_20$(0.2 g), $CaCl_2$(0.1 g), NaCl(0.1 g), $FeCl_3.6H_20$(0.01 g) and yeast extract (1.0 g) in 1 l. of distilled water.

Mycelia were cultivated in a liquid medium containing sucrose (80 g), KH_2PO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NH_4NO_3 (1.0 g) and trace elements (2.5 ml) made up to a total volume of 1 l. with distilled water. The trace element solution contained per 100 ml: $FeSO_4 \cdot 7H_2O$ (0.10 g), $CuSO_4 \cdot 5H_2O$ (0.015 g), $MnSO_4 \cdot H_2O$ (0.01 g), $ZnSO_4 \cdot H_2O$ (0.16 g) and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.01 g). The sucrose and $MgSO_4$ were autoclaved separately from the bulk of the medium and were added aseptically after cooling.

Cultural conditions.

Two culture conditions were used for Fusarium. Shake cultures were grown either in 2 l. of medium in 2.8 l. Fernback flasks or in 250 ml of medium in 500 ml flasks at room temperature with shaking (New Brunswick Scientific Co., Model No. 6635). Submerged cultures were grown in 10 l. of medium in a 14 l. fermentor (Fermentation Design Inc.) with 400 RPM of agitation and 2 l/min of aeration at 25°C.

A. vinelandii and A. globiformis were grown in 250 ml of medium in 1 l. flasks at room temperature without shaking. All stock cultures, both fungi and bacteria, were stored at 4°C.

Preparation of inocula.

Fungal cells were first transferred from stock cultures to fresh PDA slants and were incubated at room temperature

for 2 days. Then, inocula were prepared by transferring material from well grown slants into 125 ml portions of sterile culture medium in 250 ml flasks and incubating for 48 hours with shaking at room temperature. A 1 % inoculum was used to initiate growth in fresh medium for individual experiments.

A. vinelandii and A. globiformis were grown in 8 ml of broth medium in 18 ml culture tube and incubated for 2 to 3 days at room temperature. A 1 % inoculum was used to initiate growth in fresh medium.

Collection of medium and cell samples.

The fungal cultures (the quantity depending upon the individual experiment) were collected by filtration on a Buchner funnel with the aid of a vacumn (water aspirator). Circles of 10 cm Whatman No. 1 paper were used. Culture filtrates to be assayed for gibberellins were stored at 4°C. Mycelial samples used for the preparation of cell-free extracts were washed with three portions (250-500 ml) of distilled water. Mycelial samples for preparation of cell suspensions were washed with three portions (250-500 ml) of 0.02 M potassium phosphate buffer, pH 6.9. The washed samples for preparation of cell-free extracts were scraped from the paper and frozen at -20°C immediately after collection or after lyophilization.

Bacterial cells were harvested by centrifugation at 15,000 g for 20 minutes, washed twice with 250 ml portions of 0.02 M Tris-chloride buffer, pH 7.2 and stored at -20°C.

Preparation of cell-free extracts.

Fungal extracts were prepared by disrupting the cells in a modified Hughes press (Raper and Hyatt, 1963). The washed mycelial samples were pressed dry with paper toweling and frozen in cylinders shaped to fit into the upper chamber of the press. Before disruption, the cylinders were chilled further in dry ice for 15 to 30 minutes. The press was also cooled in dry ice before use. After the frozen cells were put into the upper chamber, the press was placed on the platform of the Carver Laboratory Press Model "C". The plunger was inserted into the upper chamber above the frozen cells. Pressure up to 20,000-24,000 psi was applied until the material was completely extruded through the slots of the middle section into lower chamber. The disrupted cells were resuspended with portions of 0.02 M TES(N-tris(hydroxymethyl) methyl-2-amino ethane sulfonic acid) buffer adjusted with KOH to pH 7.2, and centifuged at 45,000 g for 30 min at 0°C to remove most of the cell debris and then at 110,000 g for 60 min at 0°C to remove microsomal material. The supernatant fractions were either assayed immediately to measure enzyme activities or stored

at -20°C under nitrogen until used for assays.

Bacterial extracts were prepared from cell suspensions by disruption of the cells with the aid of a Biosonik BP III sonic probe. Suspensions were prepared by mixing cells, glass beads (0.5 mm in diameter), and buffer (5 % sucrose in 0.1 M potassium phosphate buffer, pH 7.2 containing 0.01 M 2-mercaptoethanol) in the ratio of 1:2:2 (w/w/v). Usually the volumes were about 10 ml. After sonification for a 2 min period (using an ice bath for cooling) the suspensions were centrifuged at 45,000 g for 30 min at 0°C. The supernatant fractions were either assayed immediately or stored under nitrogen until use.

Endosperm extracts were prepared as described by Graebe et al. (1965) from frozen seeds of E. macrocarpa. Lyophilized extracts were redissolved in 0.01 M potassium phosphate, pH 7, containing 0.01 M 2-mercaptoethanol.

Preparation of mycelial suspensions for direct assay.

The washed mycelial samples were pressed dry with paper toweling. Ten g portions were transferred to 50 ml beakers and were suspended in 40 ml of 0.02 M potassium phosphate buffer, pH 6.9. The dry weight of the suspended material ranged from 0.025 to 0.030 mg/ml.

Protein determination.

The amount of protein in cell-free extracts was

estimated according to the method described by Lowry et al. (1951).

Gibberellin bioassay.

To determine gibberellin activities in culture extracts, the culture filtrates were adjusted to pH 2-3 with 2 N HCl. A 2.5 ml sample of each was transferred to a 12 ml heavy walled centrifuge tube and extracted successively with 2.5 ml, 2.0 ml and 2.0 ml portions of ethyl acetate. The pooled ethyl acetate extracts were extracted four times with 1.0 ml portions of 0.5 M $NaHCO_3$. The pooled $NaHCO_3$ extracts were adjusted to pH 2-3 by the addition of 0.15 ml of 2 N HCl and were extracted with 2.0 ml, 1.5 ml and 1.5 ml portions of ethyl acetate (total volume of 5 ml). The ethyl acetate solution was evaporated to dryness under nitrogen to remove the solvent. The remaining materials were dissolved in a solution of 0.1 % Tween 20 and 2 % ethyl alcohol in water. The products from the Lily strain were dissolved in 0.2 ml of the above solvent, but the extracted material from other strains was dissolved in only 0.08 ml.

Seeds of the dwarf-5 mutant of Zea mays used for bioassay of the gibberellin activity, were obtained from the Laboratory of B. O. Phinney, Department of Botany and Plant Biochemistry, University of California, Los Angeles.

The seeds were soaked 24 hours in tap-water and planted in vermiculite at 25°C. The vermiculite was liberally watered to insure uniformity of germination. Ten days after planting, dwarf seedlings were selected and were replanted in vermiculite. The dwarf plants were nourished with half-strength Hoagland's solution (Hoagland and Arnon, 1950).

Two seedlings were used for each treatment in the cases of extracts from Fusarium strains and GA_3 standards, except for the Lily strain for which one seedling per treatment was used. A 0.02 ml portion of extract or standard was applied to the "cup" formed by the emerging leaves at the top of the seedling. Following treatment, the plants and non-treated controls were allowed to grow in a greenhouse for two weeks. At the end of the period, the seedlings were removed from the vermiculite and the lengths of the 3rd, 4th and 5th leaf sheaths were measured.

Assays for enzymatic activities.

Mevalonic acid incorporation into kaurene was measured in a procedure similar to that described by Graebe et al. 1965; Upper and West, 1967). The standard incubation mixture contained 0.02 µmoles of 2-^{14}C-(D,L)-mevalonate (ca. 3µC/µmole), 1 µmoles of ATP, 1 µmoles $MgCl_2$ and 0.25-0.5 ml of enzyme extract in a final volume of 1.0 ml. Incubations were performed in a 30° C water

bath (without shaking) for the periods indicated in individual experiments. The reactions were stopped by adding 0.08 ml of 1 M KOH. The entire mixture was extracted with three 2-volume portions of benzene: acetone (3:1).

The conversion of ^{14}C-geranylgeranyl pyrophosphate into kaurene was measured in an incubation mixture containing 0.0005 µmoles of ^{14}C-geranylgeranyl pyrophosphate, 1 µmoles of $MgCl_2$, 10 µmoles of potassium phosphate buffer, pH7, and 0.10 ml of cell-free extract in a final volume of 0.5 ml. Incubations were performed in a 25-30°C water bath (without shaking) for the periods indicated in individual experiments. The reactions were stopped by the addition of 0.25 ml acetone. Then, the mixtures were extracted once with 1.0 ml of benzene and twice with 0.75 ml portions of benzene: acetone (3:1).

Mevalonate incorporation by mycelial suspensions was measured by mixing 9.0 ml of the suspension with 0.045 ml containing 1.3×10^6 cpm of 2-^{14}C-mevalonate (0.1134 µmoles) in a 50 ml beaker. The mixture was stirred 1 min with a magnetic stirrer and then was distributed as 2.0 ml portions in 4 scintillation vials. The remaining 1 ml of suspension was extracted immediately to serve as the zero-time control. The 4 vials were incubated at room temperature for 2, 4, 8 and 24 hours with shaking.

