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Experiments were performed to determine whether a direct correlation exists between the growth rates of tall and dwarf peas grown either in the light or in the dark and endogenous gibberellin (GA) content. Three separate but related experimental approaches were utilized: (1) the determination of possible qualitative and/or quantitative differences in the GA's present in etiolated and light-grown tall and dwarf peas through extraction and bioassay techniques; (2) the detemination of possible variations in sensitivity of tall and dwarf peas to Amo-1618, a known inhibitor of GA biosynthesis; and (3) the development of a cell-free system from peas capable of synthesizing one or more GA's or tetracyclic intermediates in the pathway of GA biosynthesis. The results of the GA extraction experiments were somewhat variable, but they indicated that etiolated tall peas contained more presumptive GA_5 than did etiolated dwarf peas

and that light-grown tall and dwarf peas contained approximately equal levels of presumptive GA_5. However, results of one experiment suggested that dwarf pea seedlings grown in the light actually contained more presumptive GA_5 than did tall pea seedlings grown under the same environmental conditions. Due to the variability between experiments it was concluded that results obtained from extraction and bioassay techniques may not quantitatively reflect endogenous GA relationships.

Other results which suggested that dwarf pea seedlings synthesize less GA than tall pea seedlings when both are grown in the dark were obtained by a seed treatment with Amo-1618. The subsequent growth of seedlings of the dwarf variety was inhibited more on a percentage basis than seedlings of the tall variety. However, when the shoot tips of six-day-old etiolated tall and dwarf seedlings were treated with various dosages of Amo-1618, no difference in the sensitivity was observed for the two varieties. Thus it appeared that there was no difference in the rate of GA biosynthesis between established etiolated seedlings of tall and dwarf peas. Experiments indentical to those conducted with etiolated plants utilizing Amo-1618 were performed on light-grown peas. The results of these experiments showed that while seedlings of the tall variety were quite sensitive to the growth retardant, seedlings of the dwarf variety were hardly affected. Therefore these experiments with light-grown

plants were inconclusive in determining whether the differences exhibited in the growth rates of light-grown tall and dwarf peas are due to differences in endogenous GA biosynthesis.

A hypothesis has been proposed to explain the phenomenon of photoinhibition of stem elongation in tall and dwarf pea seedlings. According to this hypothesis, GA_1 is the limiting factor in growth and GA_5 is its immediate precursor. In complete darkness the conversion of GA_5 to GA_1 is uninhibited, but when seedlings are exposed to white or red light an inhibitor is produced which blocks the conversion of GA_5 to GA_1. Seedlings of the dwarf variety contain higher concentrations of this inhibitor than do seedlings of the tall variety, and this inhibitor is at least partially responsible for the difference in growth rates between tall and dwarf peas which are grown in the light.

The biosynthesis of (-)-kaurene from 2-^{14}C-mevalonate in cell-free extracts of pea seeds was investigated. (-)-Kaurene was identified as a product of the reactions by: (1) comparison with authentic (-)-kaurene on thin-layer and gas-liquid chromatography and (2) oxidation of the presumptive ^{14}C-kaurene and (-)-kaurene with osmium tetroxide to form the common derivative kaurane-16,17-diol. The enzyme system which synthesized (-)-kaurene required the presence of ATP, and Mg^{2+} or Mn^{2+}, with Mn^{2+} being the better divalent cation activator. The apparent

rates of (-)-kaurene biosynthesis were the same in extracts prepared from developing seeds of tall and dwarf peas. The growth retardants Amo-1618 and CCC were found to inhibit (-)-kaurene biosynthesis. CCC was a much less effective inhibitor than Amo-1618, with approximately 1000-fold higher concentrations of CCC than Amo-1618 being required to cause similar percentages of inhibition.

Comparative Gibberellin Relationships in Tall and Dwarf Peas (*Pisum sativum* L.)

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COMPARATIVE GIBBERELLIN RELATIONSHIPS IN TALL AND DWARF PEAS (PISUM SATIVUM L.)

INTRODUCTION

For many species of angiosperms, especially cultivated species, there are two or more varieties which are distinguishable at least partially by differences in the rate of shoot elongation and shoot height at maturity. A typical species which illustrates this characteristic is the common garden pea (Pisum sativum L.). The numerous varieties of this species range from dwarf types that mature at heights of 30 cm or less to tall varieties that attain a meter or more at maturity when grown in light (Moore, 1964). Interestingly, however, when grown in complete darkness, tall and dwarf varieties exhibit approximately equal growth rates (Gorter, 1961, 1964; von Abrams, 1953). There are marked differences in the rates of stem elongation exhibited by etiolated and light-grown plants of the same variety, such differences often being of greater magnitude in the case of the dwarf than in the tall varieties (Gorter, 1961, 1964; Lockhart, 1959; Lockhart and Gottschall, 1959; von Abrams, 1953). From these observations two related problems which have been identified are: (1) the biochemical bases for dwarfism, or more specifically, for differential rates of stem elongation among varieties of the same species when grown in a common

environment; and (2) the mechanism of the photoinhibition of stem elongation.

Auxin Relations

The biochemical bases for dwarfism and for light-induced inhibition of stem elongation have been investigated extensively by plant physiologists during the past 30 years. Many studies were concerned exclusively with the comparative endogenous auxin relations and how they are related to growth rate (DeHaan and Gorter, 1936; Galston, 1959; Galston and Baker, 1951; Galston and Kaur, 1961; Hillman and Galston, 1957; Kuraishi and Muir, 1962, 1964a, b; McCune and Galston, 1959; Moore and Shaner, 1968; Sastry and Muir, 1965; Scott and Briggs, 1960, 1962, 1963; van Overbeek, 1935, 1938; von Abrams, 1953). The results of van Overbeek (1935, 1938), indicated that dwarf corn (Zea mays L.) seedlings contained less auxin than seedlings of a tall variety. This difference was attributed mainly to the presence of higher levels of auxin-destroying enzymes in the dwarf variety than in the tall and secondarily to greater auxin production in the tall than in the dwarf. DeHaan and Gorter (1936) showed that green dwarf pea seedlings produced less and destroyed more auxin than did green tall seedlings. They concluded that destruction of auxin is the determining factor for the variable growth of green pea stems. Interestingly, these authors detected very little

auxin in etiolated plants, as compared to light-grown plants, an observation confirmed by other investigators (Scott and Briggs, 1960, 1962, 1963). Another investigator (von Abrams, 1953), using both etiolated tall and dwarf peas, could not detect any difference in either: (1) extractable auxin content; (2) capacity to inactivate or destroy auxin; or (3) capacity to convert tryptophan to auxin. However, von Abrams obtained a stimulation of growth in etiolated dwarf peas by the application of indole-3-acetic acid (IAA) to young seedlings. Identical treatment of etiolated tall seedlings either had no effect or inhibited growth slightly. However, the increased growth rate resulting from application of IAA to dwarf peas did not attain the growth rate of etiolated tall peas; thus, von Abrams concluded from his studies that auxin deficiency is not the fundamental cause of dwarfism in peas. Such a stimulation of growth by applying IAA to intact plants is quite difficult to show and has been demonstrated in only a few species (see Moore, 1967b).

Recently, Moore and Shaner (1968), employing extremely sensitive techniques, have shown that cell-free extracts prepared from shoot tips of etiolated and light-grown tall and dwarf peas are capable of synthesizing ^{14}C-IAA from ^{14}C-labeled tryptophan and that the apparent order of rates of net biosynthesis of IAA in preparations of the four kinds of seedlings is: light-grown tall > light-grown dwarf > etiolated tall $\simeq$ etiolated dwarf. Research currently is

being conducted by these investigators to determine whether the observed differences in the rates of net IAA biosynthesis between the two genotypes grown in the dark and light are due partially to actual differences in rates of biosynthesis or solely to differences in IAA oxidase activity.

Gibberellins as Plant Constituents

Progress towards the ultimate elucidation of the hormonal bases of dwarfism and photoinhibition of stem elongation was made when Brian and Hemming (1955) demonstrated that application of gibberellin (GA) would cause the growth rate of dwarf pea seedlings to become equal to, or even greater than, that of tall (normal) peas. This discovery was followed by the report of Phinney (1956) on the effects of GA on several dwarf corn mutants. An interesting observation was that five of the dwarf mutants tested responded very well to applied GA, four responded only slightly, and one mutant completely failed to respond. From these results, it would appear that endogenous GA itself is not the only factor limiting growth in some corn mutants, but that GA utilization and/or the absence of other essential growth factor(s) which eventually lead to the growth response are the cause of the dwarf type of growth.

Extraction of GA-like substances from angiosperms was first reported by Radley (1956) who found these substances in pea shoots.

Phinney et al. (1957) reported finding similar compounds in extracts from seeds and fruits of nine genera representing seven different families, thus suggesting the occurrence of GA in all angiosperms. Structures for at least 13 GA's are known (e.g., Paleg, 1965). The occurrence of GA-like substances is not restricted to angiosperms nor the fungus Fusarium moniliforme. Several reports indicate that GA-like substances have been found in gymnosperms, ferns, brown and green algae, and bacteria (Jackson, Brown and Burlingham, 1964; Kato, Purves and Phinney, 1962; Katznelson, Sirois and Cole, 1962; Mowat, 1963; Radley, 1961, Vančura, 1961).

Lockhart (1956), in investigations on the phenomenon of light-induced inhibition of stem elongation, showed that GA treatment would not increase the growth rate of etiolated tall peas, but would increase the growth rate of etiolated dwarf peas. He demonstrated also that light-induced inhibition of stem elongation was completely reversible for both tall and dwarf pea varieties by GA applications, but that the application of GA could not increase the growth rate above that of etiolated tall peas, which was considered to be the potential maximum rate for peas. Lockhart's initial report was followed by several other papers on the effects of GA on growth of light-grown plants (see e.g., Lockhart, 1958, 1959; Marth et al., 1956; Sale and Vince, 1960; Vince, 1967), all of which suggested that light profoundly alters endogenous GA relationships in some

manner. Lockhart (1959) concluded from his studies on the mechanism of stem growth inhibition by light that visible radiation affects stem growth of peas by reducing the natural GA level, either by causing the formation of a GA-destroying system or by retarding the biosynthesis of GA.

Photomorphogenesis

The effect of light on morphogenesis of peas has been investigated extensively (e.g., Galston, Tuttle and Penny, 1964; Lockhart, 1956; Parker et al., 1949; Thomson, 1954; Thomson and Miller, 1961; Went, 1941). The results of these investigations have shown that red or white light, while actually inhibiting shoot elongation, increases the rate of node formation and stimulates leaf expansion. The light-induced increase in node formation and stimulation of leaf expansion are not reversed by GA application (Lockhart, 1956) but are nullified by immediate subsequent irradiation with far-red light (Parker et al., 1949, Thomson, 1959; Went, 1941). The effect of light on the apparent inhibition of internode elongation was envisioned by one investigator (Thomson, 1954) as a stimulation of maturation rather than the inhibition of enlargement.

Gibberellin Relations in Tall and Dwarf Peas

Because of the discovery that GA treatment will increase the

growth rate of light-grown dwarf peas, making it equal to that of tall peas (Brian and Hemming, 1955; Lockhart, 1956; Vlitos and Meudt, 1957), and the fact that GA-like substances had been isolated from peas (Phinney et al., 1957; Radley, 1956), Brian (1957) proposed that the difference in growth rates between tall and dwarf peas could be accounted for by a GA deficiency in the dwarf plants. Lockhart's proposal (1959) that light somehow reduces the effective level of GA in the plant is compatible with the hypothesis of Brian (1957) in that the effective level of GA could be lowered by: (1) reducing the rate of biosynthesis; (2) inducing the formation of inhibitory substances; or (3) reducing the sensitivity of the tissue to GA (Kende and Lang, 1964). Experimental approaches used in attempts to elucidate the cause of differential growth rates between tall and dwarf varieties have been of two major types: (1) studies of comparative responses to applied GA and GA precursors (Brian and Hemming, 1955; Katsumi et al., 1964; Kende and Lang, 1964; Lockhart, 1956, 1959; Lockhart and Gottschall, 1959; Moore, 1967a; Phinney et al., 1964; Simpson and Wain, 1961; Tanimoto, Yanagishima and Masuda, 1967; Vlitos and Meudt, 1957); and (2) extraction and bio-assay of endogenous GA and/or inhibitors (Kende and Lang, 1964; Köhler, 1964, 1965a, b, 1966; Köhler and Lang, 1963; Radley, 1958).

Comparative Responses of Dwarf and Tall Peas to Gibberellins

Studies utilizing the comparative responses of tall and dwarf peas to applied GA have given strong support to the hypothesis that the dwarf varieties are indeed deficient in endogenous GA (e.g., Brian and Hemming, 1955; Katsumi et al., 1964; Lockhart, 1956, 1959; Lockhart and Gottschall, 1959). Brian and Hemming (1955) were able to show an inverse correlation between the response of pea plants to a given dose of GA and their growth rate before GA treatment. The results from the dwarf corn mutants were not as definitive, because some of the mutants would respond very well to GA whereas others would only yield a small response or none at all (Phinney, 1956). It appears from data of this type that at least in peas and some dwarf corn mutants, the rate of growth is limited by a deficiency of endogenous GA.

Extraction and Bioassay of Gibberellins

The extraction and bioassay data have not definitely shown significant differences in GA content between etiolated and light-grown tall and dwarf peas. Radley (1958) did not demonstrate any significant difference in GA level of light-grown tall and dwarf peas. Other investigators (Köhler and Lang, 1963) reported that tall pea seedlings grown in total darkness and in red light of low intensity

contained equivalent amounts of GA, but that no GA could be detected in a dwarf variety. In contrast to these findings, Kende and Lang (1964) tentatively identified GA_1 (fraction II) and GA_5 (fraction I) from dwarf pea seedlings which were grown in darkness or low-intensity red light. These authors did not detect any difference in the total GA content between the etiolated and red light-grown plants. More recently, Köhler (1965b) has presented data which indicate that tall pea seedlings grown in far-red light contain eight times more fraction I (GA_5) activity of Kende and Lang (1964) than do dwarf pea seedlings grown under the same conditions. Other reports (Köhler, 1965a, 1966) indicated that tall peas grown in dim red light contained ten times more fraction I (GA_5) than etiolated tall peas. These findings suggest a red-light stimulation of GA biosynthesis which can be reversed by exposing the red-light-treated plants to far-red light (Köhler, 1966). A very recent report indicated a light stimulation of GA biosynthesis also in Fusarium moniliforme (Mertz and Henson, 1967) Köhler (1965b) suggested from his work with CCC [(2-chloroethyl)-trimethyl ammonium chloride], a known inhibitor of GA biosynthesis (Harada and Lang, 1965; Kende, Ninnemann and Lang, 1963; Ninnemann et al., 1964; Zeevaart, 1965), that etiolated peas require little if any GA for growth. This conclusion was based on the fact that CCC inhibited the growth of light-grown peas but did not inhibit significantly the growth of etiolated peas.

Differential Tissue Sensitivity to Applied Gibberellins

Evidence has been presented from several laboratories (Galston and Kaur, 1961; Gorter, 1961; Kende and Lang, 1964) that light reduces the sensitivity of a tissue to applied GA. One study (Kende and Lang, 1964) revealed that red light-grown dwarf peas are incapable of responding to GA_5 whereas etiolated tall and dwarf peas, as well as red light-grown tall peas artificially dwarfed with 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methyl phenyl piperidine-1-carboxylate (Amo-1618), were very capable of responding to GA_5. Thus, these authors concluded that light somehow reduced the sensitivity of the dwarf pea to GA_5 but not to GA_1.

Light-Induced Inhibitors of Gibberellin Action

Another factor which may partially account for the dwarf habit of growth of some varieties and the photoinhibition of stem elongation is the light-induced formation of inhibitors of GA action. Several reports have presented evidence for endogenous inhibitors of GA action (Bünsow, 1961; Corcoran, West and Phinney, 1961; Köhler, 1964; Köhler and Lang, 1963; Simpson and Wain, 1961) in several angiosperm species. However, it is difficult to attribute any physiological significance to these compounds since apparently they are effective only in the presence of exogenous GA (Köhler and Lang, 1963).

Description of the Problem

Current knowledge concerning the hormonal bases of dwarfism and photoinhibition of stem elongation is very fragmentary and incomplete. It seems certain that both auxins and GA's, perhaps also other growth substances, are causally implicated in both phenomena. Nevertheless the current investigations were restricted to attempts to discover possible variations in endogenous GA relationships which are correlated with differential rates of stem growth among pea seedlings of different genotypes and between etiolated and green pea seedlings of the same genotype. Hopefully, a better understanding of the role of this one kind of hormone would contribute substantially to a partial elucidation of the hormonal bases of dwarfism and photoinhibition of stem growth.

Three separate but related experimental approaches to the problem were utilized: (1) the determination of possible qualitative and/or quantitative differences in the GA's present in etiolated and light-grown tall and dwarf peas through extraction and bioassay techniques; (2) the determination of possible variation in sensitivity of etiolated tall and dwarf peas to Amo-1618, a known inhibitor of GA biosynthesis; and (3) the development of a cell-free system from peas capable of synthesizing one or more GA's or tetracyclic intermediates in the pathway of GA biosynthesis. The

three approaches were employed because the inherent disadvantages and limitations of each of the individual methods could be overcome partially by comparing the results from all three, thus assuring a better chance of determining the role of GA's in the physiology of dwarfism and photoinhibition of stem elongation.