At the end of the incubation period, a 2 ml portion

of acetone containing HCl (0.01 M) was added to the incubation mixture. Then, the vials were directly placed in the centrifuge and centrifuged to separate the cells from the supernatant phase. The supernatant fractions were transferred to 12 ml heavy-walled centrifuge tubes. The mycelia were successively extracted twice with 2 ml portions of acid-acetone. The acid-acetone extracts were combined with the supernatant fraction and were evaporated under nitrogen to remove the acetone. Then these aqueous fractions were brought to pH 7-9 with 1 M KOH and were extracted 3 times with 2 ml portions of benzene: acetone (3:1). The aqueous phases were adjusted to pH 2-3 with 2 M HCl again and the acidic lipid components including gibberellins were extracted in the same fashion as for the direct assay for gibberellin activities in culture medium.

The total amount of radioactivity from 2-^{14}C-mevalonate or ^{14}C-geranylgeranyl pyrophosphate which was converted into extractable materials was determined by counting the radioactivity of an aliquot of the acetone, benzene-acetone, or ethyl acetate extract. The product composition was determined by thin layer chromatography of the remaining organic extract after the extract was concentrated to a small volume under nitrogen.

Thin layer chromatography systems.

The products from incubations with either ^{14}C-

mevalonate or ^{14}C-geranylgeranyl pyrophosphate as substrate were assayed by chromatography of the concentrated organic extracts on precoated thin layer plates of silica gel. The products and standards were dissolved in 0.05 to 0.10 ml of benzene or acetone and applied to plates. Diterpene products were separated by solvent system A (hexane) and system B (benzene: ethyl acetate (9:1)). The plates were developed first in hexane to 15 cm and then were redeveloped in same direction with benzene: ethyl acetate (9:1) to 12 cm as described by Graebe et al. (1965). Gibberellins were separated by solvent system C (di-isopropyl ether: acetic acid (95:5)). The plates were developed in diisopropyl ether: acetic acid (95:5) to 15 cm at room temperature (MacMillan and Sutter, 1963).

Representative Rf values of a number of diterpene reference compounds and gibberellic acid in the three systems are presented in Table 1. Reference standards in the system A and B were detected by exposing the plates to iodine vapors. Standards in system C were detected by spraying plates with ethanol: concentrated sulfuric acid (95:5) followed by heating at 100 to 120°C for 10 min. Spots were observed by examination of the plates under ultraviolet light.

After detection of the standards, the silica gel on the plates was scraped into vials either for every 0.5

Table 1. Mobilities of terpenes and gibberellins on thin layer plates of silica gel.

Compounds	Developing solvent		
	System A	System B	System C
	Rf	Rf	Rf
Kaurene	0.90	0.90	
Farnesol	0	0.35	
GerGer-ol	0	0.40	
Kaurenol	0	0.45	
Kaurenal	0	0.93a	
Kaurenoic acid	0	0.1-.2a	
GA_3			0.14
GA_4			0.37b
GA_1			0.37b

Solvent system: A) Hexane; B) Benzene: ethyl acetate (9:1); C) Di-isopropyl ether: acetic acid (95:5)

a. from Dennis and West (1966)

b. from MacMillan and Sutter (1963)

cm of length from the origin or in regions corresponding to the location of standards and other compounds.

Radioassay techniques.

Measurements of radioactivity were performed with a Beckman Model LS 250 liquid scintillation system. Samples dissolved in organic solvents or absorbed on silica gel from thin layer plates were counted in 5 ml of toluene scintillation fluid (5.5 g of Pre Mix "M" in 1 liter of toluene). Mycelial samples were counted in 5 ml of toluene scintillation fluid after dehydration by acid-acetone. Counting rates were corrected for background. Samples were counted for 10 min or until the error (95 % level of confidence) was less than 2 %.

RESULTS

Growth response of the dwarf-5 mutants of Zea mays to culture extracts of Fusarium species.

In order to detect the ability of several strains of Fusarium to synthesize gibberellins, cultures were sampled after 5 weeks of growth, extracted and applied to seedlings of the dwarf-5 mutant of Zea mays. Because the supply of dwarf-5 seeds was limited, only 2 plants were used per treatment in most cases. This number of plants was sufficient for detecting levels of gibberellins as low as 0.05 µg (Table 2 and Fig. 3), but the measurements obtained can only serve for a semiquantitative estimation of the amount of gibberellins applied. For the extracts tested, as little as 0.2 mg of GA_3 equivalents per liter would produce a significant growth response.

A photograph showing the relative sizes of the plants at the end of the assay period is presented in Fig. 3 and 4. The average of the lengths of the third, fourth and fifth leaf sheaths was used as a measure of the growth response of the plants to the material applied. The results are summarized in Table 2. The only strain which gave a strongly positive response was F. moniliforme Lily strain. This strain is a known producer of gibberellins and served as the positive control strain. The growth

Table 2. Gibberellin activity in extracts of 5 week fungal culture filtrates as measured by bioassay with the dwarf-5 mutant of Zea mays.

Species	amount per plant	average length (cm)[a]
Untreated control	none	3.65
GA_3 Standard	5.0 µg	9.6
"	0.5 µg	5.13
"	0.05 µg	5.3
	0.005 µg	2.45
F. moniliforme Lily strain	0.02 ml[b] extracts	9.31
F. solani strain 2	"	4.28
F. solani strain 3	"	3.83
F. oxysporum strain 2	"	3.80
F. oxysporum strain 8	"	3.70
F. moniliforme strain 1	"	3.83
F. moniliforme strain 2	"	2.93
F. moniliforme strain 6	"	3.0
F. moniliforme strain 8	"	2.83
F. roseum strain 46	"	3.3
F. roseum strain 22	"	3.43
F. tricinctum strain 10	"	4.40

a. The average length is based up on the 3rd, 4th, and 5th leaf sheaths of two treated plants.

b. The extracts from 2.5 ml of culture filtrates were dissolved in 0.08 ml of solvent with the exception of the Lily strain which was dissolved in 0.20 ml of solvent.

Figure 3. Growth response of dwarf-5 seedling of Zea mays to treatments with Gibberellic acid (GA_3). Seedling are shown at 15 days after treatment.

F. solani strain 2
F. solani strain 3
F. oxysporum strain 2
F. oxysporum strain 8
F. tricinctum strain 10
F. moniliforme strain 1
F. moniliforme strain 2
F. moniliforme strain 6
F. moniliforme strain 8
F. roseum strain 22
F. roseum strain 46
F. tricinctum strain 10

Figure 4. Growth response of dwarf-5 seedling of Zea mays treated with fungal extracts. Seedling at back row are the GA_3 and Fusarium moniliforme Lily strain controls with two normal seedlings at each end. Seedlings are shown at 15 days after treatment.

response with this strain was calculated as equivalent to 20 mg of GA_3 per liter of culture medium.

The observed responses to extracts from F. moniliforme strains 2, 6 and 8 and F. roseum strains 22 and 46 were not significantly different from untreated plants. A very slight response was obtained with extracts from F. tricinctum strain 10 and F. solani strain 2. However, the amounts of response were less than that obtained with 0.05 µg of GA_3.

Medium samples collected after various periods of growth by F. moniliforme Lily strain in a 10 liter fermentor were also tested. The results were summarized in Table 3. The appearance of the plants at the end of the assay period are shown in Fig. 5. The results demonstrated that gibberellins or gibberellin-like substances accumulated in the culture medium to a significant level as early as 24 hours after inoculation and increased with the culture age.

Detection of gibberellins after thin-layer chromatography of ethyl acetate extracts.

Since gibberellins can be detected on thin-layer chromatograms by a characteristic fluorescence obtained after spraying and heating, ethyl acetate extracts of various culture filtrates were applied to silica gel plates and developed.

Table 3. Influence of culture age upon the gibberellin activity in culture filtrates of *Fusarium moniliforme* Lily strain as measured with the *dwarf-5* mutant of *Zea mays*.

Samples	Amount per plant	Average length (cm)	No. of plants per treatment
Untreated control	none	3.65	2
GA_3 standard	5 µg	9.6	2
"	0.5 µg	5.13	2
"	0.05 µg	5.3	2
"	0.005 µg	2.45	2
24 hr culture age	0.02 ml[a]	4.41	1
30 hr "	extracts	5.01	1
38 hr "	"	4.54	1
44 hr "	"	6.65	1
54 hr "	"	6.68	1

a. The extracts from 2.5 ml of culture filtrates were dissolved in 0.20 ml of solvent.

Control —
GA_3, 5 ug —
GA_3, 0.05 ug —
24 hr. culture extracts —
30 hr. culture extracts —
38 hr. culture extracts —
44 hr. culture extracts —
51 hr. culture extracts —
5 week culture extracts —

Figure 5. Growth response of dwarf-5 seedlings of Zea mays treated with gibberellic acid and with culture extracts of the control strain Fusarium moniliforme Lily strain at various culture ages. Seedling are shown at 15 days after treatment.

Culture filtrates from 5 week cultures of the strains of Fusarium listed in Table 2 were examined. Only extracts from the Lily strain showed fluorescent spots. The purple and blue fluorescent spots had Rf values corresponding to GA_4-GA_7 and GA_3, respectively. Authentic reference standards of GA_3 and GA_4-GA_7 were chromatographed with the fungal extracts. The observed Rf values correspond to those reported by MacMillan et al. (1963). Extracts of culture filtrates from the other strains tested did not show any blue or purple fluorescent spots beyond the origin. This indicated the absence of gibberellins and structurally related compounds in the medium from these strains.

Influence of drying cells upon activity in extracts.

Enzymic reactions involved in gibberellin biosynthesis have been demonstrated in cell-free extracts of endosperm from immature seeds of E. macrocarpa Greene (Dennis and West, 1967) and in extracts of F. moniliforme Lily strain (M. O. Oster, personal communication). However, it has been difficult to obtain these enzyme activities in the cell-free extracts of the Lily strain with reproducibility. One proposal has been that the mycelia must be dried and rehydrated before disrupting the cells. Thus, a study was performed to detect a difference between extracts prepared from dry and wet cells. Samples of 24

hour and 31 hour cells of F. moniliforme Lily strain (from a liquid culture in a 2.8 liter flask incubated with shaking at room temperature) were filtered and washed twice with distilled water. Each cell sample was divided into two portions. One portion was dried by lyophilization and the other portion was frozen in a cylinder for disruption. The lyophilized cells were rehydrated with 0.02 M potassium phosphate buffer, pH 7.2, and frozen in cylinders for disruption. Cell-free extracts were prepared and the activity of the extracts for converting ^{14}C-geranylgeranyl pyrophosphate into kaurene was measured.

The protein content in the cell-free extracts was 9.0 mg/ml for both dried and wet mycelial extracts of the 24 hour sample, 8.85 mg/ml for the dried 31 hour mycelial extract, and 9.1 mg/ml for the wet 31 hour mycelial extract. Thus, the extracts were essentially equivalent in protein concentration. Drying seemed to have no effect upon the subsequent release of cell contents by disruption with the modified Hughes Press.

Examination of the products produced from geranylgeranyl pyrophosphate revealed no significant difference between dried and wet mycelial extracts. The amount of conversion of ^{14}C-geranylgeranyl pyrophosphate is shown in Table 4. Kaurene formation by 24 hour extracts prepared from dried cells was 466 cpm and 338 cpm from wet cells and

Table 4. Influence of lyophilization of samples from cultures of F. moniliforme Lily strain upon the activity in cell-free extracts toward geranylgeranyl pyrophosphate.

Treatment of cells used for extracts		^{14}C-Products (cpm)	
		Kaurene region	Alcohol region
24 hr age	lyophilized cells	466	1,140
	undried cells	338	1,620
31 hr age	lyophilized cells	2,260	1,180
	undried cells	2,480	1,360

by 31 hour extracts from dried cells was 2260 cpm and 2480 cpm from wet cells. Alcohol formation was 1140 cpm by extracts from dried 24 hour cells, 1620 cpm by extracts from wet 24 hour cells, 1180 cpm by extracts from dried 31 hour cells, and 1360 cpm by extracts from wet 31 hour cells.

These results seem to show that the lyophilized cells are not much different from wet cells in terms of the ability to retain enzymic activity for kaurene synthase during storage and preparation of extracts.

Effects of culture age on the enzymatic activity.

A submerged culture of the Lily strain was prepared to provide mycelial samples at various ages for assays with ^{14}C-geranylgeranyl pyrophosphate. The cell-free extracts were prepared from undried cells and were assumed to be equivalent in protein concentration because a constant weight of cells and volume of buffer was used for preparing the extracts. The conversion of ^{14}C-geranylgeranyl pyrophosphate to diterpene hydrocarbon products increased with the age of the cells as shown in Fig. 6. A biphasic curve was observed for both culture growth and activity for kaurene formation. The activity for kaurene formation leveled off during the period from 34 hour to 44 hour of culture age. After 44 hours, an increase in the activity for kaurene formation occurred which was almost the same as

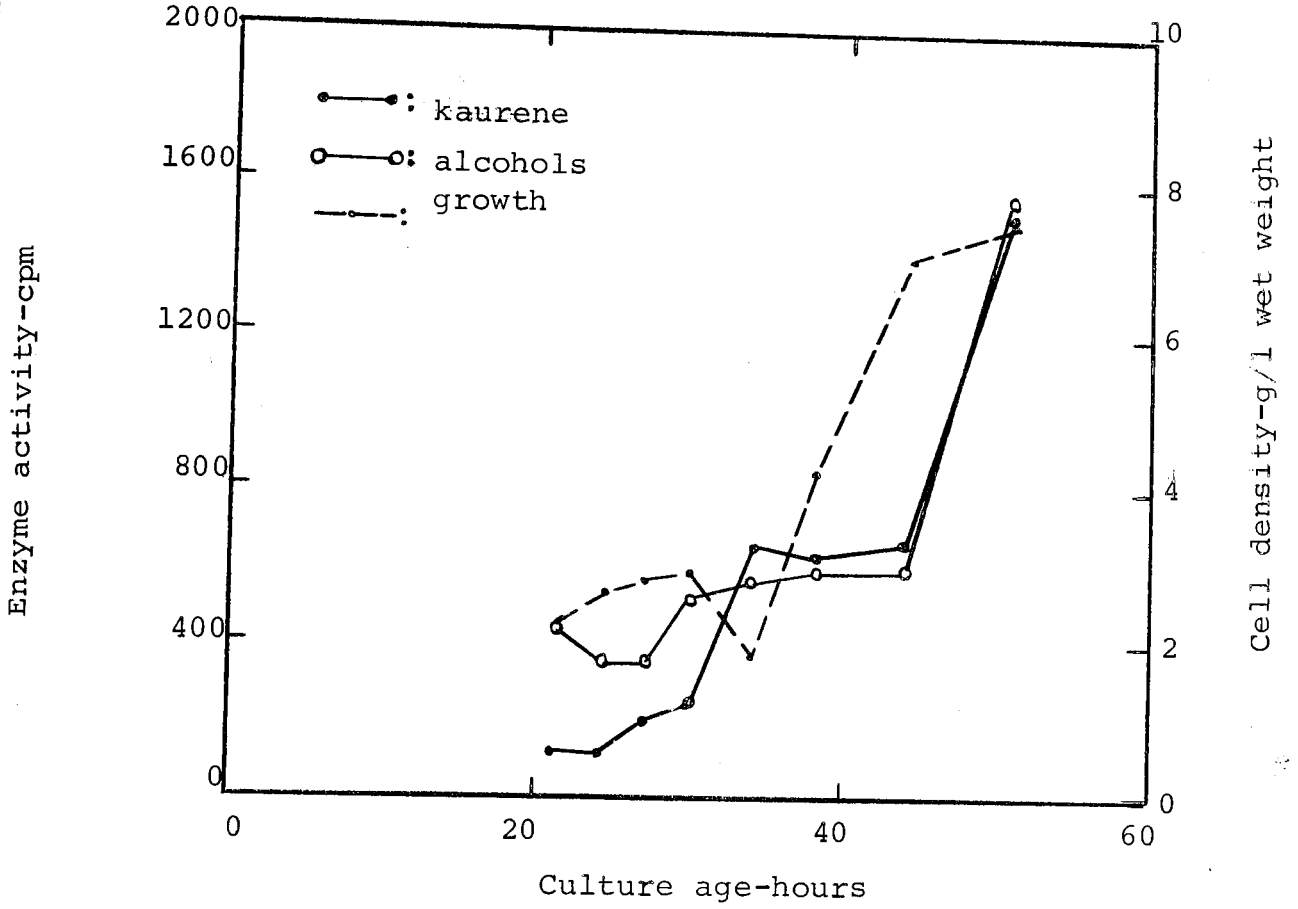


Figure 6. Effect of culture age on the enzymatic activities of cell-free extracts of F. moniliforme Lily strain. Enzymatic activity for the conversion of ^{14}C-geranylgeranyl pyrophosphate (spec. act., 13.4 Mc/umole) to kaurene and alcohol was measured in reaction mixtures containing 0.05 ml of extract (ca. 0.05 mg protein) in a final volume of 0.25 ml. The reaction time was 30 min. Other conditions were as described (see Materials and Methods).

for the period between 30 to 34 hours.

The growth curve suggests that the culture underwent lysis during the period between 30 to 34 hours of culture age (Fig. 6). After this period, the culture grew much faster than during the first phase of growth.

Studies of ^{14}C-mevalonic acid metabolism in cell-free extracts.

Attemps to measure the ability of cell-free extracts to incorporate ^{14}C-mevalonate into diterpene hydrocarbons were not very successful. A major difficulty seemed to be the preparation of active extracts from cells containing the activities of interest. As a control for the incubation system, extracts from the endosperm of immature seeds of E. macrocarpa Greene were incubated with 2-^{14}C-mevalonate. A high level of activity was observed. The conversion of ^{14}C-mevalonate by cell-free extracts of F. moniliforme Lily strain was also measured (Table 5). Kaurene formation by extracts from the Lily strain and wild cucumber alone were 76 cpm and 13,568 cpm, respectively. Upon combining extracts of the Lily strain and the wild cucumber, only 2,122 cpm of kaurene was formed. These results suggest that cell-free extracts of the Lily strain contain inhibitors which prevent kaurene synthesis.

Extracts were prepared from cells of other organisms

Table 5. Incorporation of ^{14}C-mevalonate by cell-free extracts of *F. moniliforme* Lily strain and wild cucumber.