MATERIALS AND METHODS

Source and Purity of Reagents

Mevalonic acid-2-^{14}C lactone (sp. act. 5.02 mc/mmole) in benzene was purchased from CalBiochem. The lactone was hydrolyzed by treating overnight with 100% excess NaOH equivalents. The benzene was removed in vacuo, and the mevalonate diluted with distilled water to a concentration of approximately 0.01 μc/μl. Adenosine triphosphate (ATP) and the growth retardant Amo-1618 were purchased from Sigma Chemical Company and Enomoto and Co., Redwood City, California, respectively. The growth retardant CCC was a gift of American Cyanamid Company. Samples of (-)-kaurene were generously supplied by Dr. L. H. Briggs, University of Auckland, Auckland, New Zealand and Dr. C. A. West, University of California, Los Angeles, California. Samples of GA_1 and GA_5 were supplied by Dr. G. W. Elson, Imperial Chemical Industries, Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, England. Constituents of the scintillation fluid, 2, 5-diphenyloxazole and p-bis-2'-(5'phenyloxazolyl)-benzene, were purchased from Packard Corporation. Column material for gas-liquid chromatography, SE-52 and Gas-Chrom Q (80-100 mesh), were purchased from Applied Science Laboratories. All other chemicals were of reagent grade and all organic solvents were

redistilled. Celite (diatomaceous earth) was washed with 3 N HCl and rinsed extensively with distilled water. The celite was then rinsed with methanol and dried at 100°C in an oven.

Culture of Plants

Seeds of both Tall and Dwarf Telephone peas (W. Atlee Burpee Co., Riverside, California) were surface-disinfected with 0.5% NaClO for 5 minutes after which the seeds were rinsed three times with distilled water. The surface-disinfected seeds were planted in plastic pots containing water-saturated vermiculite. The pots were placed in a growth chamber (Percival Model E-57) programmed to provide a 16-hour photoperiod at 20° ± 1°C (light intensity ≃ 7800 lux) and 8 hours of darkness at 17° ± 1°C. The same temperature regime was maintained for etiolated plants, but the lights were turned off and the chamber made light-tight. Plant measurements were taken from the cotyledonary node to the highest visible node. The growth chamber was opened to measure and water the etiolated plants only at night and then only in the presence of a dim green light (15 watt green fluorescent tube covered with eight layers of green and two layers of amber DuPont cellophane).

Pea seedlings were also grown in a greenhouse where the critical physical environmental factors were a 16-hour photoperiod at approximately 20°C and an eight-hour nyctotemperature at

approximately 17°C.

Etiolated peas to be used for extraction or for bioassay were grown in light-tight cabinets at room temperature (19-25°C). Seeds were disinfected and planted in vermiculite as described above. The plants were handled only under dim green light, as described above.

Extraction and Bioassay of Gibberellins

Extraction and Partitioning

The extraction procedure used was a modification of the procedure described by Kende and Lang (1964). Light-grown seedlings were grown in the greenhouse for 12 days at which time the shoots were excised at the cotyledonary node and immediately frozen. Etiolated seedlings were grown in light-tight cabinets for 12 days at which time the shoots were harvested as described above. Each sample of plant material extracted consisted of 1025 to 2150 seedlings. The plant material was homogenized with methanol (1 g fr wt/2 ml methanol) in a Sorvall Omni-mixer. The extract was passed through cheese cloth and the residue re-extracted. Next, the combined methanol extracts were filtered through Whatman No. 1 filter paper under reduced pressure and the methanol of the filtrate was removed under vacuum in a rotary-film evaporator. The resulting aqueous phase was adjusted to a standard volume and the pH brought

to 8.4. The pH 8.4 aqueous extracts were partitioned twice against equal volumes of ethyl acetate and twice against quarter volumes of petroleum ether (boiling range = 30-60°C). These alkaline extracts were saved for bioassay of inhibitory substances (see below). The aqueous phase then was adjusted to pH 2.5 with HCl and partitioned three times with equal volumes of ethyl acetate. The water from the combined ethyl acetate extracts was removed by freezing and the ice was removed by filtration with a Buchner funnel. The dehydrated ethyl acetate extracts were taken to dryness under vacuum at 30°C.

Partition Column Chromatography

Partition column chromatography was carried out according to the procedure described by Kende and Lang (1964). Acid-washed celite was packed under suction in a glass tube (1.5 cm diameter) to a height of 27 cm. Seventy-five ml of 0.5 M potassium phosphate buffer (K_2HPO_4-KH_2PO_4), pH 6.4, were partitioned with 150 ml of ethyl acetate. After the two phases (buffer saturated with ethyl acetate and ethyl acetate saturated with buffer) had separated, the two fractions were passed separately through the column under suction.

The dried plant extracts were re-dissolved in ethyl acetate saturated with buffer and pipetted onto the column of buffered celite. As the column was developed with ethyl acetate, ten fifty-ml

fractions were collected, each of which subsequently was evaporated to dryness under vacuum. The residue from each fraction was taken up in 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate) in 20% ethanol and stored in a freezer (-10°C) until used for bioassay.

Samples of GA_1 and GA_5 also were passed through the buffered celite column to ascertain in which fractions these known gibberellins would be found. The GA's were detected by thin-layer chromatography. Samples of each fraction from the column were applied to glass plates coated with 250 μ layers of silica gel G and were subsequently developed in isopropyl ether: acetic acid (95:5, v/v). After development the plates were sprayed with 2 N H_2SO_4 and heated several minutes at 100°C and then viewed under ultraviolet light.

Bioassay of Gibberellin-Like Substances

The bioassay used was a modification of the dwarf pea biqassay of Kende and Lang (1964), in which the growth retardant Amo-1618 is employed to chemically dwarf etiolated Dwarf Telephone peas. Surface-disinfected seeds were placed in a 0.1 mM solution of Amo-1618 (35.8 mg/l) for six hours at room temperature in darkness. The imbibed seeds were again surface-disinfected, planted in water-saturated vermiculite and placed in light-tight cabinets. After six days of growth, the artificially-dwarfed etiolated peas were

selected for uniformity, the shoot lengths were measured, and each of the 10 to 19 seedlings in each group was treated on the shoot tip with 10 μl of one of the extract fractions isolated by column chromatography. All manipulations were carried out under dim green light. Seedlings treated with Tween-20 were used as controls; plants treated with 0.001, 0.003, or 0.01 μg GA_3/plant served as other standards for comparison. Five days after treatment the plants were again measured and the data were expressed as the net increase in shoot height over the five-day period.

Extraction of Inhibitor(s) of Gibberellin Action

Extraction and Partitioning

The inhibitor of GA-action reported by Köhler and Lang (1963) was suggested to be cationic in nature; therefore, pH 8.4 was used in attempts to isolate this (these) compound(s). The alkaline extracts from the GA extraction and partitioning procedure were used to assay for the inhibitor(s). The petroleum ether extract and the alkaline ethyl acetate extracts were combined and the water removed by freezing, as described above. The organic solvents were taken to dryness in a rotary-film evaporator at 30°C. Then the residues were taken up in ethyl acetate and divided into three equal aliquots. Each individual aliquot was taken to dryness and the residue taken up

in either: (1) 0.05% Tween-20 in 20% ethanol; (2) 0.05% Tween-20 in 20% ethanol containing 0.001 μg GA_3/10 μl or; (3) 0.05% Tween-20 in 20% ethanol containing 0.01 μg GA_3/10 μl.

Bioassay

Dwarf and Tall Telephone peas were grown in the greenhouse as described previously. When ten days of age, the plants were selected for uniformity, measured, and treated with 10 μl each of: (1) extract plus Tween-20; (2) extract plus Tween-20 plus 0.001 μg GA_3; (3) extract plus Tween-20 plus 0.01 μg GA_3; (4) Tween-20; (5) Tween-20 plus 0.001 μg GA_3; or (6) Tween-20 plus 0.01 μg GA_3. Each 10 μl aliquot of extract contained the active substances from 7.5 seedlings. Five days after treatment the plants were measured and the results reported as the net increase in shoot height in five days.

Sensitivity of Tall and Dwarf Telephone Peas to Amo-1618

Seed Treatment

Seeds of Tall and Dwarf Telephone peas were surface-disinfected and treated by soaking in each of the following concentrations of Amo-1618: 0, 0.001, 0.003, 0.010, 0.032 and 0.10 mM. Plants were grown either in the light or in the dark in a growth

chamber for 12 days, at which time the plants were harvested.

Shoot Tip Treatment

To determine if a different method of application of Amo-1618 would yield results similar to those in the seed-treatment experiments, six-day-old etiolated and light-grown tall and dwarf pea seedlings were treated by applying to each shoot tip 10 μl of 0.05% Tween-20 in 20% ethanol containing Amo-1618 at one of the following concentrations: 0, 0.001, 0.003, 0.010, 0.032 and 0.10 μmole. Six days after treatment the plants were measured and the results expressed as the net increase in shoot height in six days.

Cell-Free Biosynthesis of (-)-Kaurene

Plant Material

Dwarf and Tall Telephone peas were grown in the greenhouse, as described previously. Plants were allowed to set fruit, and when seeds were approximately half to two-thirds mature size, the seeds were excised and frozen.

Preparation of Enzyme Extract

Fresh frozen seeds were homogenized in cold 0.1 M phosphate buffer (1 g fr wt/ml buffer), pH 7.4, containing 50 μg/ml each of

penicillin G and streptomycin sulfate using a Thomas teflon-to-glass homogenizer held in an ice bath. Homogenates were centrifuged at 40,000 x g for 15 minutes and the resulting supernatant was used as the enzyme source.

Reaction Conditions and Product Isolation

Routinely each reaction mixture contained 0.75 ml of enzyme extract, approximately 100 mμmoles of Na-2-^{14}C-mevalonate (~0.5 μc), 3 μmoles each of $MgCl_2$, $MnCl_2$ and ATP, and 0.1 M KH_2PO_4-K_2HPO_4 buffer, pH 7.4, in a total volume of 2.0 ml. In experiments utilizing growth retardants, the retardant solutions were prepared in phosphate buffer. Reactions were allowed to proceed for one hour at 30°C, unless otherwise noted, and were stopped by boiling for five minutes. Following centrifugal sedimentation of the precipitate, the supernatant was discarded, and the precipitate was washed with 2.0 ml of buffer and again pelleted by centrifugation. The washed pellet then was extracted with acetone (3 x 2 ml), and the combined acetone extracts were evaporated under reduced pressure using a rotary-film evaporator. Each residue was extracted with acetone (2 x 0.2 ml + 1 x 0.1 ml) and the entire 0.5 ml of extract was applied to a 5 x 20 cm glass plate coated with a 250μ layer of silica gel G. Thin-layer chromatograms were developed routinely in hexane. After the solvent front advanced 15 cm from the

origin the plates were removed from the solvent and scanned for radioactivity. After preparative isolation of (-)-kaurene, samples of (-)-kaurene were eluted from chromatograms with acetone and were chromatographed a second time on freshly prepared plates coated with $AgNO_3$-impregnated silica gel G (Bennett, Ko and Heftmann, 1966) and developed in hexane:methanol (99:1, v/v). The silica gel G was prepared by slurrying 30 g of dry gel with 62 ml of eight % (w/w) $AgNO_3$ solution.

Radioassay Procedures

Thin-layer chromatograms were scanned to detect radioactive compounds with a Vanguard Automatic Chromatogram Scanner, Model 880 equipped with glass plate attachment, Model 885. Silica gel from appropriate regions on the chromatograms was removed and placed in liquid scintillation vials containing 10 ml each of toluene containing 40 mg of 2, 5-diphenyloxazole and 0.5 mg of p-bis-2'-(5'-phenyloxazolyl)-benzene. Radioactivity measurements were made in a Packard Tricarb Liquid Scintillation Spectrometer, Model 314 EX. Liquid scintillation data are expressed in disintegrations per minute (dpm); counting efficiency ranged from 68 to 71%.

Protein Nitrogen Determinations

Protein nitrogen contents of 1.0 ml aliquots of each enzyme

extract were measured by a micro-Kjeldahl procedure (Horwitz, 1965).

Identification of ^{14}C-Product

The ^{14}C-product, which was suspected to be (-)-kaurene from preliminary extraction and thin-layer chromatographic behavior, was co-chromatographed with authentic (-)-kaurene using thin-layer and gas-liquid chromatography. Samples of authentic (-)-kaurene and ^{14}C-product were applied at common origins on thin-layer plates and were developed in hexane. An aliquot of authentic unlabeled (-)-kaurene and ^{14}C-product in benzene was injected onto a column (6.35 mm x 1.22 m) packed with 10% SE-52 on Gas-chrom Q (80 to 100 mesh) and developed at a column temperature of 170°C with an argon flow rate of 50 ml/min on an F&M Gas Chromatograph equipped with dual hydrogen flame detectors. Injection port and detector temperatures were 270°C and 170°C respectively.

When samples were to be collected from the gas chromatograph for ^{14}C determination, a column splitter equipped with a micrometer was employed. This allowed a portion of the effluent to go to the mass detector and the remainder to be trapped for liquid scintillation counting. Samples were collected by connecting micropipettes to the column with rubber adapters. The micropipettes

were packed with glass wool which was moistened with the counting solution. The tips of the micropipettes were then submersed in the scintillation fluid and effluent allowed to bubble through. After collection of the fractions, the scintillation fluid in the vials was drawn up through the glass wool several times to rinse residual radioactive material into the vials.

As a third method of confirming the identity of the ^{14}C-product as (-)-kaurene, aliquots of the authentic material and the ^{14}C-product were subjected to oxidation with osmium tetroxide (Briggs et al., 1963). A 0.3 ml aliquot of 2% osmium tetroxide in water was partitioned against 10 ml of diethyl ether. A 5.0 ml sample of the ether-osmium tetroxide solution was added to 800 μg of (-)-kaurene in a glass-stoppered 12 ml conical centrifuge tube. An additional 4.5 ml of the ether-osmium tetroxide solution were added to 500 μg of (-)-kaurene which also contained 5430 dpm of ^{14}C-product. The tubes were placed in a plastic bag to prevent evaporation and kept at room temperature for 48 hours. At the end of this time H_2S was bubbled through the solutions, the ether allowed to evaporate to dryness, and the resulting residue extracted with acetone and used for thin-layer chromatography. The solvents used were hexane, hexane:acetone (7:3, v/v), and ethyl acetate. After development, the plates were either scanned for radioactivity or sprayed lightly with 2 N sulfuric acid followed by heating at 100°C

for a few minutes, and then viewed under ultraviolet light. After prolonged heating the compounds of interest were detectable by distinctive coloration so that inspection in ultraviolet light was not necessary.

RESULTS

Growth curves for light-grown and etiolated Tall and Dwarf Telephone peas through 14 days of growth are found in Figure 1. Marked differences in rates of stem elongation between etiolated and green plants of the same variety and between dwarf and tall varieties, green or etiolated, were observable soon after emergence and became increasingly apparent throughout 14 days of development.

From these data the arbitrary decision was made to use 12-day-old seedlings for extraction of GA's, since at this age all four kinds of seedlings clearly were exhibiting a nearly constant rate of stem elongation. Seedlings of Tall Telephone peas which were grown in the light entered the linear phase of growth after approximately nine days following planting, whereas light-grown seedlings of the Dwarf Telephone variety did not enter this phase of growth until after 15 days following planting; etiolated peas of both varieties entered the linear phase of growth somewhere between the fourth and seventh day after planting.

Bioassay of the Gibberellins from Peas

The results of the bioassays of GA-like substances extracted from etiolated tall and dwarf peas and fractionated on a buffered celite column which was eluted with ethyl acetate are found

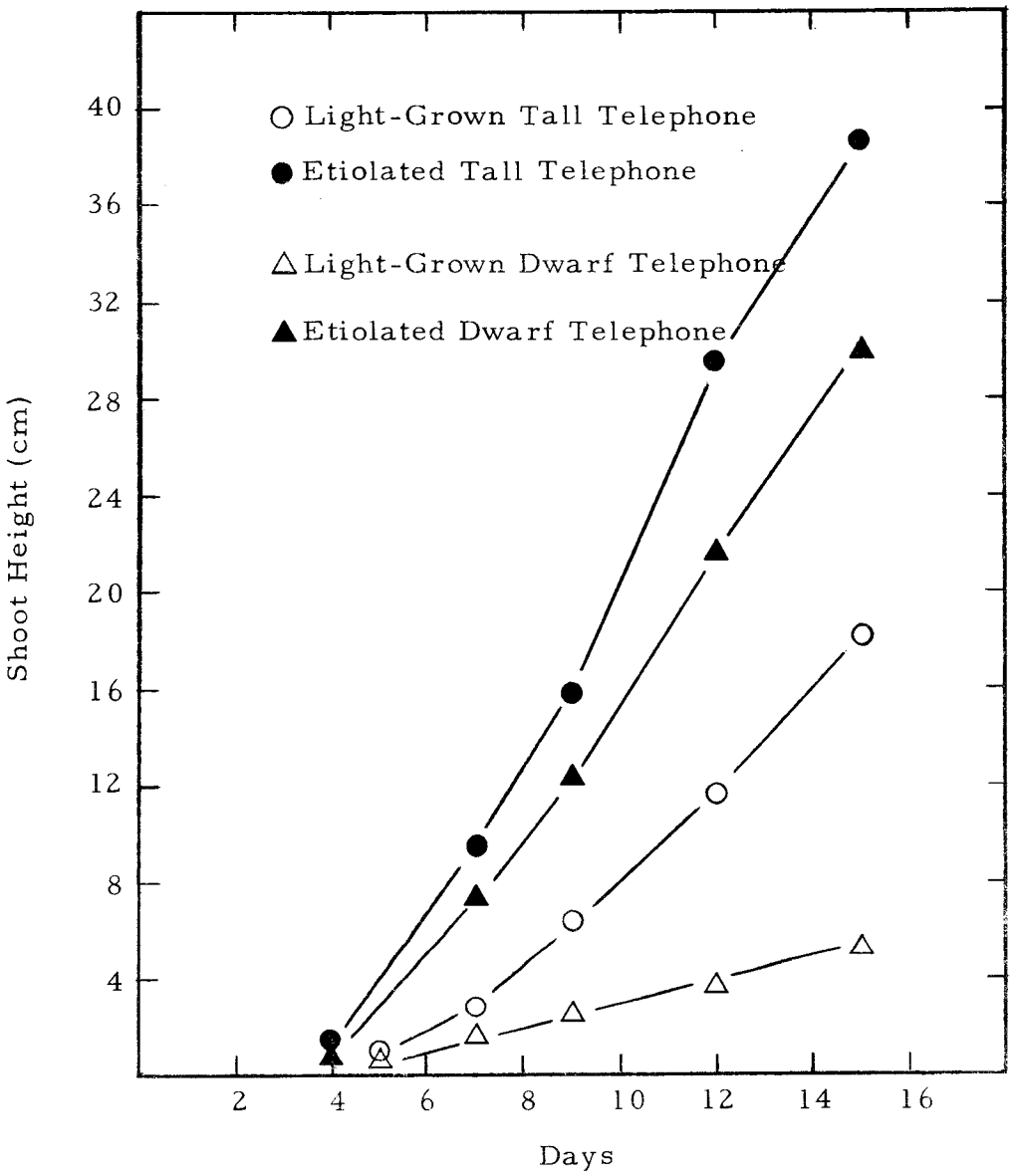


Figure 1. Growth curves of etiolated (closed symbols) and light-grown seedlings (open symbols) of Tall (circles) and Dwarf (triangles) Telephone peas grown in growth chambers. Each point represents the mean value for 20 plants.

in Figures 2 and 3, respectively. The distribution of GA-like activity in the various fractions of extracts of etiolated tall and dwarf peas was very similar; however, it appeared that extracts of etiolated tall peas contained more GA-like activity than did extracts of etiolated dwarf peas. Authentic GA_5 was found to elute from the column in fractions 2, 3 and 4; authentic GA_1 was eluted in fractions 8 and 9, with approximately equal distribution between the two fractions. The relative distribution of GA_5 in the earlier fractions was 3 > 4 > 2. It is assumed that the GA-like activity in the extracts of etiolated peas is analogous to that of fraction I of Kende and Lang (1964), which they identified as GA_5. Conceivably the sequence of biologically active fractions may have contained one or more active substances in addition to GA_5. However, in view of the lack of evidence in support of this possibility, the sequence of fractions is presumed to have contained a single active substance, which will henceforth be called presumptive GA_5. Only one other GA has been identified from peas, namely GA_1 (Kende and Lang, 1964), and all available evidence would indicate that the sequence of biologically active fractions isolated in the present work could not have contained GA_1. In analogous experiments, the level of GA-like activity was much lower than the activity reported here, but there did appear to be more GA-like activity in the etiolated tall seedlings than in the etiolated dwarf plants.

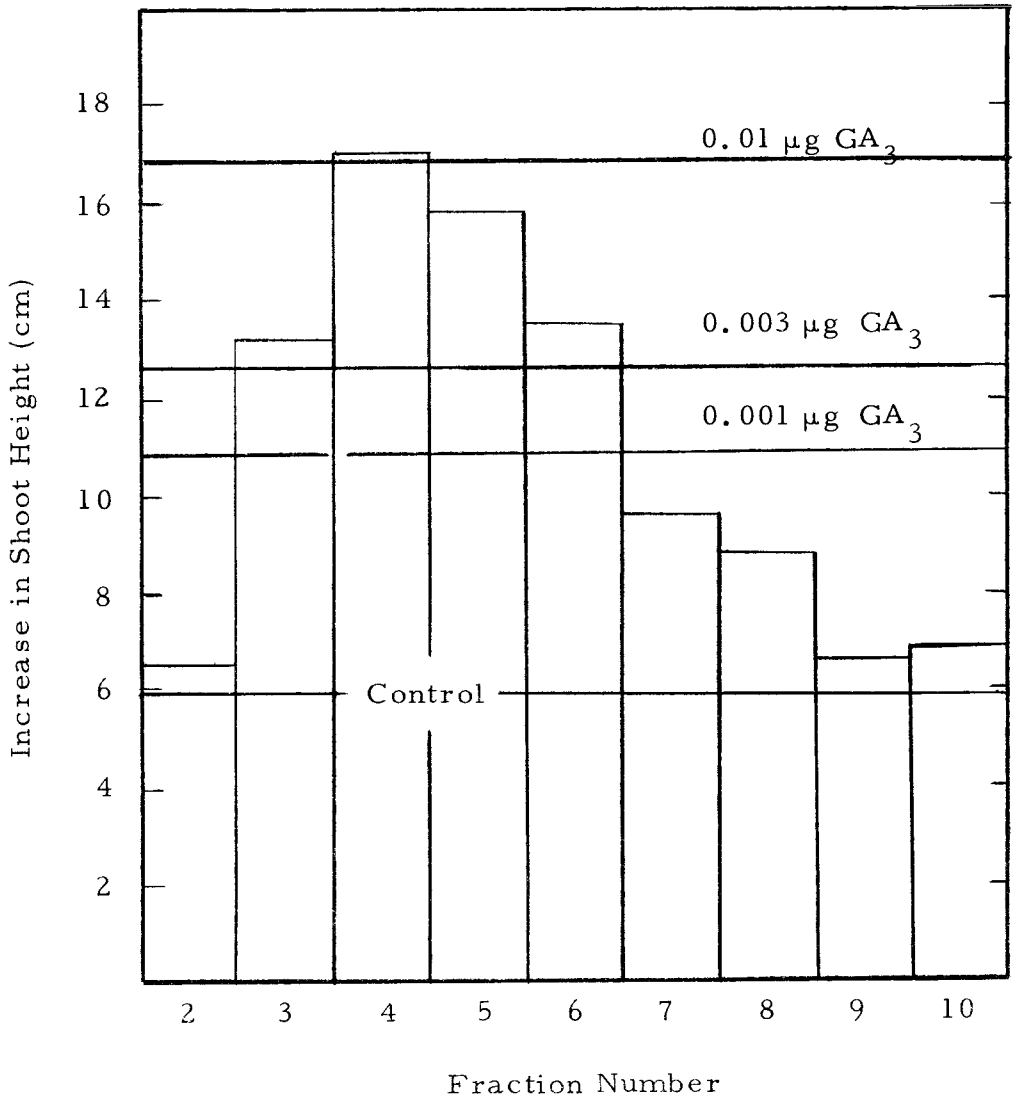


Figure 2. Histogram of the gibberellin-like activity from the acid fraction extract from etiolated Tall Telephone peas. The first fraction from the column proved lethal to the bioassay plants. Each bioassay plant was treated with extract from 30 seedlings. The number of plants in each treatment group was 8 to 11.

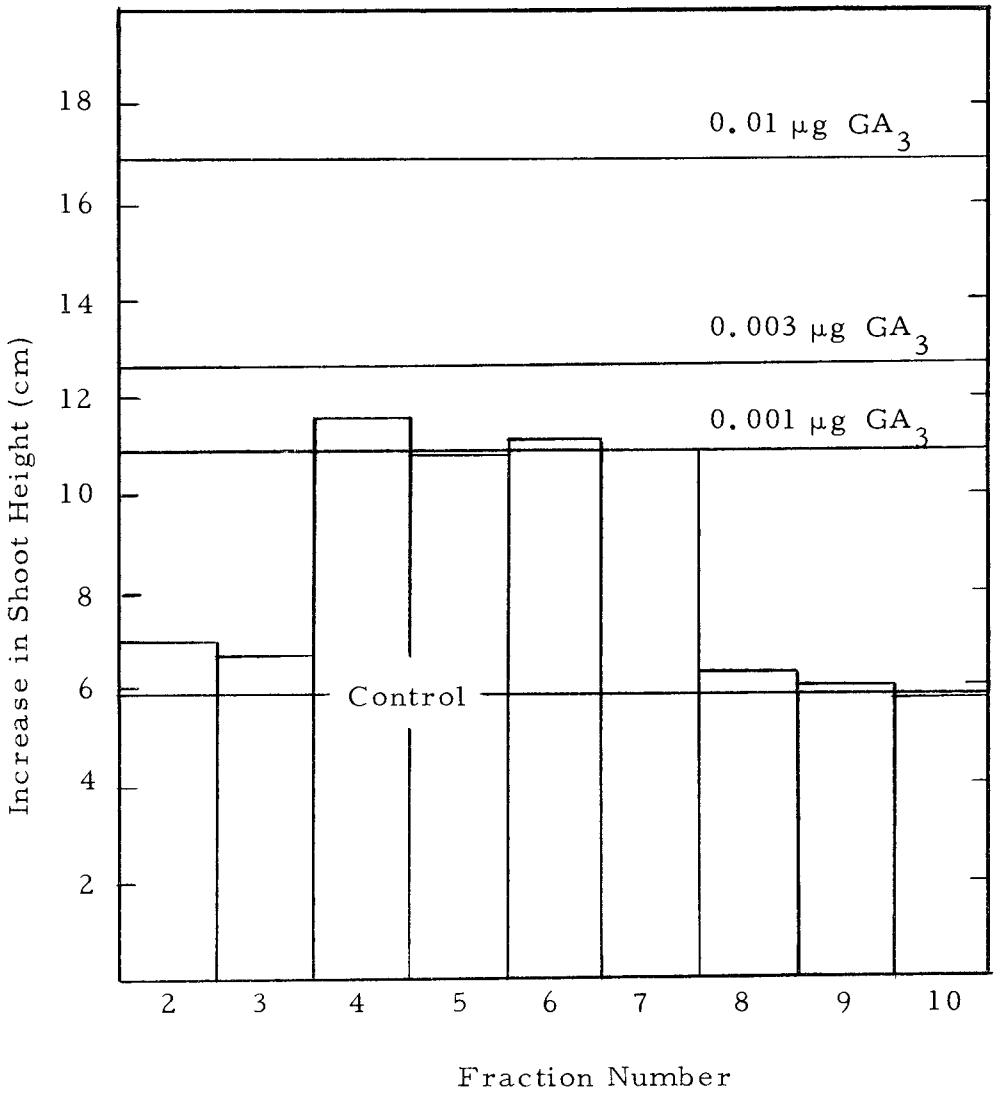


Figure 3. Histogram of the gibberellin-like activity from the acid fraction extract of etiolated Dwarf Telephone peas. The first fraction from the column proved lethal to the bioassay plants. Each bioassay plant was treated with extract from 30 seedlings. The number of plants in each treatment group was 6 to 11.

The results of the GA bioassays for light-grown Tall and Dwarf Telephone peas are given in Figures 4 and 5, respectively. There appeared to be approximately equal amounts of GA-like activity in light-grown tall and dwarf peas. This GA-like activity also is assumed to be analogous to that of Kende's and Lang's (1964) fraction I, for reasons given above, and the presumably single active substance will henceforth be called presumptive GA_5. The bioassay data from another experiment indicated that the dwarf variety contained substantially higher levels of presumptive GA_5 than did the tall variety.

A comparison of the GA-like activity in the different fractions of light-grown and etiolated peas indicates possible qualitative and quantitative differences in the GA's present in the seedlings. However, the other experiments indicated that such large differences in distribution and level of GA-like activity in the different fractions of etiolated and light-grown peas probably do not exist.

Bioassay of Inhibitor of Gibberellin Action

The results of bioassaying the alkaline fraction (pH 8.4) from the GA extracts on light-grown Dwarf and Tall Telephone peas are found in Figures 6 and 7, respectively. A preliminary bioassay for the inhibitor was carried out by applying extract from 30 plants to the shoot tip of each bioassay plant; however, almost all extracts

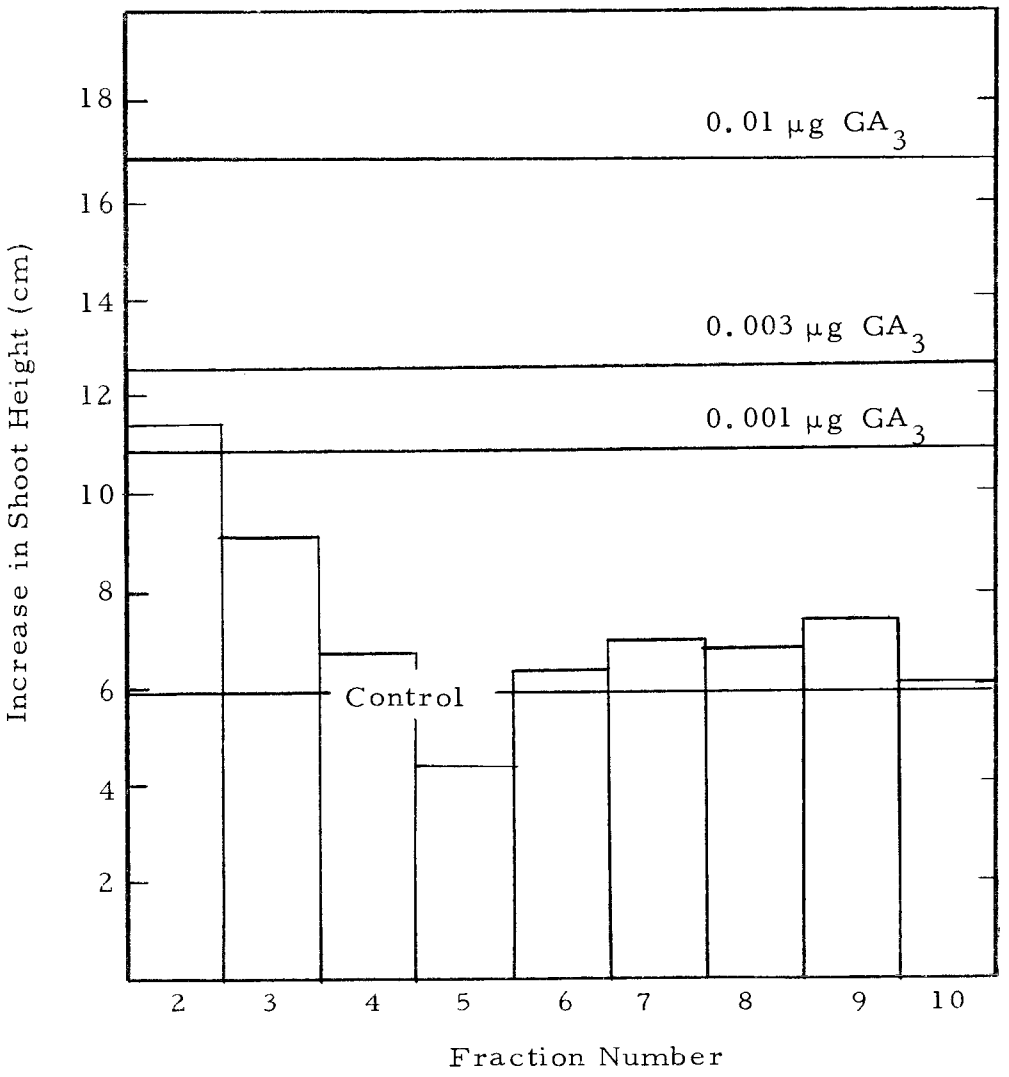


Figure 4. Histogram of gibberellin-like activity from the acid fraction extract of light-grown Tall Telephone peas. The first fraction from the column proved lethal to the bioassay plants. Each bioassay plant was treated with extract from 30 plants. The number of plants in each treatment group was 8 to 14.

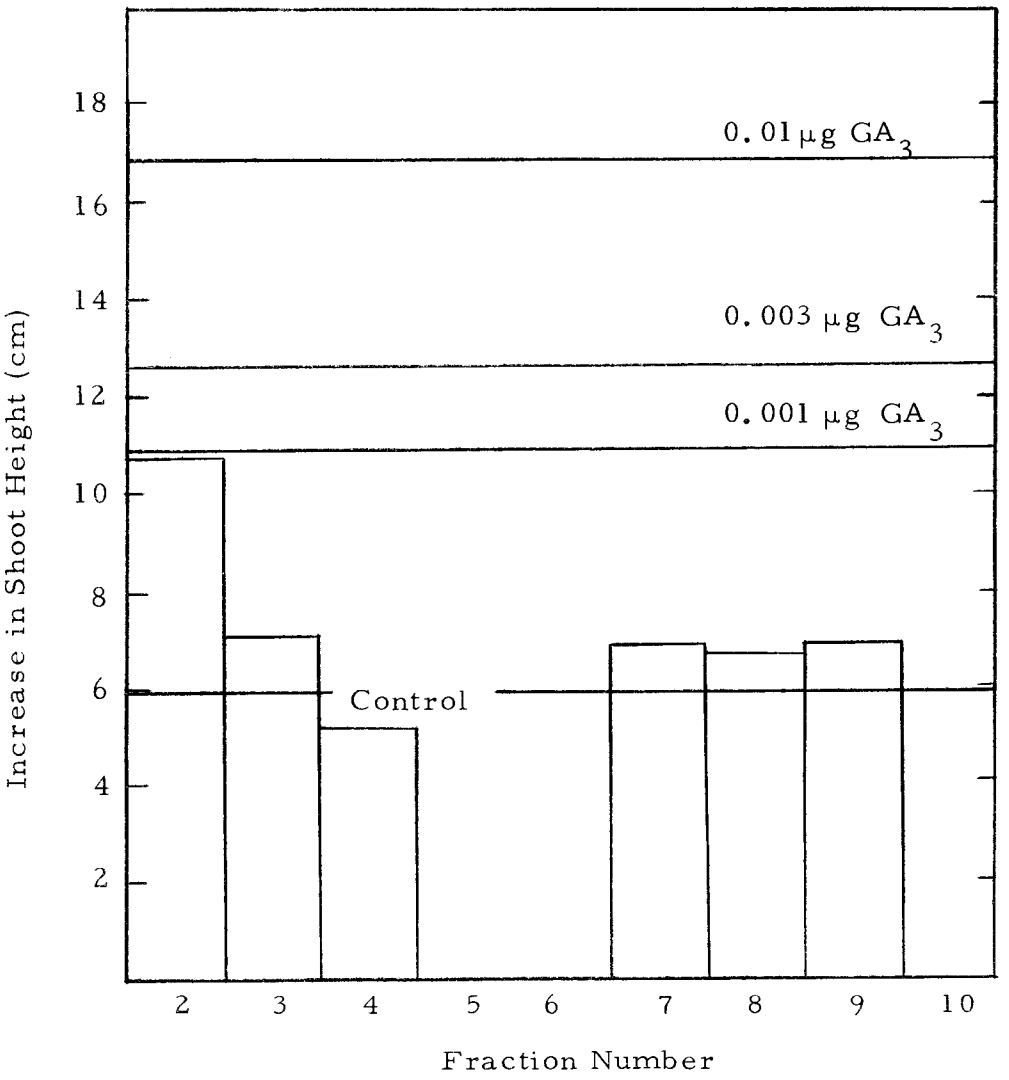


Figure 5. Histogram of gibberellin-like activity from the acid fraction extract of light-grown Dwarf Telephone peas. The first, fifth and sixth fractions from the column proved lethal to the bioassay plants. Each bioassay plant was treated with extract from 30 plants. The number of plants in each treatment group was 9 to 12.