Cell-free extracts		^{14}C-Products in kaurene region	Total lipid ^{14}C-Free products
Source	ml	cpm	cpm
F. moniliforme Lily strain 31 hr. cells	0.4	76	159
Wild cucumber	0.2	13,568	15,517
F. moniliforme Lily strain 31 hr. cells	0.2	2,122	2,557
Wild cucumber	0.2		

and were assayed with 2-^{14}C-mevalonate and with ^{14}C-geranylgeranyl pyrophosphate. The organisms used were F. tricinctum, F. moniliforme strain 1 and two species of bacteria, A. vinelandii and A. globiformis. The amount of radioactivity detected in the kaurene region was less than 10 cpm. In the alcohol region, more than 1000 cpm was present with all of the tested organisms. These results suggest that either the activity for kaurene synthesis was absent in these cell-free extracts or inhibitory materials were present which mask the activity (as seen with the Lily strain).

Incorporation studies with mycelial suspensions.

Since earlier results indicated that the amount of activity in cell-free extracts depended upon factors in the preparation of the extracts which could not be easily controlled, an assay system was developed for use with suspensions of the mycelia. To test this system for measuring incorporation of 2-^{14}C-mevalonate into free lipid compounds, a suspension of cells (harvested from a culture of the Lily strain after 12 hours of growth) was prepared and incubated with 1.3 x 10^6 cpm of 2-^{14}C-mevalonate in a total volume of 9 ml. Immediately upon mixing, a 1 ml aliquot was removed and extracted with benzene-acetone (3:1) to serve as the zero-time control. The remaining

mixture was incubated with shaking at room temperature. Samples were collected after 2, 4, 8, and 24 hours of incubation and were extracted in the same fashion as the zero-time control.

The amount of radioactivity obtained in the extracts increased with increased time of incubation. The observed cpm were 293, 1767, 3483, 4225, and 3839 for the 0, 2, 4, 8, and 24 hour samples, respectively. The benzene-acetone extracts were concentrated and applied to silica gel plates to separate the components. (Fig. 7 and 8).

The amount of radioactive products in the kaurenal region increased up to 8 hours and then decreased. After 24 hours, most of the radioactive materials were in the polar regions of the plate. These results follow the predicted sequence of incorporation of mevalonate into diterpene procursors of gibberellins. Thus, the assay system with mycelial suspensions appeared to be satisfactory for detecting the presence of activity for mevalonate incorporation into free lipids.

To further test the assay system and to establish the optimum period for harvesting cultures of the Lily strain (with respect to activity for diterpene synthesis) samples from a submerged culture were collected throughout the period of cell growth and were assayed for conversion of mevalonate to free-lipid products.

The conversion of ^{14}C-mevalonate to total

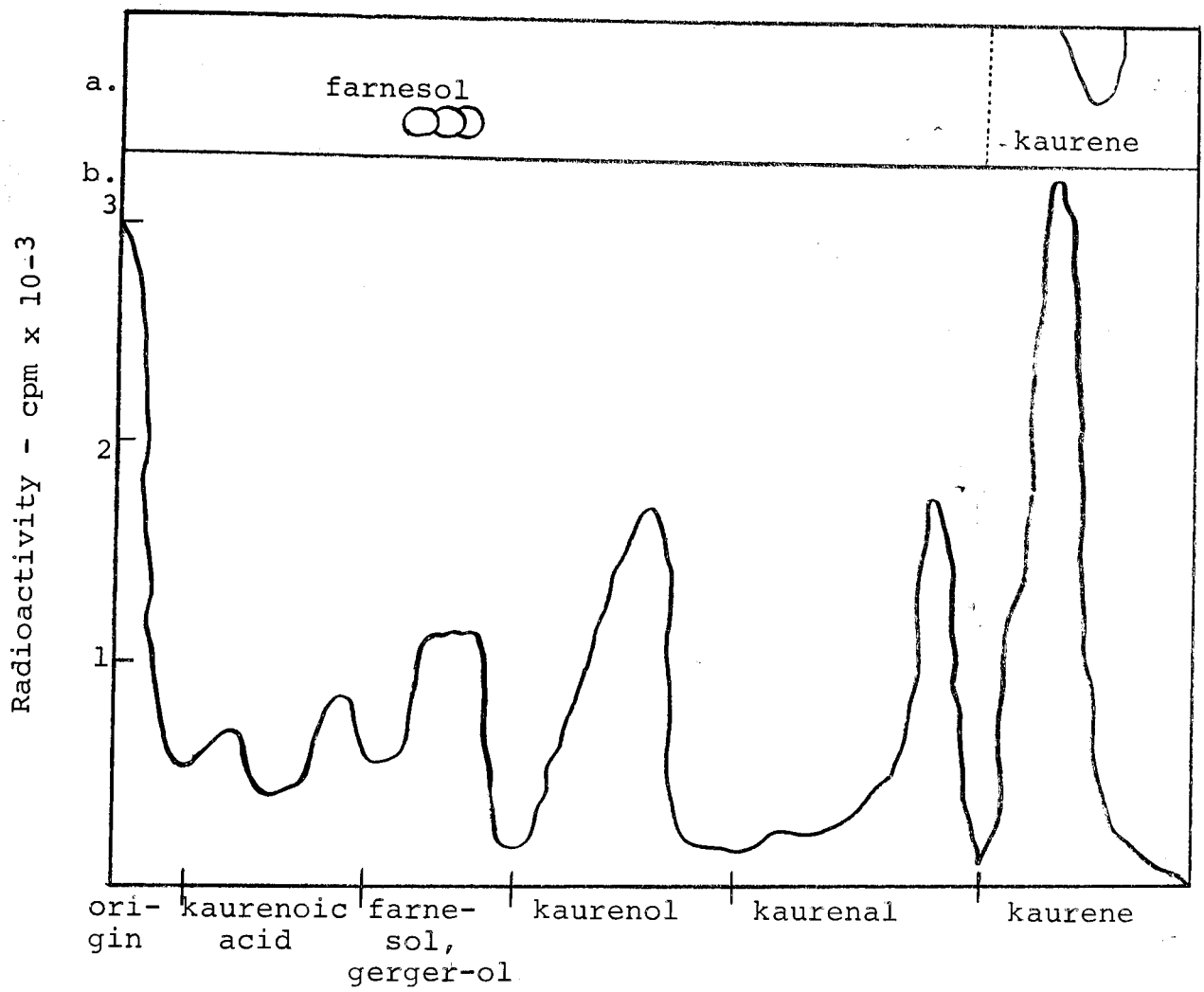


Figure 7. Resolution of ^{14}C- products from a reaction mixture with cells of F. moniliforme Lily strain. (a) thin layer chromatography plate, (b) distribution of radioactivity on plate. The plate was developed in solvent system A and then in solvent system B. Sections of the plate, 0.5 cm wide, were counted to measure radioactivity.

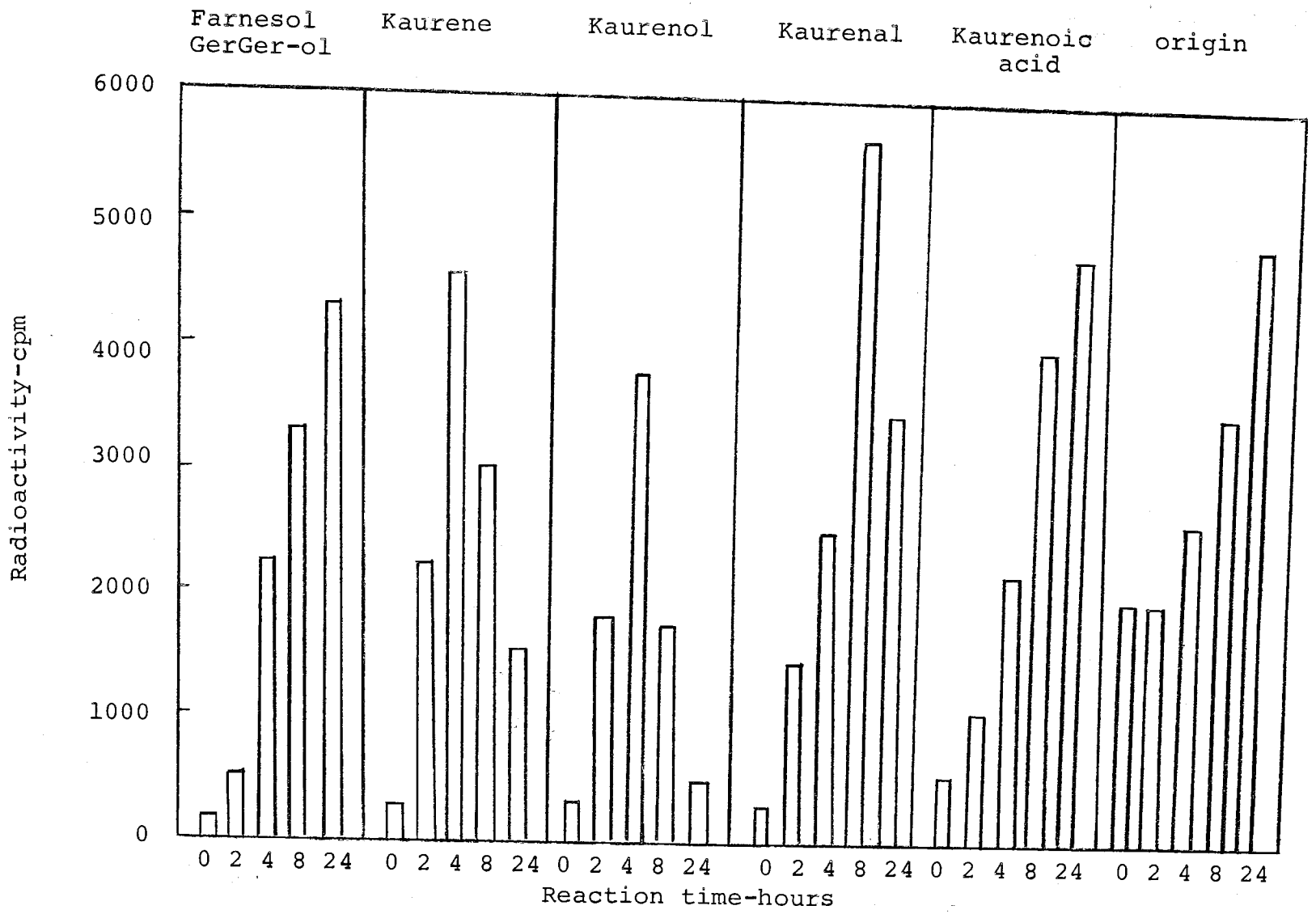


Figure 8. The sequence of formation of "diterpene" products by cells suspensions of F. moniliforme Lily strain.
Cells from a 12 hour culture were used. The radioactive products from 1 ml reaction mixtures were resolved by thin layer chromatography as shown in Fig. 7.