Figure 6

The effects of the alkaline extracts (pH 8.4) on the growth response of light-grown Dwarf Telephone peas to applied GA_3 where A, is the extract from light-grown Dwarf Telephone; B, is the extract from light-grown Tall Telephone; C, is the extract from etiolated Dwarf Telephone and D, is the extract from etiolated Tall Telephone peas. The horizonal lines denote the growth increments for plants which were not treated with alkaline extract but which were treated only with 0, 0.01 and 0.10 μg GA_3. Extract from 7.5 plants was applied to each bioassay plant. The number of plants in each treatment group was 7 to 14.

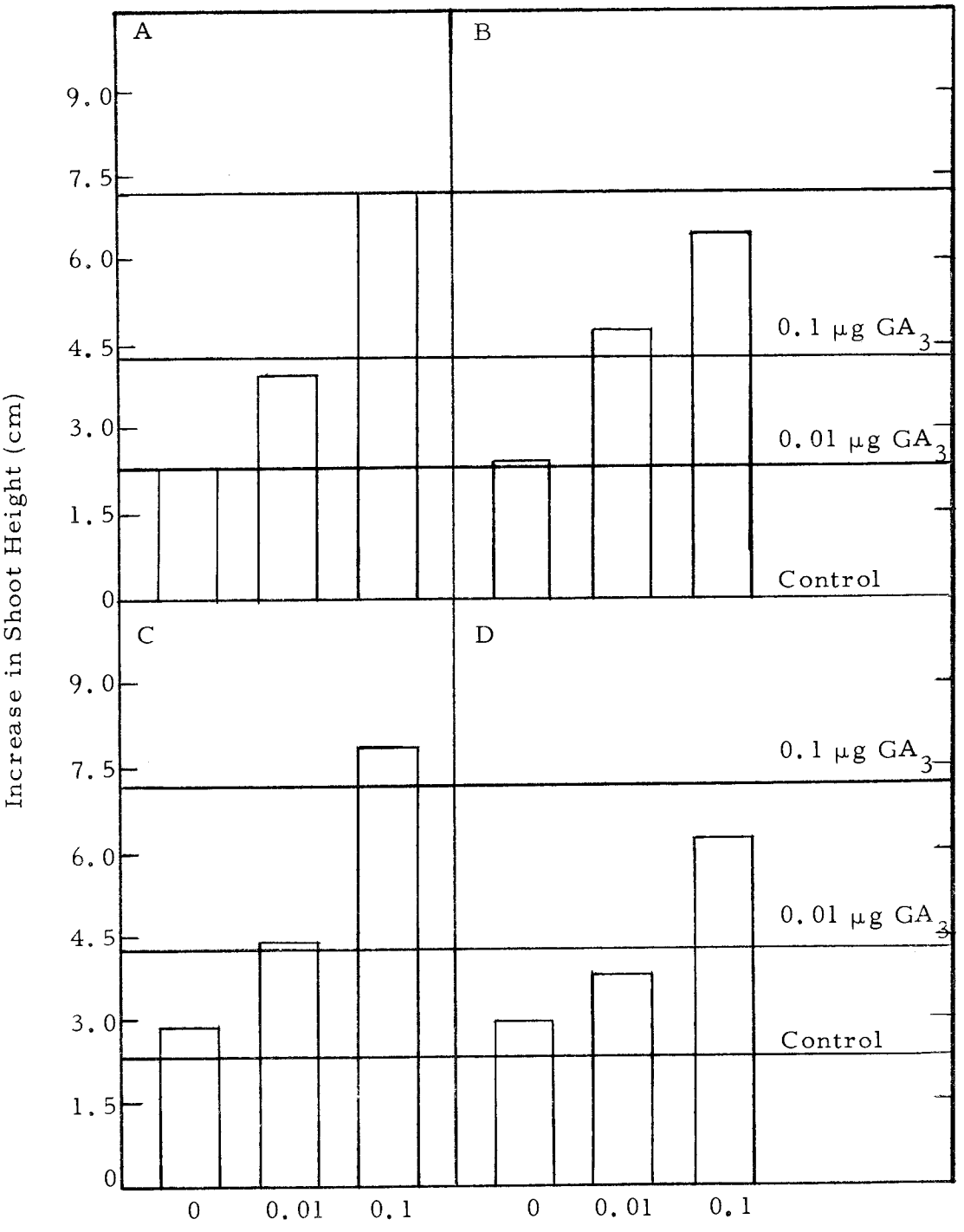
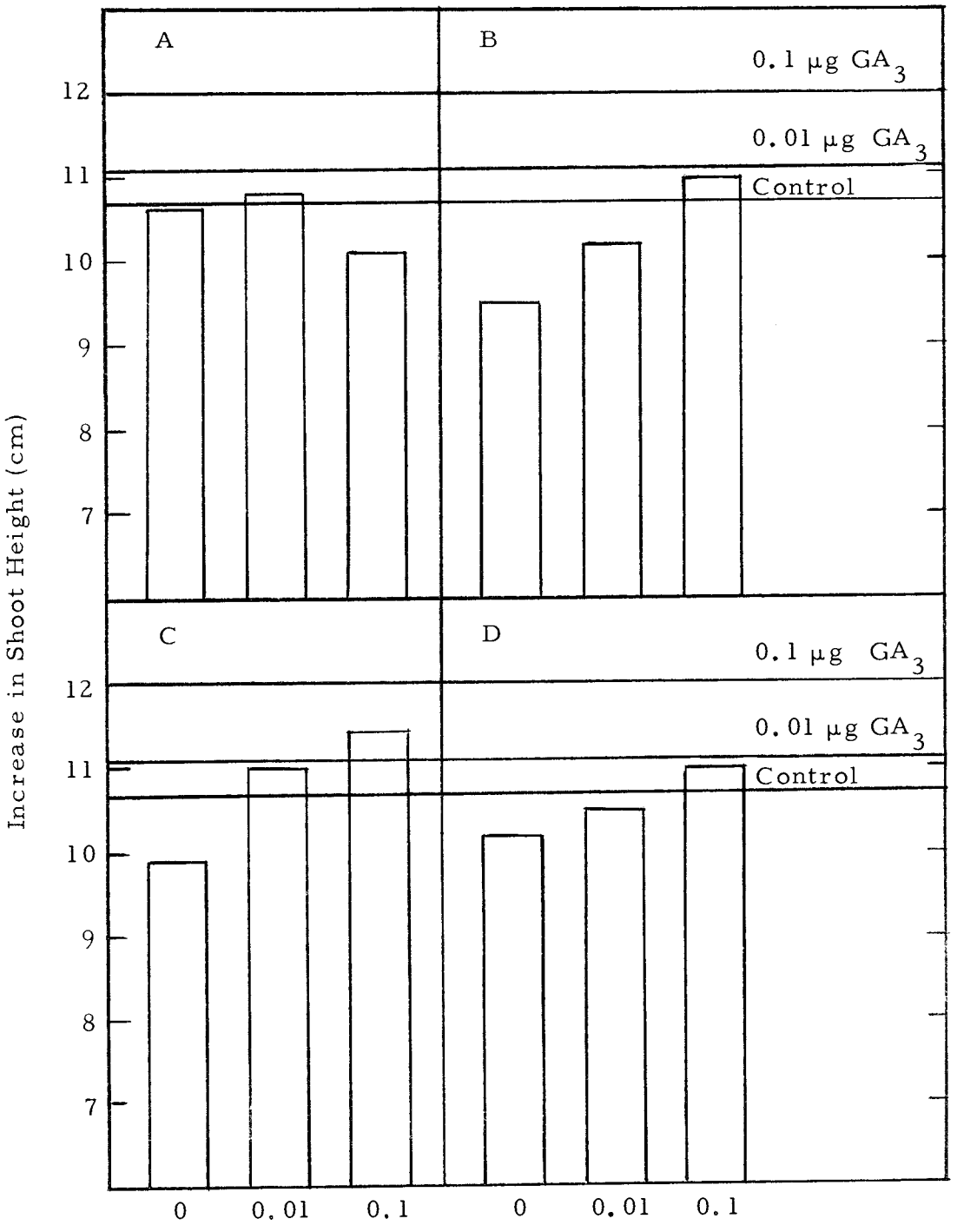

A
B
C
D
9.0
7.5
6.0
4.5
3.0
1.5
0
0.1 μg GA3
0.01 μg GA3
Control
0
0.01
0.1
Increase in Shoot Height (cm)
μg of GA3 Applied to Bioassay Plants Treated with Alkaline Extracts

Figure 7

The effects of the alkaline extracts (pH 8.4) on the growth response of light-grown Tall Telephone peas to applied GA_3. A, B, C and D are the same as in Figure 6. The horizontal lines denote the growth increments for plants which were not treated with alkaline extract, but which were treated only with 0, 0.01 or 0.10 μg GA_3. Extract from 7.5 plants was applied to each bioassay plant. The number of plants in each treatment group was 9 to 13.


A
B
0.1 μg GA3
0.01 μg GA3
Control
C
D
0.1 μg GA3
0.01 μg GA3
Control
Increase in Shoot Height (cm)
12
11
10
9
8
7
0
0.01
0.1
0
0.01
0.1
μg of GA3 Applied to Bioassay Plants Treated with Alkaline Extracts

were toxic or lethal and lower levels of extract had to be used. The results presented in Figure 6 are the effects of the alkaline fraction on the growth response of Dwarf Telephone peas to applied GA_3. The results presented here do not indicate the presence of an inhibitor of GA action. When these same extracts were applied to light-grown Tall Telephone seedlings (Figure 7), there was some inhibitory effect, even in the absence of exogenous GA. However, the amount of inhibition was small and no definite correlation could be shown between the amount of inhibition induced by an extract and growth rate of the plants from which the inhibitor was extracted. Perhaps the inhibition of GA action was somewhat greater by extracts from light-grown dwarf seedlings than by the other extracts.

Effect of Amo-1618 on the Growth of Etiolated and Light-Grown Tall and Dwarf Peas

The results of treatment of seeds of Tall and Dwarf Telephone peas with Amo-1618 on subsequent etiolated shoot elongation are presented in Figure 8. The results indicate that Amo-1618 inhibited etiolated shoot elongation of both Tall and Dwarf Telephone peas, presumably because GA biosynthesis is inhibited, and that the dwarf variety was more sensitive to a lower level of the retardant than was the tall variety. This is most easily seen at 0.001 and 0.003 mM Amo-1618. At these concentrations the dwarf variety was inhibited

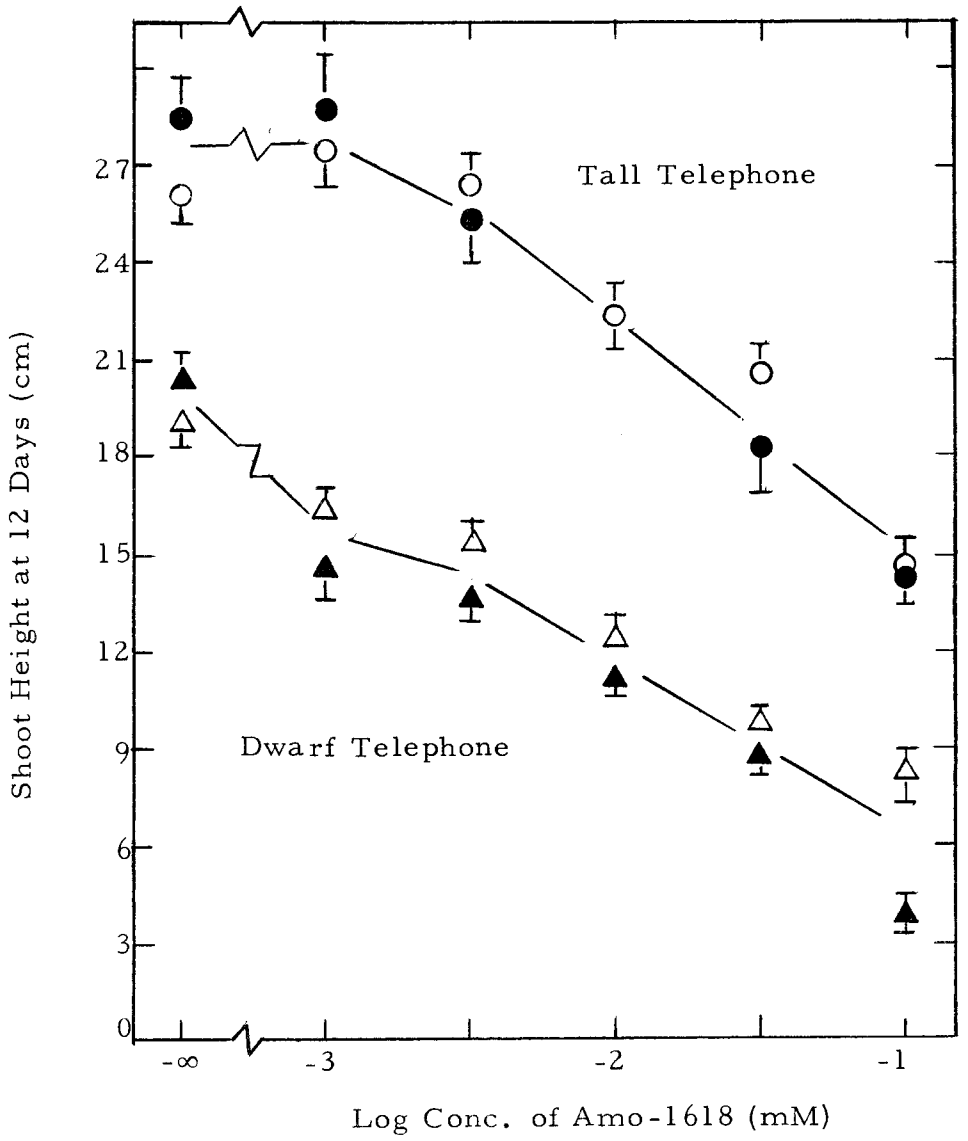


Figure 8. The effect of soaking seeds of Tall (circles) and Dwarf (triangles) Telephone peas in serial dilutions of Amo-1618 for six hours on the subsequent 12 days growth in the dark. The open and closed symbols represent different experiments. Each point represents the mean value for 9 to 22 plants. The vertical bars represent the standard errors of the means.

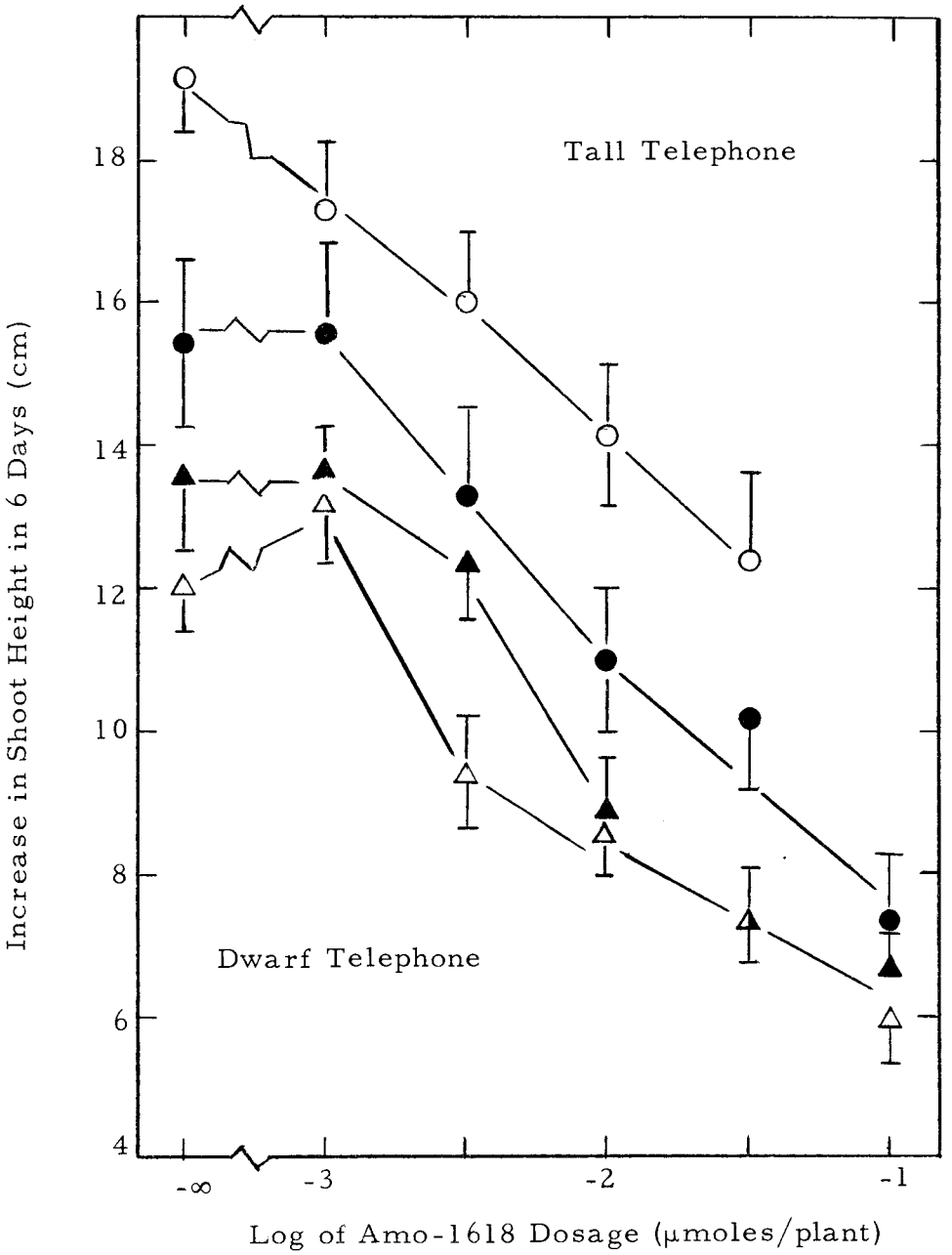
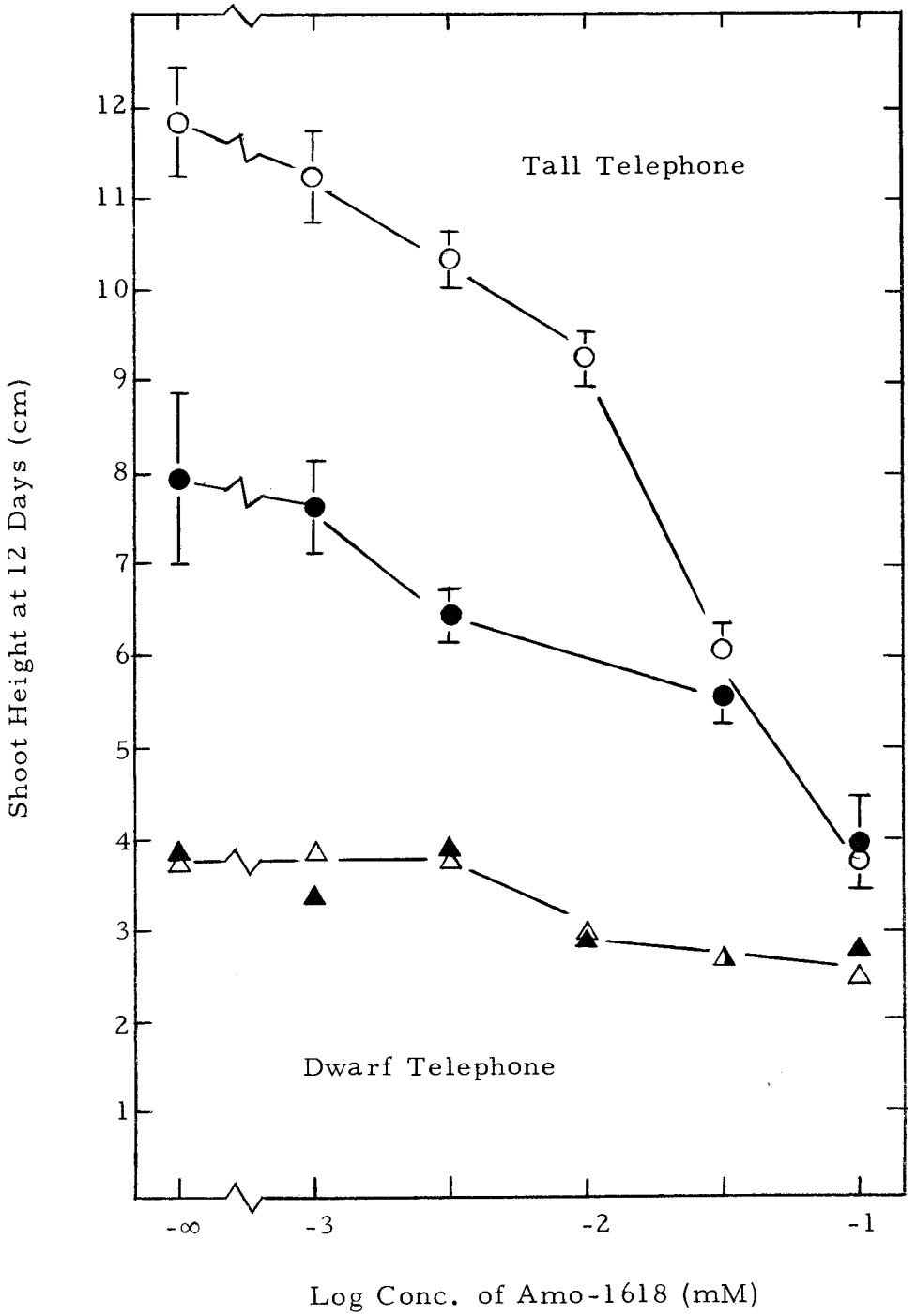


Figure 9. The effects of applications of Amo-1618 to shoot tips of six-day-old etiolated Tall (circles) and Dwarf (triangles) Telephone peas on the subsequent six days growth in the dark. Each point represents the mean value of 7 to 19 plants. The open and closed symbols represent different experiments. Vertical bars represent the standard errors of the means.

Figure 10

The effect of soaking seeds of Tall (circles) and Dwarf (triangles) Telephone peas in serial dilutions of Amo-1618 for six hours on the subsequent 12 days growth in the light. The open and closed symbols represent different experiments. The growth rates in one experiment with Tall Telephone was low due to the seeds being treated with the fungicide Captan. Each point represents from 5 to 15 plants. Vertical bars represent the standard errors of the means. The standard errors of the means are not reported for Dwarf Telephone because they were too small to plot.


Shoot Height at 12 Days (cm)
12
11
10
9
8
7
6
5
4
3
2
1
Tall Telephone
Dwarf Telephone
-∞
-3
-2
-1
Log Conc. of Amo-1618 (mM)

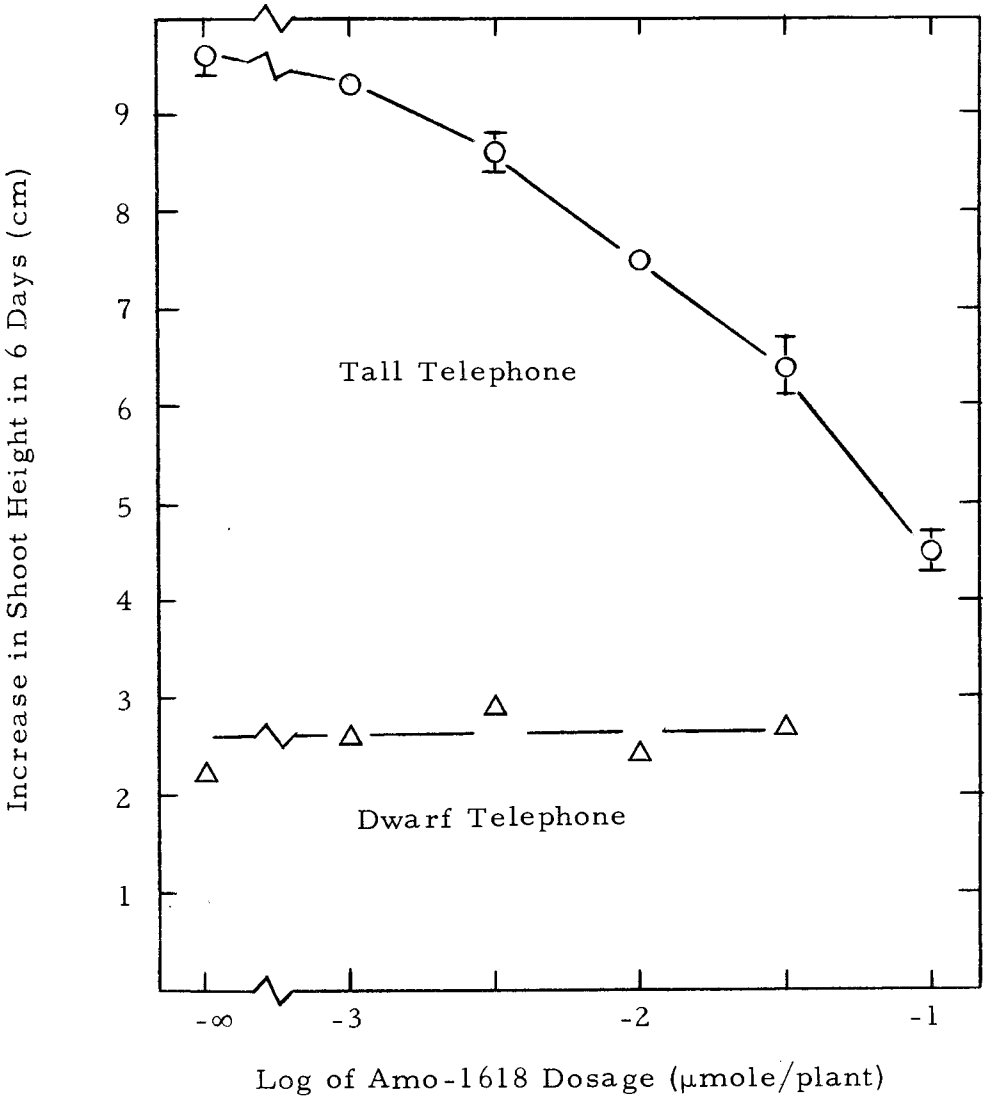


Figure 11. The effects of applications of Amo-1618 to shoot tips of six-day-old [illegible] tall (circles) and dwarf (triangles) peas on the subsequent [illegible]. Each point represents the mean value of [illegible] except the 0.032 μmoles point of Dwarf Telephone which represents only eight plants. Vertical bars represent standard errors of the means.

but the tall variety was not. Also, when the percentage inhibition was plotted against Amo-1618 concentration, the percentage inhibition of growth of dwarf seedlings exceeds percentage inhibition of tall seedlings at all concentrations. In order to confirm the validity of these results another method of treatment was employed. For this series of experiments the shoot tips of six-day-old etiolated Tall and Dwarf Telephone peas were treated with various dosages of Amo-1618 (Figure 9). These experiments showed that by this method of treatment both cultivars were equally inhibited by all dosages of Amo-1618 which were tested.

Experiments identical to those described for etiolated seedlings were performed on light-grown peas. The results of the seed treatment are presented in Figure 10. Here it can be seen that Amo-1618 did indeed inhibit the growth of light-grown Tall Telephone peas, but very little inhibition was observed in Dwarf Telephone peas. The effects of shoot tip applications of Amo-1618 on the growth of light-grown peas are illustrated in Figure 11. In these experiments the growth of tall peas was inhibited by Amo-1618, but that of dwarf peas was not.

(-)-Kaurene Biosynthesis

Product Isolation and Preliminary Identification

Strip chart scans of thin-layer chromatograms of radioactive products isolated from reaction mixtures revealed three major areas of radioactivity (Figure 12). On some chromatograms there was an indication that the peak of radioactivity nearest the origin represented two or more compounds. The second component, which moved to an R_f of approximately 0.4, also represents at least two unidentified compounds. The third peak, $R_f \sim 0.9$, which will be referred to as the ^{14}C-product or presumptive (-)-kaurene, migrated with unlabeled (-)-kaurene when the two compounds were co-chromatographed on thin-layer plates coated with silica gel G and developed in hexane. The R_f values determined for the ^{14}C-product agree well with the R_f values reported for (-)-kaurene by Graebe et al. (1964) and Dennis et al. (1964).

Experiments with gas-liquid chromatography indicated that the presumptive ^{14}C-kaurene was homogeneous (Figure 13B). The labeled product exhibited the same retention time as one component of the unlabeled (-)-kaurene supplied by Prof. L. H. Briggs (Figure 13). The discovery upon gas chromatography that the (-)-kaurene was resolvable into two compounds was not surprising

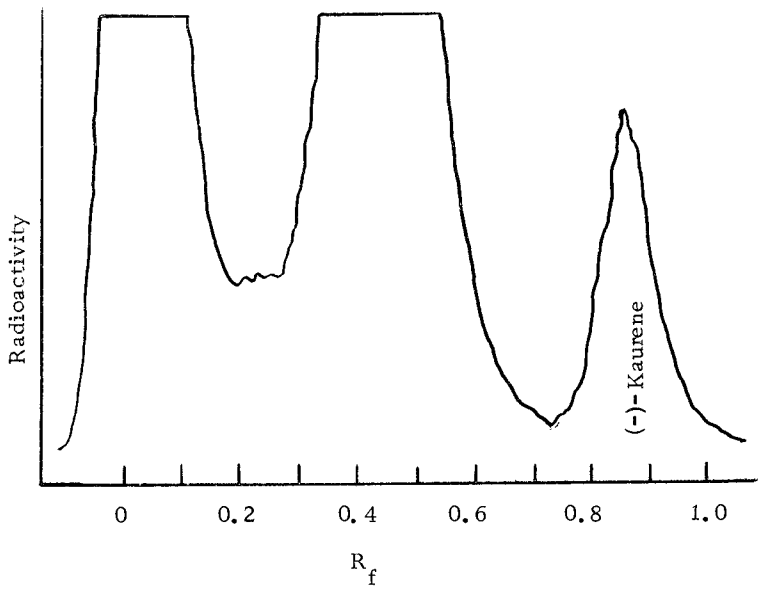


Figure 12. Strip chart tracing of a typical thin-layer chromatogram illustrating resolution of products isolated from cell-free reaction mixtures after development in hexane on silica gel G. The peak of radioactivity resolved at approximately R_f 0.9 coincided with the position of authentic unlabeled (-)-kaurene.

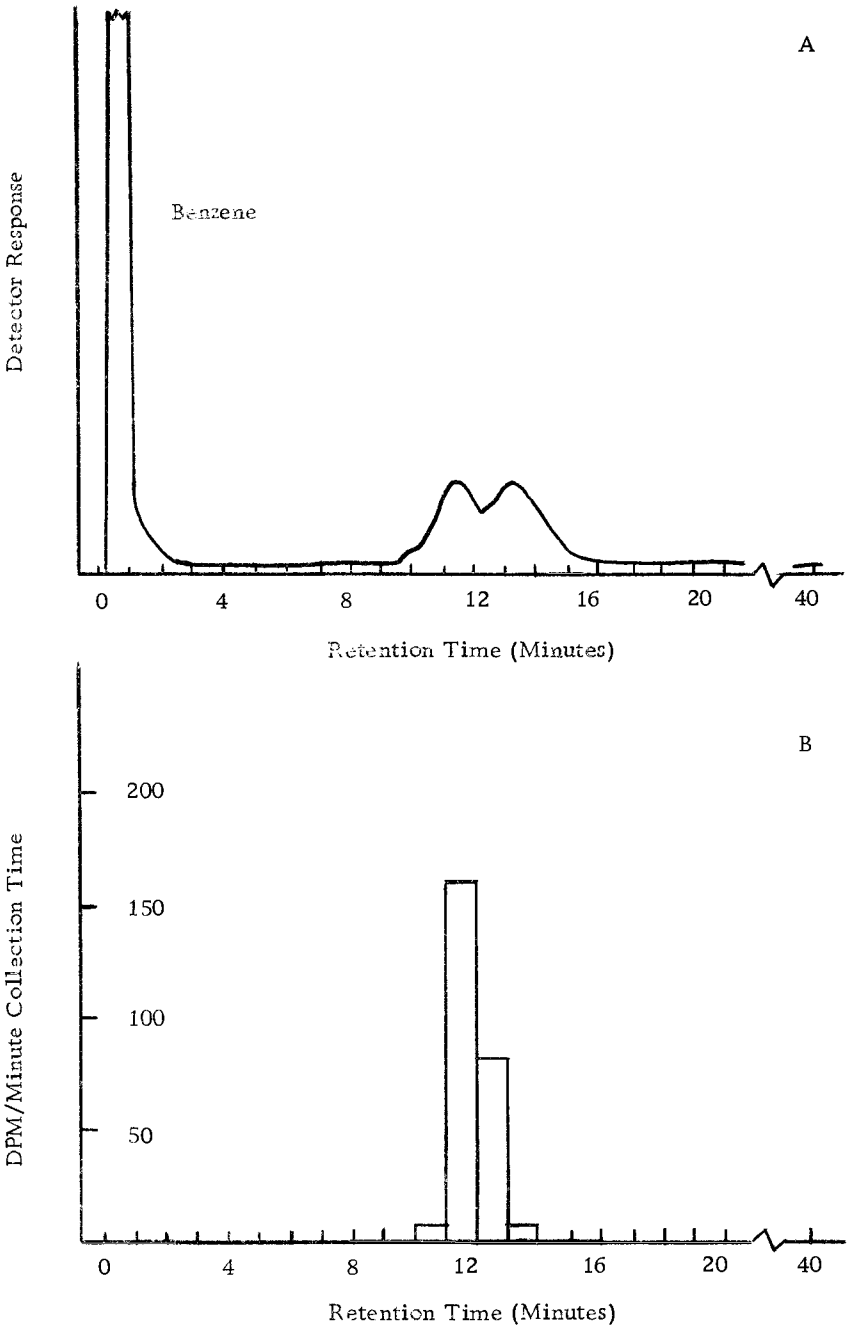


Figure 13. A, gas chromatogram of a sample of unlabeled authentic material showing two components identified as (-)-kaurene (briefest retention time) and presumptive isokaurene. B, gas chromatographic data illustrating coincident retention time of ^{14}C-product with (-)-kaurene in A.

due to the fact that Briggs[1] related that the sample of (-)-kaurene was contaminated with isokaurene; thus it seemed a likely possibility that kaurene and isokaurene were the two compounds resolved by gas chromatography. The fact that (-)-kaurene will isomerize to (-)-isokaurene and that these two isomers can be separated on silver nitrate-impregnated silica gel G plates and by gas-liquid chromatography was recently reported (Appleton et al., 1966).

In an attempt to verify the identification of the compounds in the authentic material as kaurene isomers, portions of the kaurene standard were spotted on $AgNO_3$-impregnated silica gel G plates and developed in hexane: methanol (99: 1, v/v). The result of these investigations was the resolution of two distinct spots with quite different R_f values. The more mobile compound was suspected to be (-)-kaurene, and the less mobile compound was assumed to be isokaurene. Isokaurene would be expected to migrate more slowly than kaurene[2]. The ^{14}C-product, when chromatographed on $AgNO_3$-impregnated silica gel G plates developed in hexane: methanol,

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[1] Personal communication from Professor L. H. Briggs, Department of Chemistry, University of Auckland, Auckland, New Zealand, 1967.

[2] Personal communication from Associate Professor D. J. Baisted, Science Research Institute, Oregon State University, 1967.

migrated identically to (-)-kaurene.