benzene-acetone extractable products and to kaurene is shown in Fig. 9. The cpm in total free lipid products and cpm in the kaurene region apparently changed in the same manner during the testing periods. With a 2 hour reaction time, the apparent activity for kaurene synthesis reached a peak at 72 hours of culture age and then decreased. With an 8 hour reaction time, the cpm in the kaurene region generally increased with culture age. The cpm in the total benzene-acetone extracts was initially high (at 12 hours), rapidly decreased until 24 hours of culture age, and then followed the same pattern as the cpm in the kaurene region. With a 24 hour reaction time, the distribution of radioactivity in both total benzene-acetone extracts and kaurene region in the culture sample up to 48 hours of age was similar to that observed with an 8 hour reaction time. Older culture samples (after 48 hours of age) were similar in activity to that observed with a 2 hour reaction time.

Incorporation of 2-^{14}C-mevalonate into acidic products.

The previous study suggested that a significant amount of conversion of kaurene to polar or non-extractable products (with respect to benzene-acetone) was occurring during the longer periods of incubation. To extract more polar and acid components, the reaction mixtures (after an initial extraction with benzene-acetone) were adjusted to

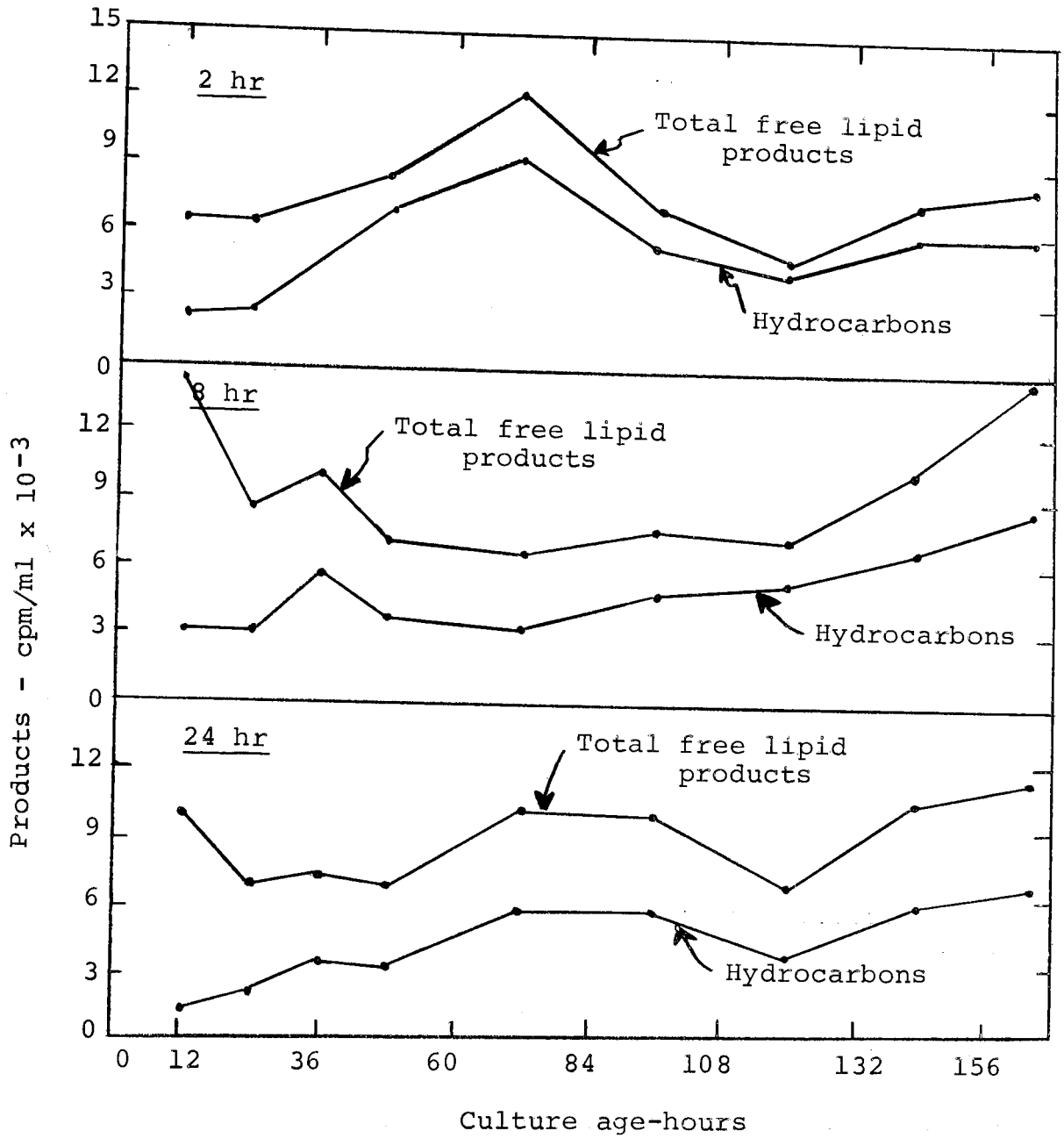


Figure 9. Influence of culture age upon the formation of diterpene hydrocarbons and total free lipids by washed mycelial suspensions of F. moniliforme Lily strain. The reaction mixtures were incubated for the times indicated. Hydrocarbons are those products which migrate to the kaurene region.

pH 2-3 and were extracted with ethyl acetate. This extraction procedure was expected to transfer gibberellins to the organic phase. The ethyl acetate extracts were concentrated, applied to thin-layer plates, and developed in the solvent system for separation of gibberellins.

The amount of radioactivity in the region for GA_4-GA_7, increased as a function of the time of incubation as shown in Fig. 10. The cpm in this region apparently was almost maximal after an 8 hour incubation period for all of the mycelial samples. With a 2 hour incubation time, the cpm in this region was 2,500 cpm for the 12 hour cells. It gradually decreased to 1,000 cpm for the 72 hour cells and gradually increased again to 2,600 cpm for cells collected at the end of culture period. With an 8 hour and 24 hour incubation period, the cpm in the GA_4-GA_7 region was 7,000 cpm for the 12 hour cells. The cpm was 1,600 cpm for the 72 hour cells and was 6,000 cpm for cells collected at the end of the culture period.

Incorporation of 2-^{14}C-mevalonate into products "bound" to the mycelia.

To determine whether the extraction procedure was effective for removing all of the radioactivity associated with the cells, the residual cell material was counted after extraction with acidified acetone.