Another sample of (-)-kaurene, which was supplied by Prof. C. A. West, was co-chromatographed also with the ^{14}C-product on $AgNO_3$-impregnated silica gel G in hexane: methanol. When the plates were scanned a single major peak of radioactivity was seen, which coincided exactly with the position of authentic unlabeled (-)-kaurene on the chromatograms.

Preparation and Chromatography of Oxidized Derivatives

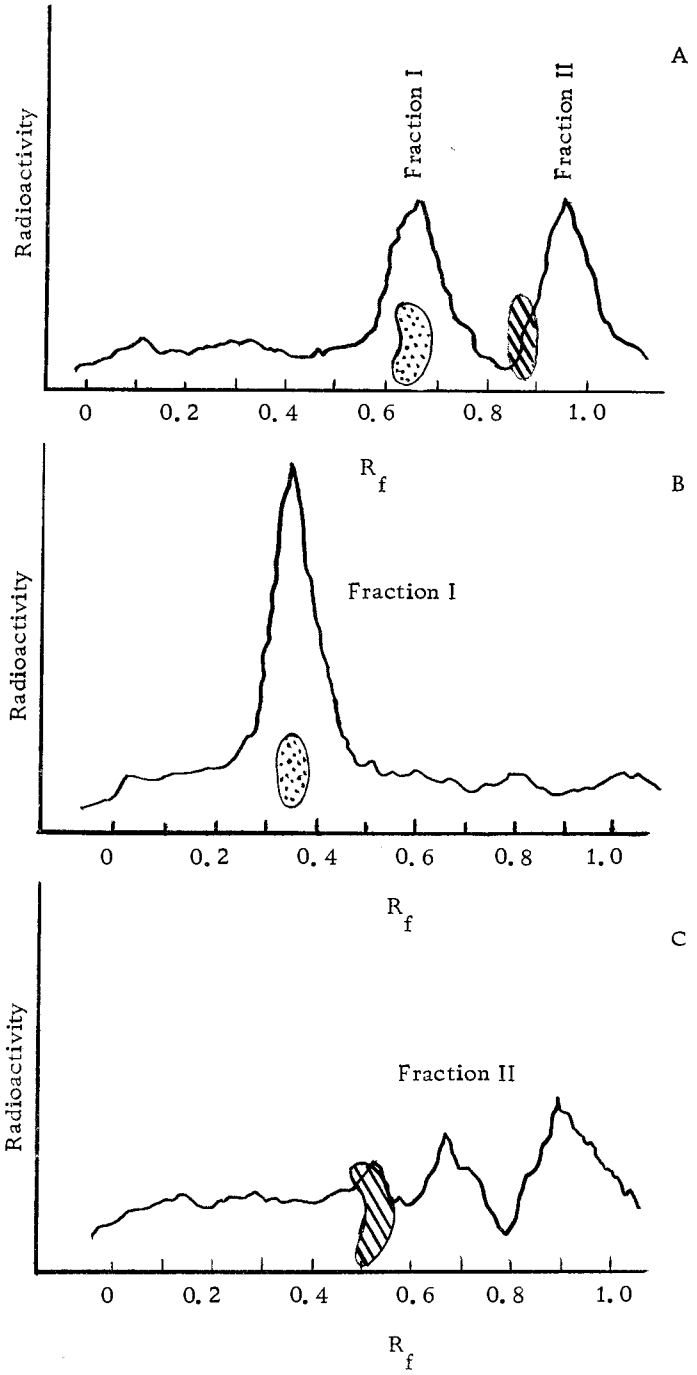
Another procedure utilized in efforts to confirm the identification of the ^{14}C-product as (-)-kaurene-^{14}C was the preparation of oxidized derivatives of both ^{14}C-product and unlabeled (-)-kaurene and co-chromatography of the derivatives. Osmium tetroxide oxidation of the exocyclic methylene group of authentic unlabeled and presumptive ^{14}C-kaurene to form kaurene-16, 17-diol was carried out according to the procedure of Briggs et al. (1963). The oxidized derivatives were chromatographed on thin-layer plates developed in hexane to remove any remaining hydrocarbon. No detectable material from either preparation was found to migrate from the origins of the chromatograms. This result indicated that no residual (-)-kaurene was present after the oxidation procedure, because (-)-kaurene exhibits an R_f of approximately 0.9 when chromatographed in hexane (Figure 12). However, examination of

chromatograms to which were applied oxidized products from authentic unlabeled (-)-kaurene and which were developed in ethyl acetate revealed two spots, indicating the presence of more than one oxidized derivative. One substance was purple and moved to R_f 0.87, and the other substance was brown and moved to R_f 0.65. When the oxidized ^{14}C-product was chromatographed also in this solvent, two areas of radioactivity appeared on the strip chart scans (Figure 14A). One peak of radioactivity (fraction I) coincided with the brown spot, R_f 0.65, and the second peak (fraction II) moved to R_f 0.96, well in advance of the purple-colored component of the unlabeled material.

Radioactive fraction I was located by scanning of plates immediately after chromatography, eluted with acetone, and was re-chromatographed in hexane:acetone (7:3, v/v). Fraction I moved to R_f 0.35, which coincided in position exactly with the co-chromatographed brown-colored compound made visible by H_2SO_4 treatment of the chromatograms (Figure 14B). When fraction II was eluted and re-chromatographed in hexane:acetone (7:3, v/v), three minor peaks of radioactivity were observed on the strip chart scans (Figure 14C). The compound from the authentic material which turned purple after treatment with H_2SO_4 coincided with one of these peaks, but the amount of radioactivity was very small. The peaks of radioactivity which appear in Figure 14C are presumed to represent products of side reactions resulting from

Figure 14

A, strip chart scan tracing of a typical thin-layer chromatogram of derivatives formed by the oxidation of ^{14}C-product with osmium tetroxide after development in ethyl acetate on silica gel G. The stippled and lined spots denote the locations of authentic, unlabeled kaurane-16, 17-diol and presumptive isokaurane-diol, respectively. The compounds denoted by stippled and lined spots exhibited brown and purple color reactions, respectively, upon being sprayed lightly with H_2SO_4 and heated. Fraction I was identified as kaurane-16, 17-^{14}C; fraction II contains unidentified products. B, strip chart scan tracing of fraction I after elution and re-chromatography in hexane:acetone (7:3, v/v) on silica gel G. Note coincident R_f with stippled spot denoting positions of authentic, unlabeled kaurane-16, 17-diol which was co-chromatographed on the same plate. C, strip chart scan tracing of fraction II after elution and re-chromatography in hexane:acetone as in B. Note minor peak at approximate R_f of presumptive (-)-isokaurane-diol, the position of which is denoted by the lined spot.


A
Radioactivity
Fraction I
Fraction II
0
0.2
0.4
0.6
0.8
1.0
B
Radioactivity
R_f
Fraction I
0
0.2
0.4
0.6
0.8
1.0
R_f
C
Radioactivity
Fraction II
0
0.2
0.4
0.6
0.8
1.0
R_f

osmium tetroxide oxidation of ^{14}C-product. The second compound present with (-)-kaurane-16, 17-diol in the oxidized unlabeled sample perhaps is the corresponding oxidized derivative of iso-kaurene.

Confirmation of the identification of fraction I as ^{14}C-kaurane-16, 17-diol was achieved by co-chromatography of oxidized ^{14}C-product with oxidized samples of unlabeled (-)-kaurene supplied by Prof. C. A. West. These studies also confirmed the identification of the unlabeled compound moving to R_f 0.65 in ethyl acetate and turning brown upon treatment with H_2SO_4 as kaurane-16, 17-diol.

Partial Characterization of the Enzyme System

Reaction rate for (-)-kaurene synthesis was found to be directly proportional to enzyme concentration in the range of 0.25 to 1.0 ml of enzyme extract/2.0 ml total incubate volume (Figure 15). Based on these results each reaction mixture contained 0.75 ml of enzyme extract/2.0 ml total volume of reaction mixture in all subsequent experiments. Investigations of the requirements for the reactions revealed that the system is heat labile and dependent on the presence of ATP and either Mn^{2+} or Mg^{2+} (Table 1). While the reaction mixtures routinely contained equimolar amounts of both Mg^{2+} and Mn^{2+}, Mn^{2+} was found to be a

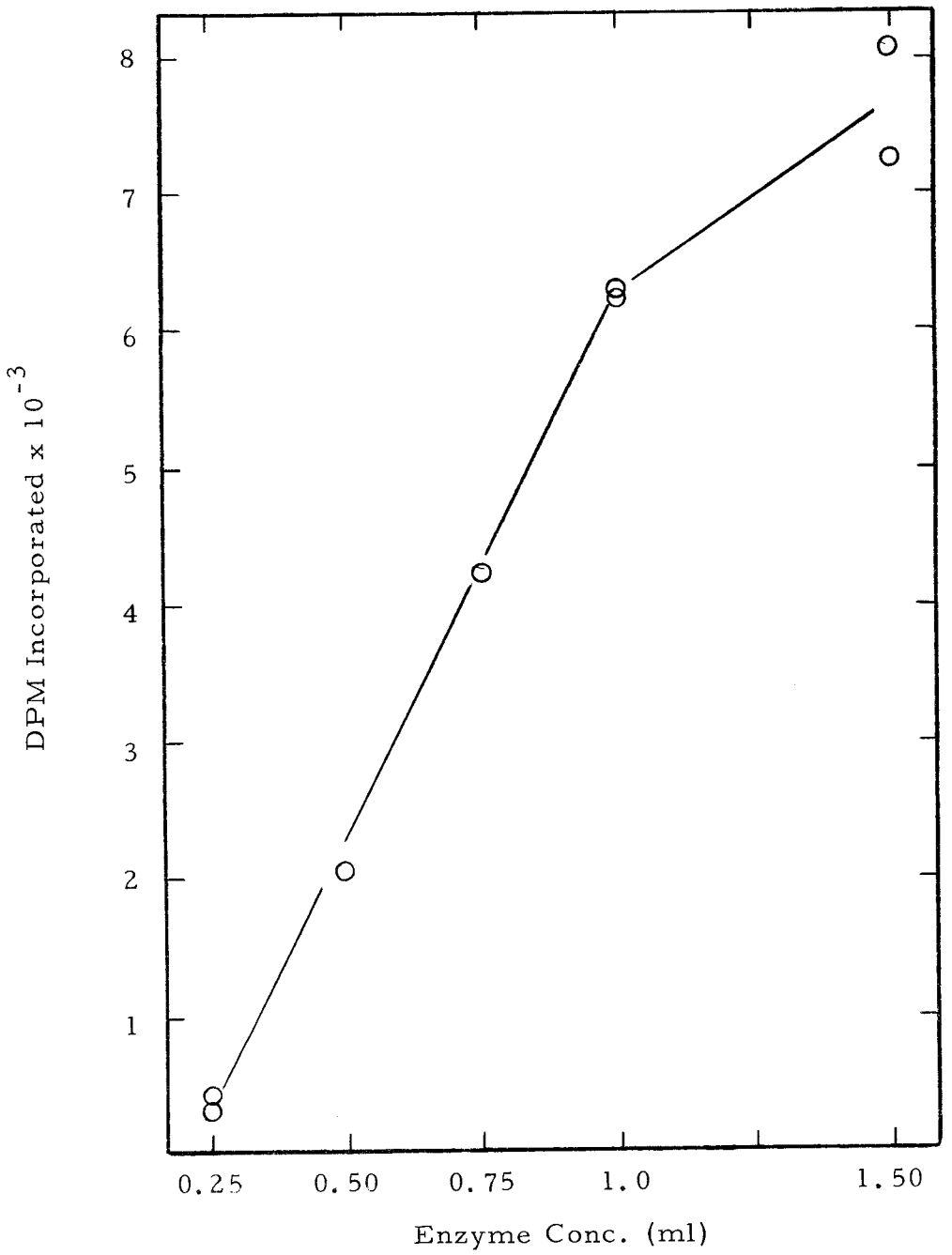


Figure 15. Incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C at various enzyme concentrations. Incubations were for one hour at 30°C. The Kjeldahl nitrogen concentration was 3.6 mg/ml of enzyme extract. Each point represents radioactivity in ^{14}C-kaurene from one reaction mixture. Radioactivity measurements were by liquid scintillation counting.

Table 1. Requirements for (-)-kaurene-^{14}C synthesis from mevalonate-^{14}C in cell-free enzyme system.

Complete reaction mixtures contained 0.75 ml of enzyme extract (3.1 mg N), 109 mμmoles of Na 2-^{14}C-mevalonate (0.548 μc), 3 μmoles each of $MgCl_2$, $MnCl_2$, and ATP made up to a total volume of 2.0 ml with 0.1 M phosphate buffer, pH 7.4. Incubations were for 1 hour at 30°. Values are the means of two replications, except the complete value which was one determination in this experiment but representative of typical values obtained in other experiments.

Conditions	Yield of ^{14}C-product dpm/mg protein N
Complete	2051
Complete minus Mg^{2+}	1905
Complete minus Mn^{2+}	497
Complete minus Mn^{2+} but with 27 μmoles Mg^{2+}	910
Complete minus Mg^{2+} and Mn^{2+}	51
Complete minus ATP	25
Complete (Boiled enzyme)	3

better activator than Mg^{2+}.

Reaction rate was linear with time up to 120 minutes (Figure 16). The rate of ^{14}C-kaurene synthesis was apparently identical for the enzyme systems isolated from both dwarf and tall peas (Figure 16).

Effects of Growth Retardants on ^{14}C-Kaurene Biosynthesis

The effects of two growth retardants, Amo-1618 and CCC, on (-)-kaurene biosynthesis were investigated. Results of the present investigation (Figure 17) revealed that Amo-1618 is a potent inhibitor of (-)-kaurene biosynthesis in cell-free extracts of immature pea seeds. An unexpected result from the present work was that CCC also inhibited (-)-kaurene production (Figure 17). CCC was a much less effective inhibitor than Amo-1618, with approximately 1000-fold higher concentrations of CCC than Amo-1618 being required to cause similar percentages of inhibition.

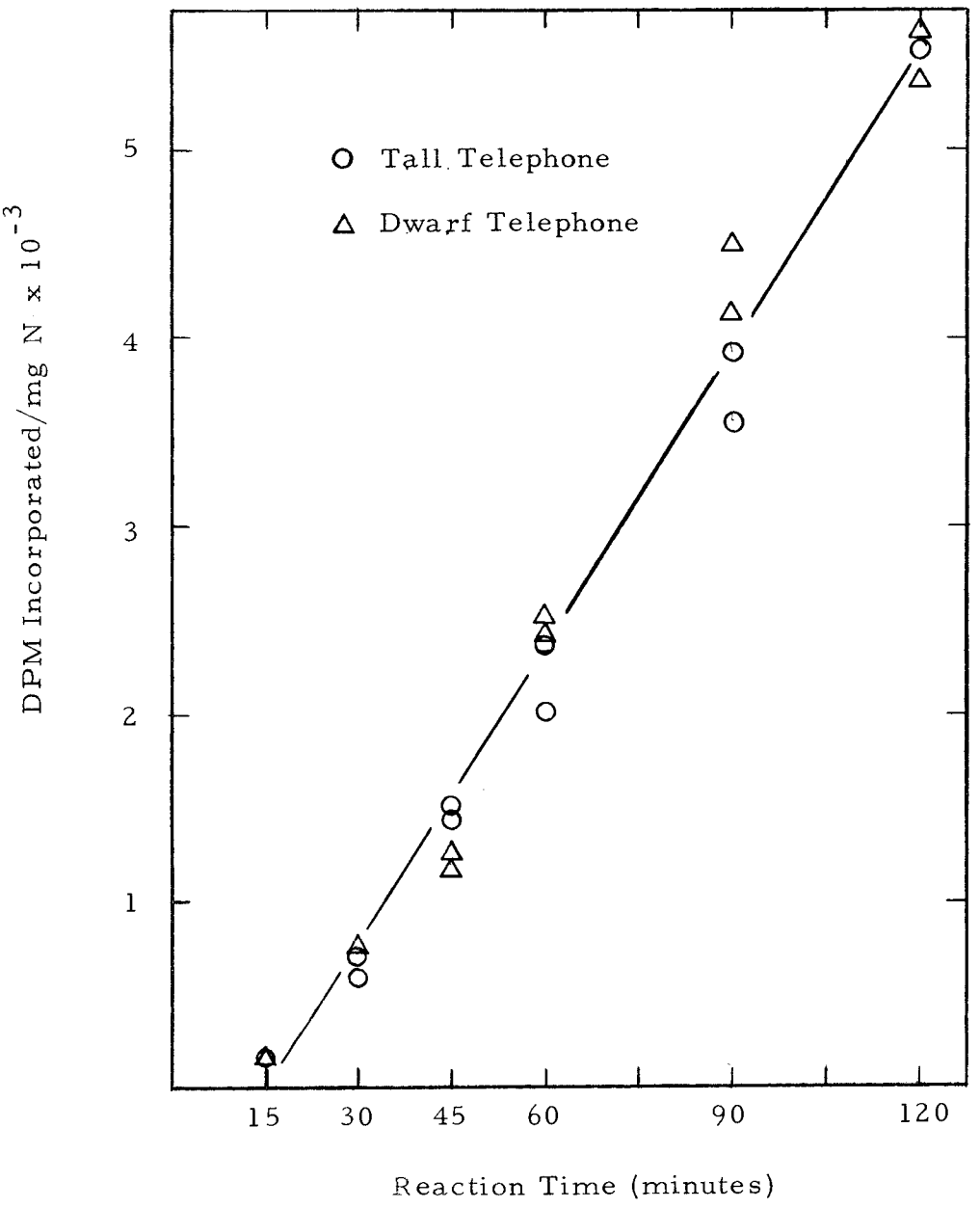
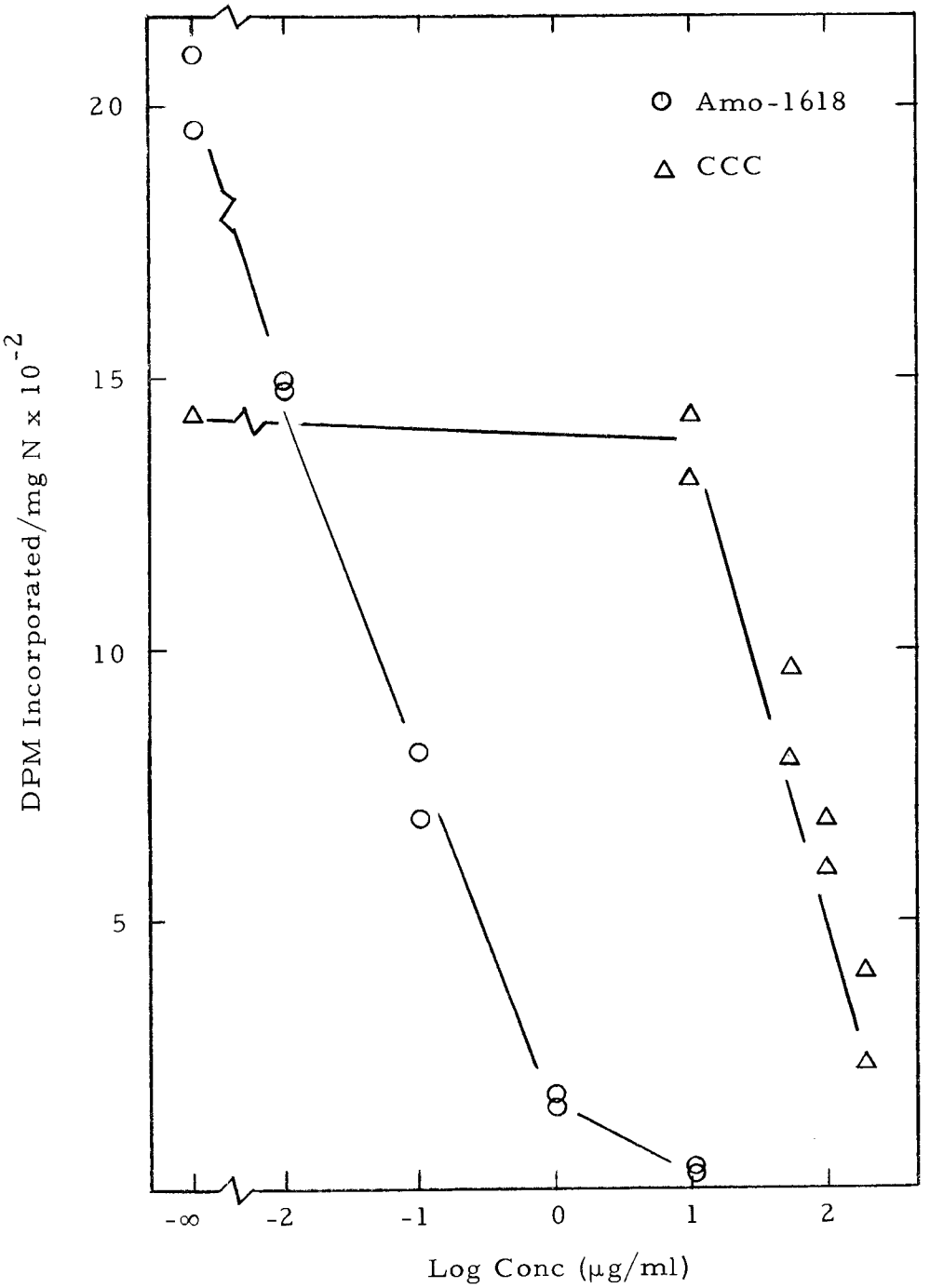


Figure 16. Time-course of incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C in cell-free extracts of Dwarf and Tall Telephone pea seeds. Each point represents radioactivity in ^{14}C-kaurene from one determination. There were two replicates for each time period for each variety. Each 2.0-ml reaction mixture contained 0.75 ml of enzyme extract (2.93 and 3.08 mg N for Tall and Dwarf Telephone, respectively), 3 μmoles each of $MgCl_2$ and $MnCl_2$ and ATP, 98 mμ moles of Na 2-^{14}C-mevalonate (0.493 μc) and pH 7.4 phosphate buffer. Measurements of radioactivity were by liquid scintillation counting.

Figure 17

Effects of Amo-1618 and CCC on incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C in standard cell-free reaction mixtures. Reaction mixtures were prepared as described in legend for Figure 16 and were incubated for one hour at 30°C. The extract in the Amo-1618 and CCC experiments contained 2.88 and 2.81 mg N, respectively. Each point represents radioactivity in ^{14}C-kaurene from one determination; there were two replicates for each determination. Measurements of radioactivity were by liquid scintillation counting.


Amo-1618
CCC
DPM Incorporated/mg N x 10^{-2}
20
15
10
5
-∞
-2
-1
0
1
2
Log Conc (μg/ml)

DISCUSSION

Comparative Growth Rates of Etiolated and Green Dwarf and Tall Pea Seedlings

A comparison of the growth rates of etiolated and green pea seedlings revealed the following order: etiolated tall > etiolated dwarf > light-grown tall > light-grown dwarf. The growth rate of etiolated tall pea seedlings has been shown to be the maximum growth rate attainable for tall genotypes; etiolated tall seedlings do not respond to exogenous GA (Lockhart, 1956). Etiolated dwarf peas exhibit a comparatively rapid growth rate but these seedlings do respond slightly to applied GA, so that their growth rate approaches but does not exceed that of etiolated tall peas (Lockhart, 1956, 1959). Light-grown tall peas exhibit the next fastest growth rate and will respond to exogenous GA if they are treated either before they enter the "logarithmic" phase of growth or immediately after the "logarithmic" phase of growth (Moore, 1967a). A comparison of etiolated tall plants and light-grown tall plants shows that the growth rates of these two kinds of plants eventually become approximately equal (Lockhart, 1959). Light-grown dwarf peas have the slowest rate of growth and will respond very markedly to applied GA (e.g., Brian and Hemming, 1955). The growth rate of dwarf peas will approach that of light-grown tall plants when the former are

treated with GA. All of these observations support the hypothesis that endogenous GA is rate-limiting for stem elongation in all but etiolated pea seedlings of tall genotypes.

Extractable Gibberellins in Peas

Seedlings from which gibberellins were extracted were harvested when they were 12 days of age, because at this stage of development all the seedlings were exhibiting nearly constant rates of stem elongation and the differences in shoot length among the four kinds of seedlings were marked (Figure 1). It is the opinion of the author that peas in the "logarithmic" phase of growth contain higher concentrations of GA than at any other time in ontogeny. This hypothesis is supported by the fact that light-grown tall pea varieties respond to exogenous GA_3 before entering the "logarithmic" phase of growth, but once they have entered the "logarithmic" phase of growth they no longer respond, presumably because the in vivo system is already saturated with GA (Moore, 1967a). In contrast, light-grown dwarf peas respond to applied GA_3 any time throughout ontogeny.

The results of bioassaying the different extract fractions from etiolated tall and dwarf peas for GA-like activity (Figures 2 and 3), indicated that the tall peas contained more presumptive GA_5 activity than did the dwarf plants. But, as pointed out previously, a duplicate

experiment yielded substantially lower GA-like activity than those reported here. The other experiment also indicated, however, that extracts of etiolated tall peas contained more GA-like activity than did extracts of the dwarf plant. Further evidence which could suggest that etiolated tall peas synthesize more biologically active GA than do etiolated dwarf peas can be found in Figure 8. These results showed that etiolated Dwarf Telephone peas are more sensitive to any given concentration of Amo-1618 than are etiolated Tall Telephone peas, especially at the lower concentrations where the tall variety is not inhibited but the dwarf variety is. These results could be interpreted to signify that etiolated dwarf peas do indeed synthesize less GA than the tall. To confirm this hypothesis another method of treatment with Amo-1618 was employed. In these experiments, the shoot tips of six-day-old etiolated Tall and Dwarf Telephone peas were treated with various concentrations of the growth retardant. The results (Figure 9) were somewhat surprising in that etiolated plants of both cultivars were inhibited to the same degree (percentage inhibition). A possible interpretation of these results is that established etiolated tall and dwarf peas synthesize GA at the same rate. However, in view of the differences exhibited in the growth rates of the two etiolated cultivars (Figure 1), the apparent differences in extractable GA (Figures 2 and 3), and the difference in sensitivity to Amo-1618 when applied as a seed

treatment (Figure 8), it would seem that a different interpretation is necessary. The difference in results of the two methods of Amo-1618 application possibly could be explained by the fact that with the shoot tip applications all centers of GA biosynthesis were not treated. It is generally accepted that the shoot tip, including the young developing leaves is the primary site of GA biosynthesis (Jones and Phillips, 1966; Jones, 1967; Lockhart, 1957), but some limited evidence is available which implicates the root system also as a site of GA biosynthesis (Butcher, 1963; Carr, Reid and Skene, 1964; Jones and Phillips, 1966; Reid and Carr, 1967; Sitton, Richmond and Vaadia, 1967).

The conclusions that one could make from these results would be that etiolated tall peas do contain more extractable, biologically active GA than do etiolated dwarf peas, presumably because the tall plants manifest a greater rate of biosynthesis than the dwarf plants. Furthermore, it seems clear that GA is essential for the growth of etiolated peas, despite the suggestion by Köhler (1965b) that etiolated peas need little if any GA for growth. This proposal by Köhler (1965b) was based on the fact that the growth retardant CCC would inhibit the growth of light-grown tall peas, but would inhibit only to a small extent the growth of etiolated tall peas. Because of the findings of the present investigation that the growth retardant Amo-1618 does inhibit the growth of dark-grown peas and that this

inhibition can be reversed by GA application (Figures 2 to 5), it would seem that GA is indeed required for the growth of peas grown in the dark. The fact that Amo-1618 will inhibit the growth of etiolated peas and that this inhibition can be reversed by GA application has been shown also by Kende and Lang (1964).

The results of bioassaying the different fractions of the GA extracts from light-grown tall and dwarf peas are given in Figures 4 and 5. The results of these experiments were somewhat surprising in that no detectable difference could be found in the presumptive GA_5 content of light-grown tall and dwarf peas, as was reported by Köhler (1965b). Köhler reported finding eight times more presumptive GA_5 activity in red-light-grown tall peas than he did in red-light-grown dwarf peas. In another experiment not reported here in detail, extracts from light-grown dwarf peas were found to contain much more presumptive GA_5 activity than did light-grown tall peas, or just the opposite of what Köhler (1965b) reported. Köhler (1965a) has also reported that red-light-grown tall peas contain ten times more GA_5 activity than do etiolated tall peas. Hence, the results of the present investigation are not in agreement with the findings of Köhler (1965a, b).

At least two different GA's (GA_1 and GA_5) are known to be found in peas (Kende and Lang, 1964). The results of the present investigation showed that only one sequence of fractions isolated by

partition column chromatography exhibited GA-like activity. All of this activity is presumably attributable to GA_5. However, in one experiment, there was an indication of some GA-like activity in the fractions where GA_1 would be expected to come from the column. The fact that only one fraction of GA-like activity could be detected in most of the acid extracts is in good agreement with the findings of Köhler (1965b) who also found just one sequence of fractions with GA-like activity from column chromatography, which he assumed to be due to GA_5. McComb and Carr (1958) isolated a GA-like substance from extracts of Tall Telephone pea shoots which was not GA_1 or GA_3. Several possible explanations exist as to why GA_1 activity could not be detected. One possibility could be varietal differences. In the present investigation both the dwarf and the tall cultivars were late flowering varieties (Moore, 1964) whereas the pea variety used by Kende and Lang (1964) was a dwarf variety which flowered early. The flowering habits of the varieties used by Köhler (1965a, b, 1966) are not known to this author. There is the possibility that different flowering varieties contain different GA's. A second possibility could be the difference in ages of the plants that were extracted. In the present investigation 12-day-old seedlings were used, whereas, the seedlings used by Kende and Lang (1964) were only seven days old and those of Köhler were ten days (1965b) or seven days (1965a, 1966). The in vivo rate of

GA_1 synthesis in the plants used in this investigation may have been such that the GA_1 was immediately metabolized in the growth process to some inactive compound as described by Kende and Ginzburg (1967), or it may be found in some unextractable form. These authors fed ^3H-GA_1 to dwarf peas and after three days, 50% of the ^3H from ^3H-GA_1 was found in some acidic compound which was biologically inactive. Thus, it is conceivable for the rate of GA_1 biosynthesis to be such that all the GA_1 that is being synthesized in vivo is used up in the growth process and, therefore, one would not be able to extract any detectable GA_1 by standard procedures.

It is known that GA_5 will evoke a very small growth response in light-grown dwarf peas, but that etiolated tall and dwarf peas, as well as light-grown tall peas which are artificially dwarfed with Amo-1618, will respond quite well to applied GA_5 (Kende and Lang, 1964). The explanation of these results was simply that light somehow decreased the sensitivity of light-grown dwarf peas, and to a lesser extent that of light-grown tall peas, to GA_5. Instead of a decrease in tissue sensitivity the possibility does exist for light to induce an inhibitor of GA_5 action. Inhibitors which lower the growth response of dwarf peas to applied GA have been found in peas and the level of these inhibitors was the highest in dwarf peas grown in red-light (Köhler and Lang, 1963). However, these inhibitory

substances have been bioassayed only with GA_3 and it is not known whether these compounds will lower the growth response of peas to other GA's. The results of Simpson and Wain (1961) also indicated that light induces an inhibitor of growth in dwarf pea seedlings and the action of this inhibitor could be readily overcome by GA_3 treatment. Thus, the observation that GA_5 will not induce a large growth response in light-grown dwarf pea seedlings may be explained on the basis that an inhibitor prevents its action instead of the tissue becoming less sensitive.

In view of recent findings, the possibility exists that Kende and Lang (1964) misinterpreted their results. Some recent evidence strongly suggests that GA_5 is not an active GA itself but, acts as a precursor to GA_1 in peas (Jones, 1967) and that GA_1 is rate limiting for the growth of all but etiolated tall peas. The author believes that an alternative hypothesis, which accommodates all the relevant data, is warranted. If GA_1 is the active GA in the growth of peas or can be readily transformed into the active GA hormone, a possible control mechanism could operate in the conversion of GA_5 to GA_1. According to this hypothesis, the conversion of GA_5 to GA_1 in etiolated plants is uninhibited; however, in green plants a light-induced inhibitor is produced which inhibits the enzyme(s) responsible for the conversion of GA_5 to GA_1. The tall varieties contain less inhibitor than do the dwarf varieties, and this inhibitor is at least

partially responsible for the difference in growth rates between light-grown tall and dwarf peas. Also contributing to the lower growth rate of the dwarf would be the apparent lower level of GA_5 (Figures 2 and 3) and GA_1 biosynthesis in etiolated dwarf peas than in etiolated tall peas (Figure 8). Therefore, it would seem that a combination of a lower rate of GA_5 biosynthesis and a lower rate of conversion of GA_5 to GA_1 would be the cause for the slower rate of growth in light-grown dwarf peas than in light-grown tall peas. The difference in rates of growth of etiolated peas would be due to lower rates of GA_5 biosynthesis in the dwarf as compared to the rate in the tall.

The actual levels of total and biologically active GA in tall and dwarf peas still remain to be definitely clarified. Even though the results presented here indicate higher GA levels in etiolated (Figures 2 and 3) than in light-grown (Figures 4 and 5) peas, the experiments not reported in detail indicated approximately the opposite relationship. The same type of variation has been reported by Kende and Lang (1964); that is, one experiment indicated that red-light-grown peas contained more GA than did peas grown in the dark and the next experiment indicated the opposite. Consequently, the actual validity of such data must be scrutinized very carefully. Possibly a quantitative correlation does not exist between the growth rate of a particular plant and the extractable GA. This idea is

supported in part by some of the results presented in this investigation and those of Radley (1958), Köhler and Lang (1963), Kende and Lang (1964) and Köhler (1965a, 1966). Other data, however, would not agree with this idea (Köhler, 1965b).

Comparative Responses of Etiolated and Green Dwarf and Tall Pea Seedlings to a Growth Retardant

When seeds of Tall and Dwarf Telephone peas were treated with the growth retardant Amo-1618, the subsequent 12 days of etiolated growth of the dwarf variety was inhibited more than that of the tall variety (Figure 8). These results indicated that seedlings of the dwarf variety grown in the dark synthesize less biologically active GA than do dark-grown seedlings of the tall variety. Also it was shown that established seedlings of tall and dwarf peas respond the same to a given dosage of Amo-1618 (Figure 9).

Experiments identical to those conducted with etiolated peas were performed on light-grown peas. The results of the seed treatment on light-grown tall and dwarf peas are given in Figure 10. The variability between the two experiments utilizing Tall Telephone peas is due to application of a fungicide, Captan, to the imbibed seeds (closed circles). However, the results obtained with the two experiments were qualitatively the same. The growth of light-grown Tall Telephone peas was markedly inhibited (> 50%); whereas light-grown dwarf peas were barely inhibited. The original rationale of

the experiments with Amo-1618 was that if the difference in growth rates exhibited by tall and dwarf peas is due to differences in the rate of GA biosynthesis then there should be an inverse relationship between sensitivity of peas to a given concentration of Amo-1618 and the rate of GA biosynthesis. Using this criterion, one could suggest that because the light-grown tall variety is much more sensitive to Amo-1618 than is the light-grown dwarf variety, the tall synthesizes less GA than the dwarf. However, a different and more plausible interpretation is possible when one considers the striking differential effect of light on internode elongation in dwarf and tall plants. Because of differential photoinhibition of internode elongation, one probably would not expect to obtain the same results with light-grown plants as with etiolated plants, because of the anatomical limitations involved with light-grown dwarf varieties; that is, light-grown dwarf plants simply cannot be inhibited much more than they already are. Consequently, at least with light-grown dwarf pea varieties, there is little opportunity to study the shoot inhibition caused by Amo-1618 when light induces so much inhibition itself.