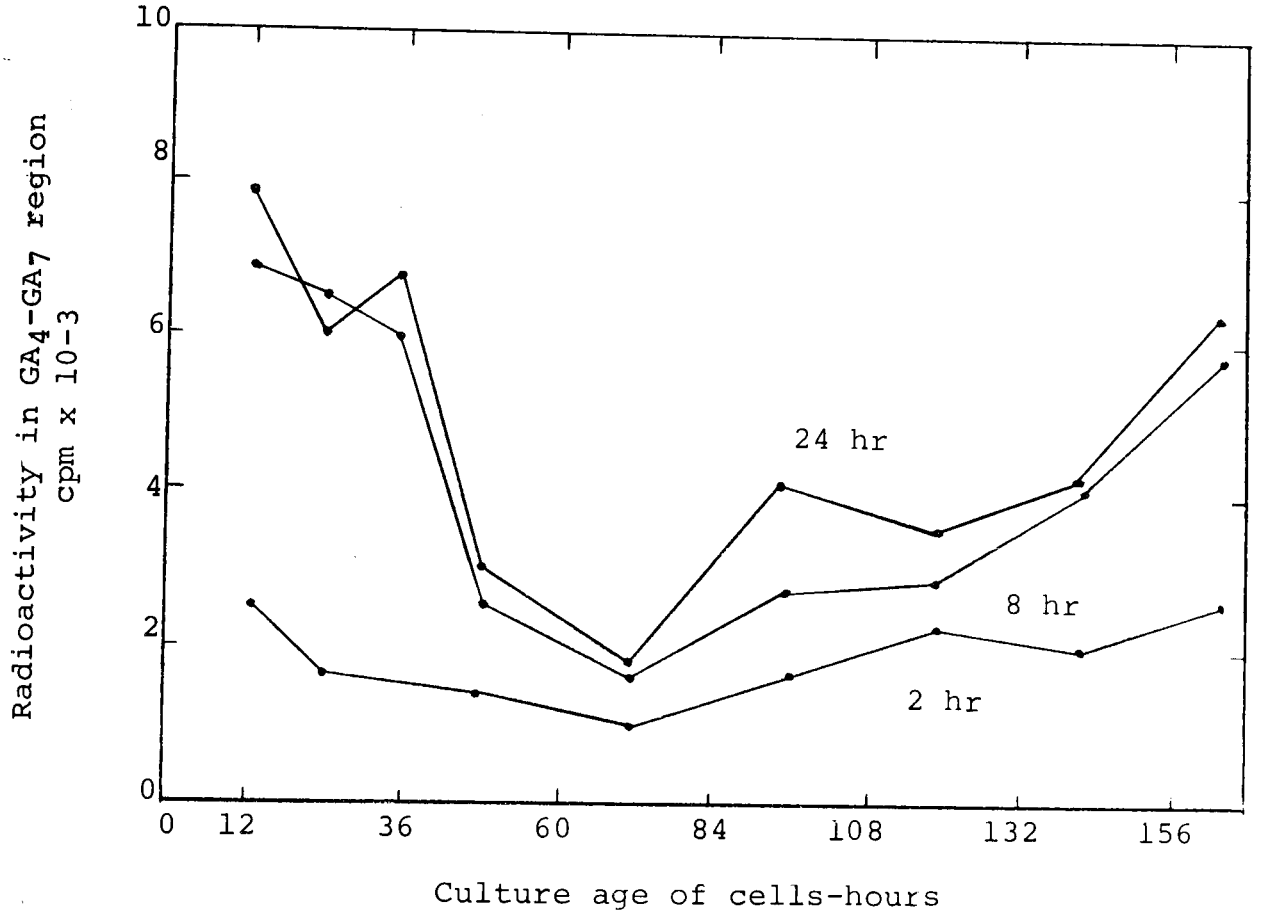


Figure 10. Formation of polar products from 2-^{14}C-mevalonate by mycelial suspensions of F. moniliforme Lily strain as a function of culture age of the cell samples. Radioactivity in the GA_4-GA_7 region on thin layer plates was determined from 1 ml reaction mixtures incubated for the times indicated.

The results are summarized in Fig. 11. It was found that radioactivity did remain in the cells. The amount of "bound" cpm increased during the first 8 hours of incubation and then usually decreased with further incubation. The nature of the "bound" products was not studied.

Survey of Fusarium strains for activity for diterpene synthesis from 2-^{14}C-mevalonate.

Samples from several strains of Fusarium were collected from 7 day cultures grown with shaking in 500 ml flasks at room temperature. The volume of medium in each flask was 250 ml. Suspensions were prepared and assayed according to the procedure developed in the initial studies with cell suspensions of F. moniliforme Lily strain. Following development, the thin-layer plates were divided into regions as shown in Fig. 7 and the gel was scraped into vials for the determination of the radioactivity in each region. The total amount of benzene-acetone extractable products and the distribution of products in regions on the thin layer plates is presented in Table 6.

Each of the strains tested converted mevalonate into several products. The distributions of radioactive products on thin-layer plates are summarized in Table 6. These products were not characterized further. However, it can be seen that the bulk of the radioactivity migrated to

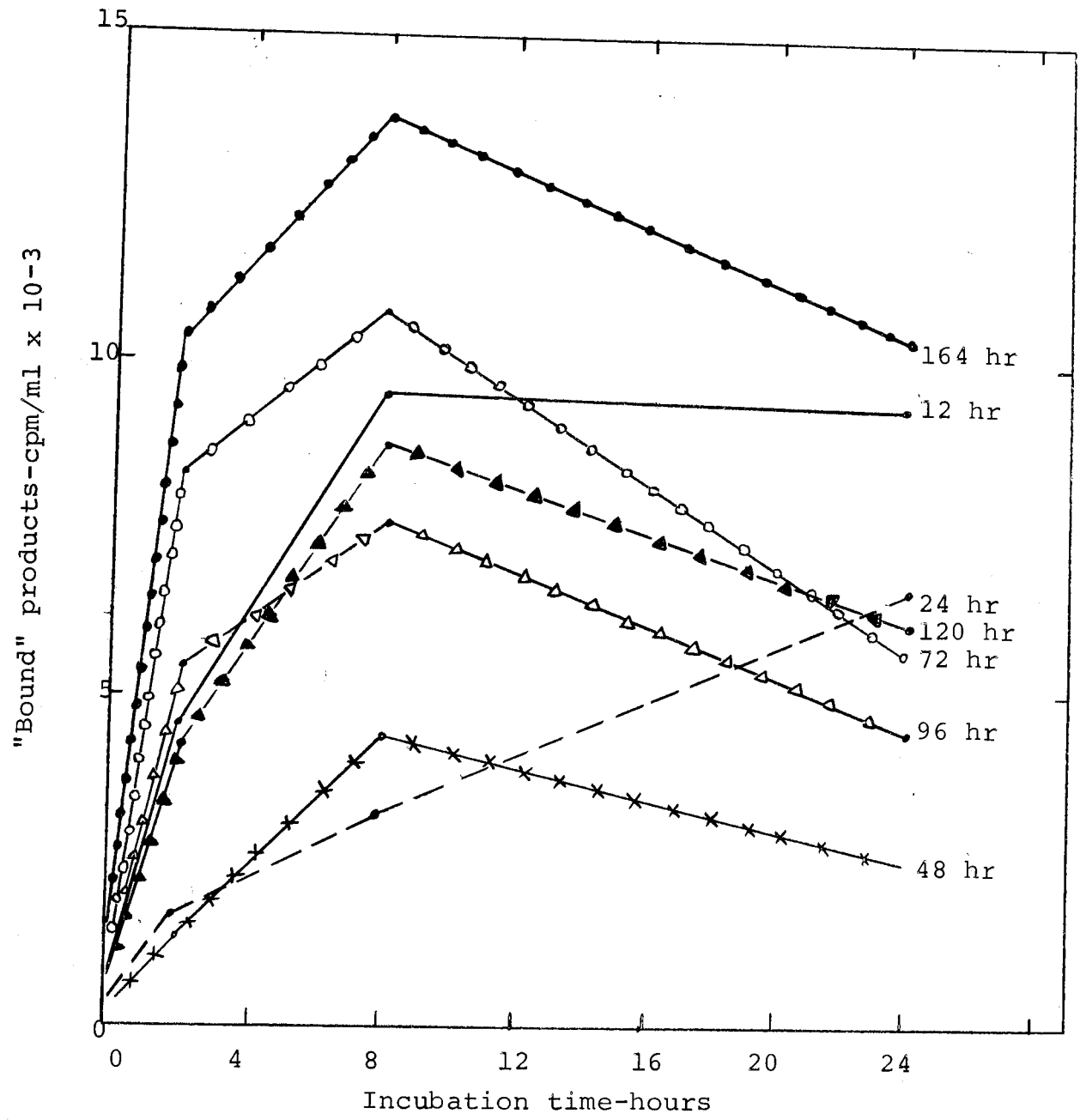


Figure 11. Incorporation of 2-^{14}C-mevalonate into products "bound" to cells of Fusarium moniliforme Lily strain as a function of the culture age.

Table 6. Distribution of products from 2-^{14}C-mevalonate synthesized by mycelial suspensions of Fusarium species. Mycelial suspensions (2 ml) were incubated with 2-^{14}C-mevalonate (2.9×10^5 cpm) for 24 hours at room temperature with shaking.

Organism	Total ^{14}C-products per ml	Regions on thin-layer plates (Solvent system A, then B)					
		Origin region	Kaurenoic acid	Farnesol GerGer-ol	Kaurenol	Kaurenal	Kaurene
F. moniliforme Lily strain	cpm 5,377	cpm 930	cpm 572	cpm 691	cpm 364	cpm 752	cpm 2,065
F. roseum strain 22	22,383	7,986	8,055	1,531	2,399	792	1,613
F. oxysporum strain 8	2,606	822	307	297	276	756	146
F. moniliforme strain 1	4,509	1,815	898	757	623	387	30
F. moniliforme strain 2	1,295	900	152	94	87	48	13
F. moniliforme strain 6	4,788	1,778	1,171	788	537	478	33
F. moniliforme strain 8	2,434	1,088	603	194	304	243	5
F. moniliforme strain 10	12,825	6,280	4,013	1,535	669	282	41
F. oxysporum strain 2	1,593	884	465	85	56	96	6
F. solani strain 2	7,294	2,080	4,011	972	114	114	4

regions of the plates which are characteristic of more polar compounds such as acids and alcohols. The amounts of radioactive products produced by F. roseum strain 22 and F. tricinctum strain 10 were 3 to 4 times greater than by the Lily strain.

The presense of radioactive products in the kaurene region is regarded as significant. The results are retabulated in Table 7 to permit a closer examination of the conversion of mevalonate into hydrocarbon compounds. Three strains produced significant amounts of radioactive products which migrated to the diterpene hydrocarbon region. The observed cpm were 146, 1613 and 2065 for F. oxysporum strain 8, F. roseum strain 22 and F. moniliforme Lily strain, respectively. The other species tested produced no significant amounts of diterpene hydrocarbon products.

Table 7. Diterpene hydrocarbon formation from 2-^{14}C mevalonate by Fusarium species. Conditions are described in Table 6.