The results of shoot tip application of Amo-1618 to light-grown Tall and Dwarf Telephone peas are given in Figure 11. These results are very similar to those obtained with the seed treatment, in that the tall variety is quite sensitive to Amo-1618 and the dwarf does not appear to be sensitive at all. Hence, the results of treating

light-grown tall and dwarf peas with Amo-1618 are not conclusive in determining whether the differences exhibited in the growth rates of light-grown tall and dwarf peas are due to differences in endogenous GA biosynthesis.

Hypothetical Inhibitors of Gibberellin Action

The hypothetical role of inhibitors of GA action in the physiology of dwarfism in peas has been discussed by Köhler and Lang (1963). These authors have correlated the amount of inhibitor present in peas to growth rates of etiolated and light-grown tall and dwarf peas and found approximately an inverse relationship. The results obtained in the present investigation, with Dwarf Telephone peas as the bioassay plant, give no conclusive indication of an inhibitor of GA-action (Figure 6). When Tall Telephone peas were used as the bioassay plant (Figure 7) some apparent inhibition was evident, but the amount of inhibition could not be correlated with the growth rates of the plants from which the inhibitor was extracted. To determine whether inhibitors of GA action play a physiological role in dwarfism, more research must be carried out. However, it is doubtful from the data of Figures 6 and 7, that a direct correlation could be made between growth rate and the content of an inhibitor(s) whose properties would allow it to partition into an organic solvent at an alkaline pH. There is the possibility, however, that other

types of inhibitors do exist; these were not looked for in this investigation.

Biosynthesis of (-)-Kaurene from Mevalonate-^{14}C in Cell-Free Extracts of Pea Seeds

Another approach useful in attempts to compare the rates of GA biosynthesis in tall and dwarf peas would appear to be investigation of cell-free biosynthesis of GA's from radioactive isotopically labeled substrates. Preliminary experiments using ^{14}C-mevalonic acid as a substrate for enzyme extracts prepared from shoots of light-grown peas showed no detectable incorporation into any of the known GA's or (-)-kaurene. Because of the lack of success of these preliminary experiments, developing pea seeds, which are known sites of GA biosynthesis (Baldev, Lang and Agatep, 1966), of both Tall and Dwarf Telephone were used as a source of enzyme and instead of pursuing biosynthesis of GA itself, the biosynthesis of (-)-kaurene, a known C-20 intermediate in GA_3 biosynthesis (Cross et al., 1964) was investigated. The results of these experiments are found in Figures 12 to 17. Strip chart scans of thin-layer chromatograms of radioactive products isolated from reaction mixtures revealed three major areas of radioactivity (Figure 12). On some chromatograms there was an indication that the peak of radioactivity nearest the origin represented two or more compounds.

This finding is consistent with the results of Graebe et al. (1965), who found both geranylgeraniol and (-)-kaurenol at the origin on their chromatograms which were developed in hexane. The second peak, which moved to an R_f of approximately 0.4, also represents at least two unidentified compounds. The systems of Graebe et al. (1965) and Dennis et al. (1965) did not synthesize these compounds. However, the system of Robinson and West (1967) synthesized a compound which possessed chromatographic characteristics very similarly to the way the compounds isolated in this research moved. This compound of Robinson and West was identified as squalene[3]. Thus, there is a good possibility that at least one of the compounds which moves to an R_f of approximately 0.4 is squalene. The third peak, $R_f \sim 0.9$ which was believed to be (-)-kaurene, was definitely identified as (-)-kaurene by: (1) comparison with authentic (-)-kaurene on thin-layer and gas-liquid chromatography (Figures 12 and 13) and (2) oxidation of the presumed ^{14}C-kaurene and (-)-kaurene with osmium tetroxide to form the common derivative kaurane-16, 17-diol (Figure 14).

In peas the transformation of mevalonic acid through the various phosphorylated intermediates to higher isoprenoid

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[3]Personal communication from Professor C. A. West, Department of Chemistry, University of California, Los Angeles, 1967.

compounds follows the same metabolic pathways as in animals and other plants (Pollard et al., 1966). Figure 18 shows the metabolism of mevalonic acid to geranylgeranyl pyrophosphate and its cyclization to (-)-kaurene. Figure 19 illustrates the conversion of (-)-kaurene to the various GA's as proposed by Geissman et al. (1966) and Verbiscar et al. (1967).

The enzyme system reported here which synthesized (-)-kaurene from ^{14}C-mevalonate required the presence of ATP and Mg^{2+} or Mn^{2+}, with Mn^{2+} being the better divalent cation activator (Table 1). The fact that Mn^{2+} was a better activator than Mg^{2+} was not surprising in that Loomis and Battaile (1963) demonstrated that Mn^{2+} was a better activator than Mg^{2+} for mevalonic acid kinase extracted from pumpkin (Cucurbita pepo) seedlings; also, Nandi and Porter (1964) reported that Mn^{2+} is a better activator than Mg^{2+} for the carrot root and swine liver enzymes which catalyze the synthesis of geranylgeranyl pyrophosphate from isopentenyl and farnesyl pyrophosphates.

The apparent rate of (-)-kaurene biosynthesis was the same in developing seeds of tall and dwarf peas (Figure 16). Hence, if a difference in the rate of GA biosynthesis exists between tall and dwarf peas at the immature embryo stage of development, the difference would have to occur at a point in the biosynthetic pathway beyond (-)-kaurene. However, it is realized that quantitative

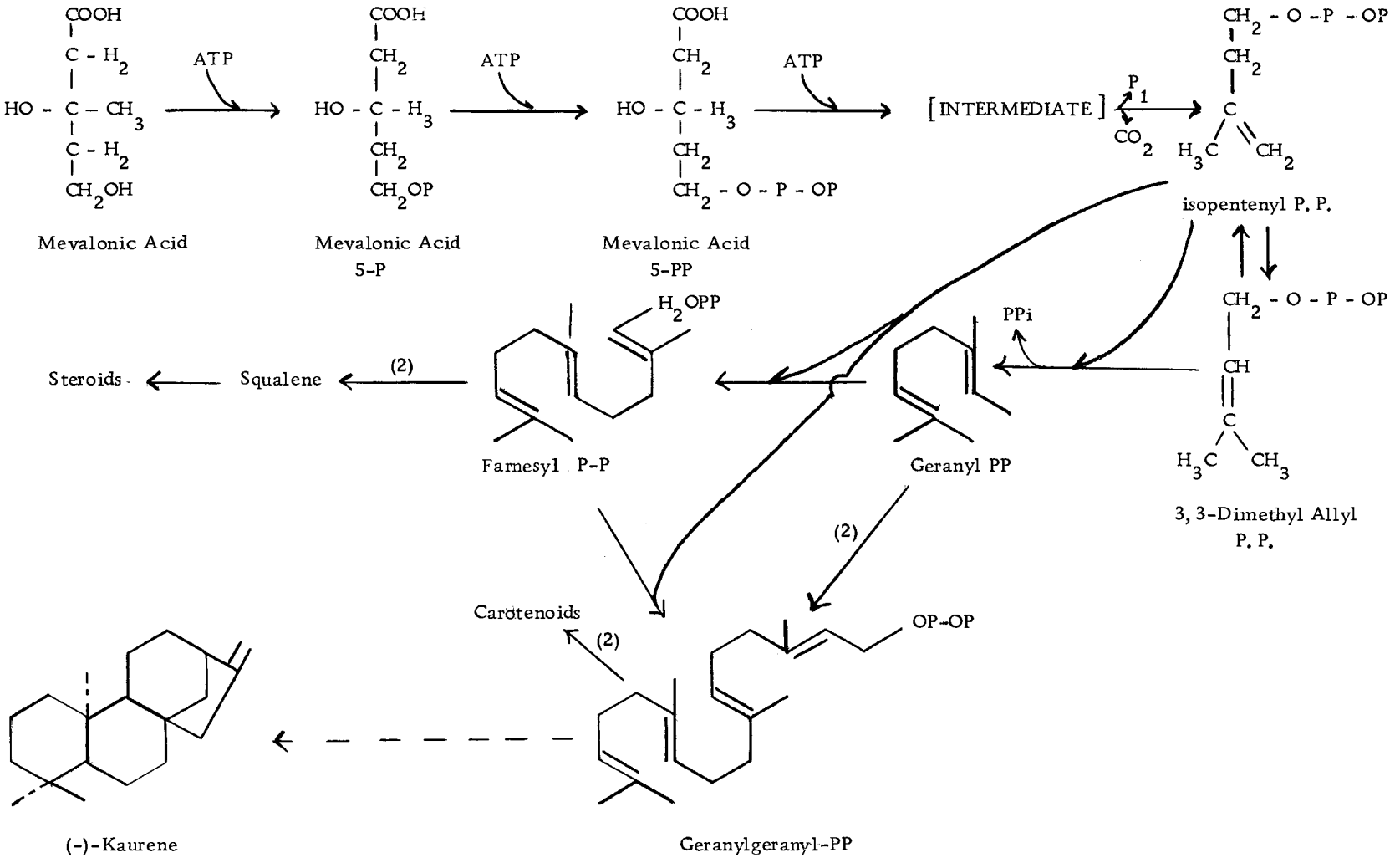

Figure 18. Metabolic transformation of mevalonic acid to (-)-kaurene through the various phosphorylated intermediates.

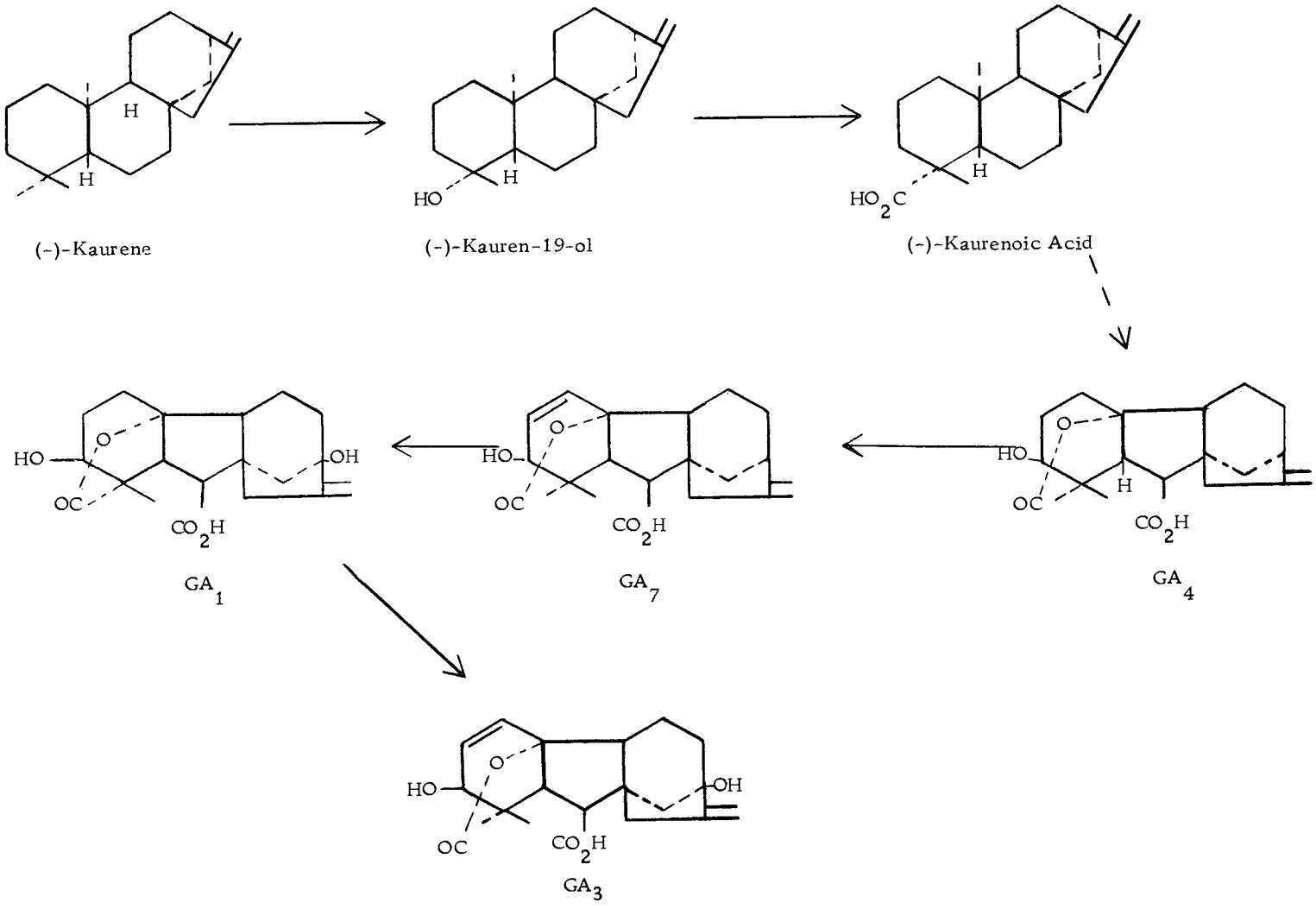

Figure 19. Metabolic transformation of (-)-kaurene to GA_3 by the fungus Fusarium moniliforme.

extrapolation of the findings from cell-free preparations to whole embryos cannot reasonably be made with absolute certainty on the basis of the evidence here presented. And, of course, genetic factors associated with differences in gibberellin metabolism might be inoperative in immature embryos but become active later in ontogeny. Very recently, Graebe (1967) reported that an enzyme system prepared from shoot tips of peas did not synthesize (-)-kaurene from ^{14}C-mevalonic acid, confirming the author's observations of preliminary experiments, but did synthesize other isoprenoids. The reason Graebe (1967) and the present author were unable to detect any (-)-kaurene biosynthesis in vitro could be due to the possibility that shoots do not synthesize (-)-kaurene. However, this would seem highly unlikely because of the data presented above on the effects of shoot tip treatment with Amo-1618 on the subsequent growth of peas.

The effects of two growth retardants, Amo-1618 and CCC, on (-)-kaurene biosynthesis were investigated. Amo-1618 is well documented as a potent inhibitor of gibberellin biosynthesis in Fusarium moniliforme (Kende et al., 1963; Zeevaart and Osborne, 1965), developing pea seeds (Baldev et al., 1965) and in Echinocystis macrocarpa endosperm-nucellus (Dennis et al., 1965). This retardant specifically inhibits the enzymic cyclization of trans-geranylgeranyl pyrophosphate to form (-)-kaurene (Dennis

et al., 1965). Ruddat (1966) has further shown that Amo-1618 inhibits the biosynthesis of the diterpenoid steviol in Stevia rebaudiana. The results of the present investigation (Figure 17) revealed that Amo-1618 is also a potent inhibitor of (-)-kaurene biosynthesis in preparations of immature pea seeds.

An unexpected result from the present work was that CCC also inhibited (-)-kaurene production (Figure 17). CCC was a very much less effective inhibitor than Amo-1618, with approximately 1000-fold higher concentrations of CCC being required to evoke similar percentages of inhibition evoked by Amo-1618. CCC has been reported to inhibit GA biosynthesis in Fusarium (Harada and Lang, 1965; Kende et al., 1963; Ninnemann, et al., 1964) in pea shoots (Köhler, 1965a) and to reduce the GA content of Pharbitis seeds harvested from previously treated parent plants (Zeevaart, 1966). However, CCC failed to inhibit kaurene synthesis significantly in Echinocystis endosperm-nucellus (Dennis et al., 1965). Therefore, it has been proposed (Dennis et al., 1965; Harada and Lang, 1965) that CCC acts at a site in the biosynthetic pathway beyond (-)-kaurene. The results reported here would suggest that either: (1) there is a difference in sensitivity of Pisum and Echinocystis enzyme systems to CCC; or (2) that the ratio of the concentrations of CCC to protein was substantially higher in the present investigations than in those reported by Dennis et al. (1965).

In any case, the present results do not disagree with the evidence that the primary site of action of CCC is beyond (-)-kaurene in the biosynthetic pathway. However, the effect described in this thesis would support the idea that perhaps CCC acts at more than one site in the pathway leading to GA production. Furthermore, there is the limited evidence for an interference of CCC with indole compound metabolism (Kuraishi and Muir, 1963; Moore, 1967b; Norris, 1966). Perhaps the mode of action of CCC is more complex than previously suspected.

SUMMARY

Experiments to determine if a correlation exists between the growth rates of tall and dwarf pea seedlings grown in the light or in the dark and the endogenous GA relations are reported. The results are summarized as follows:

1) The results of standard GA extraction and bioassay techniques indicated that: (a) only one GA-like component was resolved by column chromatography and this is presumed to be GA_5; (b) dark-grown Tall Telephone pea seedlings appeared to contain more presumptive GA_5 than did seedlings of Dwarf Telephone peas grown under the same environmental conditions; (c) there did not appear to be more presumptive GA_5 in the seedlings of Tall Telephone peas grown in the light than comparable Dwarf Telephone pea seedlings; however, some limited evidence indicated the opposite relationship; (d) the data are too variable to determine definitively whether etiolated peas contain more extractable GA than do light-grown peas or vice versa. Due to this variability, it was suggested that results obtained from extraction and bioassay techniques may not quantitatively reflect in vivo GA relationships.

2) The results of the Amo-1618 seed-treatment experiments indicated that etiolated seedlings of Dwarf Telephone peas are more sensitive to the retardant than are seedlings of Tall Telephone peas. The reason for this could be due to a lower rate of GA_1 biosynthesis in the dwarf variety. However, when established seedlings were treated, no difference in the amount of inhibition resulted, which may indicate that there is no difference in the rates of GA biosynthesis in established etiolated tall and dwarf pea seedlings.

3) A light-induced inhibitor which prevents the conversion of GA_5 to GA_1 has been hypothesized. This hypothetical inhibitor would at least partially explain the light induced inhibition of stem elongation in peas, particularly in the dwarf phenotypes.

4) A cell-free system was obtained which synthesized (-)-kaurene, a known intermediate in GA biosynthesis. The enzyme system required the presence of ATP, Mg^{2+} or Mn^{2+}, with Mn^{2+} being the better cation activator. The growth retardants, Amo-1618 and CCC both were found to inhibit (-)-kaurene biosynthesis.

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