Organisms	Kaurene region (cpm)
F. moniliforme Lily strain	2065
F. roseum strain 22	1613
F. oxysporum strain 8	146
F. moniliforme strain 1	30
F. moniliforme strain 2	13
F. moniliforme strain 6	33
F. moniliforme strain 8	5
F. tricinctum strain 10	41
F. oxysporum strain 2	6
F. solani strain 2	4

DISCUSSION

The bioassay for gibberellins with dwarf mutants of Zea mays is one of the most specific, sensitive quantitative methods available. In the present study, the dwarf-5 mutant was used to measure the activity present in culture filtrates of fungal cultures. The application of as little 0.05 µg of GA_3 to the dwarf seedling produced a measureable response in this study. By using a sufficient number of plants (10 or more), quantities less than 0.01 µg can be detected (Phinney and West, 1961), however, only 2 plants per treatment were used in the present study.

The lowest level of gibberellin activity that was detected in extracts from the culture filtrates of the known producer, F. moniliforme Lily strain, was equivalent to a concentration in the medium of 0.2 mg per liter. This value was obtained for a culture which had been incubated only 24 hours after inoculation. A five-week culture was found to contain 20 mg per liter as GA_3 equivalents. These values are similar to those reported by others.

The amounts of gibberellins produced vary according to the medium used, the cultural conditions, and the age of the cells. Kitamura et al. (1953) obtained 8 mg per liter in cultures grown in shake flasks. Stodola et al. (1955) obtained 20 mg per liter in aerated cultures after growth

had ceased. Borrow et al. (1955) reported 40 mg per liter for surface cultures and 200 mg per liter for an 18 day-old submerged culture. The patent application by Imperial Chemical Industries reports yields as high as 544 mg per liter in stirred cultures (Stowe and Yamaki, 1957).

In Fig. 6 the accumulation of gibberellins follows the growth of the culture. Previous studies have shown that gibberellin production is at its maximum after the cultures have exhausted the nitrogen source from the medium and carbon source remains (Stowe and Yamaki, 1957). Brown et al. (1964) found that the amount of gibberellic acid present in the culture medium increased linearly with time and that the maximum amount which accumulated was proportional to the initial level of nitrogen source provided. Thus, the results in Fig. 6 correspond to those of previous workers and demonstrate that gibberellins can be detected in culture filtrates during the later period of growth.

This finding is important for evaluating the negative results obtained in the dwarf-5 assay with culture filtrates from other strains of Fusarium. All strains were grown under the same conditions and assayed identically. No detectable response was observed with extracts from culture filtrates of strains 2, 6, and 8 of F. moniliforme. Although these strains belong to the same genus as the Lily strain, they are isolates from corn rather than from

infected rice. Similar results have been observed with other isolates of this species. Only those strains from infected rice have been found to produce gibberellins (Stowe and Yamaki, 1957).

Extracts from culture filtrates of strains 22 and 46 of F. roseum also were without effect upon elongation of leaf sheaths of dwarf-5 seedlings. A very slight growth response was observed for extracts from F. tricinctum strain 10 and F. solanii strain 2. The significance of these effects is difficult to assess because too few plants were used per treatment to obtain statistical significance of such small responses.

Another method for detecting gibberellins involves their separation on thin-layer plates of silica gel followed by spraying and heating. Although this procedure is less specific for gibberellins than bioassays, it is not subject to interference by anti-gibberellins which can produce a false negative response in the dwarf-5 assay system. A characteristic fluorescence is produced by compounds possessing the gibbane ring system. The fluorescence observed under ultraviolet light after spraying with ethanolic sulfuric acid permits the detection of as little as 0.00025 µg of GA_3 and 0.01 for GA_1, GA_2 and GA_4 to GA_9 (MacMillan and Sutter, 1963). Gibberellins A_1, A_3, A_5, A_6, and A_8 which contain a 7-hydroxyl group give blue

fluorescent spots of differing intensities. Gibberellins A_2, A_4, A_7, and A_9 which lack an hydroxyl group in the 7-position give purple spots.

On the basis of migration on thin-layer plates and the color of the spots, extracts of the Lily strain were found to contain material corresponding to gibberellins A_3 and A_4-A_7. This finding confirms the results from the bioassay. Extracts from culture filtrates of other strains did not produce visible fluorescent spots after chromatography, thus also confirming results obtained with the bioassay.

The conversion of geranylgeranyl pyrophosphate to kaurene is a key reaction in the pathway to gibberellins. This activity has been detected in cell-free extracts of E. macrocarpa Greene (Upper and West, 1967), Ricinus communis L. (West et al. 1968), and F. moniliforme (M. Oster, personal communication). The soluble fraction obtained after centrifugation contains the activities. In the present study, the specificity of this system was used in an effort to detect similar activities in other strains of Fusarium and bacterial species. Initial studies with the Lily strain indicated that the activity in extracts could not be obtained reproducibly. The difficulty was partially over come in later studies by centrifuging extracts at 105,000 x g to remove the bulk of phosphatase activity.

The influence of culture age upon the activity for kaurene formation by cell-free extracts of the Lily strain was measured and found to correspond roughly to the accumulation of gibberellins in the culture medium. Some activity for kaurene synthesis was present in all cell samples of the Lily strain examined; therefore the age of the cells did not seem to be critical for detecting activity.

A biphasic increase of activity as a function of culture age was obtained (Fig. 6). A similar biphasic growth curve was observed. This result suggests that the activity for kaurene synthesis is associated with "mature" cells. It is uncertain whether the activity detected during the first phase was synthesized de novo or whether it was present in the cells used as inoculum. Since the inoculum size was only 1%, it is likely that the activity represents new enzyme synthesis during the early period of growth. The second phase of growth may have arisen from germinating conidia produced during the first phase of growth. The decrease in cell concentration during the transition from one phase to another suggests a period of lysis. The rapid increase in activity after a lag is consistent with the development of this activity by mature hyphae. Similar phenomena have been described in a number of fungi (Yanagita, 1966).

The phosphatase activity of the extracts also

followed a biphasic curve during culture growth. Since phosphatases are generally less easily inactivated during extract preparation, the pattern obtained for kaurene synthesis seems to be a valid indication of the activity present in the cells.

No conversion of geranylgeranyl pyrophosphate was obtained with extracts prepared from bacterial cells (A. vinelandii and A. globiformis) nor from cells of F. moniliforme strain 1 and F. tricintum. However, phosphatase activity was present in the extracts. Thus, some enzyme activity was released from the cells by disruption. The absence of kaurene forming activity in extracts is not always conclusive evidence of an absence in the cells. Extracts of pine seedling have been shown to be inactive for terpene synthesis from mevalonate in the crude form and to be active after fractionation with ammonium sulfate (Beytia et al., 1969).

Measurement of the conversion of mevalonate into free lipid compounds revealed that F. moniliforme strain 1 and F. tricintum strain 10 possess some activities for terpene synthesis. Alcohols and more polar compounds were formed. The nature of these materials was not studied. The activity in extracts from the Lily strain was low compared to an extract from E. macrocarpa. Furthermore, the fungal extract was inhibitory to the plant extract.

This observation suggests that the presence of inhibitors in fungal extracts may obscure activities for diterpene and gibberellin synthesis which are present in an active form within organized cells.

Since the results of incorporation of mevalonate in cell-free extracts indicated that the amount of activity measured in cell-free extracts depended on factors in the preparation of extracts which could not be easily controlled, an assay system was developed for use with suspensions of mycelia to avoid problems associated with cell extracts (Birch et al., 1959). The incorporation of mevalonate into diterpene precursors of gibberellins (Fig. 8) corresponded to the synthesis of diterpenes in the sequence kaurene, kaurenol, kaurenal, kaurenoic acid. The highest concentrations of kaurene, kaurenol, kaurenal and kaurenoic acid were observed at 4 hour, 4 hours, 8 hour and 24 hour of reaction time, respectively. Dennis and West (1967) proposed a sequence of oxidation of kaurene to gibberellins on the basis of the metabolism of kaurene in endosperm which occurred in the sequence kaurene to kaurenol to kaurenal to kaurenoic acid. Also, they found that the rate of kaurenol formation was more rapid than the later steps. Although the products synthesized from mevalonate by the Lily strain were not identified, their chromatographic behavior and sequence of formation are consistent with the

results obtained by Dennis and West (1967).

An unexpected finding was that radioactivity from the 2-^{14}C-mevalonate remained in the cells after extraction with acidified acetone. None of the known intermediates beyond isopentenyl pyrophosphate would be expected to remain with the cells. The amount of "bound" products increased up to 8 hours of reaction time and then decreased. This suggests that the "bound" material may serve as precursor for the synthesis of free lipid products. One possibility for the identity of this material is that it includes the intermediates between mevalonate and dimethylallyl pyrophosphate, namely, mevalonate phosphate, mevalonate pyrophosphate, and isopentenyl pyrophosphate. In this case, the evidence would suggest that the enzyme for isomerization of the five-carbon intermediates is rate limiting. The accumulation of bound products in fungal cells may provide a useful measure for certain activities of terpene metabolism.

In the survey of Fusarium strains for incorporation of 2-^{14}C-mevalonate, it was found that all of the strains examined incorporated 2-^{14}C-mevalonate into free lipid products. The total activity of incorporation of 2-^{14}C-mevalonate was not restricted to activities for gibberellins biosynthesis. Kaurene, an important intermediate in gibberellin biosynthesis, is considered in this study to be

an indicator compound for the pathway. Since diterpene hydrocarbons are the only compounds which have been previously found to be both formed from mevalonate and to migrate to the kaurene region on thin layer plates, the presence of radioactivity in this region can be regarded as presumptive evidence for diterpene biosynthetic activity.

On this basis, it concluded that F. roseum strain 22 and F. oxysoprum strain 8 possess the activity for incorporating mevalonate into diterpene hydrocarbons. The other strains tested did not produce significant amounts of hydrocarbons. Studies of life cycles in Fusarium species (Toussoun, 1966) have shown that the perfect stage of F. moniliforme and F. roseum is Gibberella. No perfect stage is known for F. oxysporum and F. tricinctum. Although only a few species have been examined, the present studies indicate that those with the perfect stage of Gibberella are potential diterpene hydrocarbon producers. Further studies will be necessary before a relationship between diterpene synthesis and classification within the genus Fusarium can be made.

LITERATURE CITED

Beytia, E., P. Valenzuela and O. Cori. 1969. Terpene biosynthesis: Formation of nerol, geraniol, and other prenols by an enzyme system from Pinus radiata seedlings. Arch. Biochem. Biophys. 129, 346-356.

Birth, A. J., R. W. Richards, H. Smith, A. Harris and W. B. Whalley. 1959. Studies in relation to biosynthesis-XXI, Rosenonolactone and gibberellic acid. Tetrahedron 7, 241-251.

Borrow, A., S. Brown, E. G. Jefferys, R. H. J. Kessel, E. C. Lloyd, P. B. Lloyd, A. Rothwell, B. Rothwell and J. C. Swait. 1964. The kinetics of metabolism of G. fujikuroi in stirred cultures. Can. J. Microbiol. 10, 407-444.

Brown, M. E., and S. K. Burlingham. 1968. Production of plant growth substances by Azotobacter chroococcum. J. Gen. Microbiol. 53, 135-44.

Cavell, B. D., J. MacMillan, R. J. Pryce and A. C. Sheppard. 1967. Plant hormones-V. Thin-layer and gas-liquid chromatography of the gibberellins, Direct identification of the gibberellins in a crude plant extract by gas-liquid chromatography. Phytochem. 6, 867-874.

Cross, B. E., J. F. Grove, J. MacMillan and T. P. C. Mulholland. 1956. Gibberellic acid. Part IV. The structures of gibberic and allogibberic acids and possible structures for gibberellic acid. Chem. and Ind. (London) 1956, 954-955.

Cross, B. E., R. H. B. Galt, J. R. Hanson and W. Klyne. 1962. Some new metabolites of Gibberella fujikuroi and the stereochemistry of (-)-kaurene. Tetrahedron Letters 1962, 145-150.

Cross, B. E., R. H. B. Galt, J. R. Hanson, P. J. Curtis, J. F. Grove and A. Morrison. 1963. New metabolites of Gibberella fujikuroi Part II. The isolation of fourteen new metabolites. J. Chem. Soc. 1963, 2937-2943.

Cross, B. E., R. H. B. Galt and J. R. Hanson. 1964. The biosynthesis of gibberellins. Part I. (-)-Kaurene as a precursor of gibberellic acid. J. Chem. Soc. 1964, 295-300.

Cross, B. E., R. H. B. Galt and K. Norton. 1968. The biosynthesis gibberellins-II. Tetrahedron 24 231-238.

Cross, B. E. and P. L. Myers. 1969. The effect of plant growth retardants on the biosynthesis of diterpenes by Gibberella fujikuroi. Phytochem. 8, 79-83.

Dennis, D. T., and C. A. West. 1967. Biosynthesis of gibberellins. III. The conversion of (-)-kaurene to (-)-kauren-19-oic acid in endosperm of Echinocystis macrocarpa Greene. J. Biol. Chem. 242, 3293-3300.

Galt, R. H. B. 1968. New Metabolites of G. fujikuroi-XIV. Gibberellin A_{16} methyl ester. Tetrahedron 24, 1337-1340.

Geissman, T. A., A. J. Verbiscar, B. O. Phinney and G. Cragg. 1966. Studies on the biosynthesis of gibberellins from (-)-kaurenoic acid in cultures of Gibberella fujikuroi. Phytochem. 5, 933-947.

Graebe, J. E., D. T. Dennis, C. D. Upper and C. A. West. 1965. Biosynthesis of gibberellins. I. The biosynthesis of (-)-kaurene, (-)-kauren-19-ol and trans-geranylgeraniol in endosperm-nucellus of Echinocystis macrocarpa Greene. J. Biol. Chem. 240, 1847-1854.

Hanson, J. R. 1967. The chemistry of the tetracyclic diterpenoids VI. The stereochemistry of some reactions of (-)-kaurene from Gibberella fujikuroi. Tetrahedron 23, 801-804.

Harrison, D. M., J. MacMillan and R. H. B. Galt. 1968. GA_{24}, An aldehydic gibberellin from G. fujikuroi. Tetrahedron Letters 27, 3137-3139.

Henning, U., E. M. Moslein, and F. Lynen. 1959. Biosynthesis of terpenes V. Formation of 5-pyrophospho-mevalonic acid by phospho-mevalonic kinase. Arch. Biochem. Biophys. 83, 259-267.

Hoagland, D. R., and D. I. Arnon, Revised by D. I. Arnon. 1950. The water culture method for growing plants without soil. California Agricultural Experiment Station Circular 347.

Jackson, R. M., M. E. Brown and S. K. Burlingham. 1964. Similar effects on tomato plants of Azotobacter inoculation and application of gibberellins. Nature, London 203, 851.

Katsumi, M., B. O. Phinney, P. R. Jefferies and C. A. Henrick. 1964. Growth responses of the d-5 and an-1 mutants of maize to some kaurene derivatives. Science 144, 849-850.

Kandutsh, A. A., H. Paulus, E. Levin and K. Bloch. 1964. Purification of geranylgeranyl pyrophosphate synthetase from Micrococcus lysodeikticus. J. Biol. Chem. 239, 2507-2515.

Katznelson, H., and S. H. Cole. 1965. Production of gibberellin-like substances by bacteria and actinomycetes. Can. J. Microbiol. 11, 733-741.

Lochhead, A. G., and M. O. Burton. 1955. Qualitative studies of soil microorganisms. XII. Characteristics of vitamin-B_{12}-requiring bacteria. Can. J. Microbiol. 1, 319-330.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

MacMillan, J., and N. Takahashi. 1968. Proposed procedure for the allocation of trivial names to the gibberellin. Nature 217, 170-171.

Neely, P. M. 1959. The development and use of a bioassay for gibberellins. Doctoral Dissertation, University of California, Los Angeles.

Oster, M. O., and C. A. West. 1968. Biosynthesis of trans-geranylgeranyl pyrophosphate in endosperm of Echinocystis macrocarpa. Arch. Biochem. Biophys. 127, 112-123.

Paleg, L. G. 1965. Physiological effects of gibberellins. Ann. Rev. Plant Physiol. 16, 291-322.

Phinney, B. O., P. R. Jefferies, M. Katsumi and C. A. Henrick. 1964. The biological activity of kaurene and related compounds. Plant Physiol. 39, (suppl), xxvii.

Phinney, B. O., and C. A. West. 1961. Gibberellins and plant growth. in Encyclopedia of Plant Physiology. W. Ruhland. ed., Springer, Berlin, Vol. XIV, 1188-91.

Raper, J. R. M., and E. A. Hyatt. 1962. Modified press for disruption of microorganism. J. Bacteriol. 85, 712-713.

Silver, W. S., Y. M. Centifanto and D. J. D. Nicholas. 1963. Nitrogen fixation by the leaf-nodule endophyte of Psychotria bacteriophila. Nature 199, 396-397.

Stowe, B. B., and Y. T. Yamaki. 1959. The early phase of gibberellin research. Science 129, 807.

Stowe, B. B., and T. Yamaki. 1957. The history and physiological action of the gibberellins. Ann. Rev. Plant Physiol. 8, 181-216.

Toussoun T. A., and P. E. Nelson. 1968. A pictorial guide to the identification of Fusarium species. The Pennsylvania State University Press, University Park and London.

Upper, C. D., and C. A. West. 1967. Biosynthesis of gibberellins. II. Enzymic cyclization of geranylgeranyl pyrophosphate to kaurene. J. Biol. Chem. 242, 3285-3292.

Vancura, V. 1961. Detection of gibberellic acid in Azotobacter cultures. Nature, London 192, 88.

Verbiscar, A. J., G. Cragg, T. A. Geissman and B. O. Phinney. 1967. Studies on the biosynthesis of gibberellins--II. The biosynthesis of gibberellins from (-)-kaurenol and the conversion of gibberellins ^{14}C-GA_4 and ^{14}C-GA_7 into ^{14}C-GA_3 by Gibberella fujikuroi. Phytochem. 6, 807-814.

West, C. A., M. O. Oster, D. Robinson, F. Lew, and P. Murphy. 1968. Biosynthesis of gibberellin precursors and related diterpenes. In "Biochemistry and Physiology and Plant Growth substances" Edited by F. Wrightman and G. Setterfield. Runge Press. Ltd., Ottawa.

Wilson, P. W., and S. G. Knight. 1952. Experiments in Bacterial Physiology, 3rd ed., Burgess Publishing Co., Minneapolis.

Yanagita, T. 1966. Dynamic aspects of fungal growth with special reference to enzyme formation. J. Ferm. Technol. 44, 313-320.