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Biosynthesis of (−)-Kaurene in Cell-free Extracts of Immature Pea Seeds[1]

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Summary. Mevalonate-^{14}C was incorporated into (−)-kaurene-^{14}C in cell-free extracts of immature pea (*Pisum sativum* L.) seeds. The identification of ^{14}C-product as (−)-kaurene was based on: A) comparison with authentic (−)-kaurene on thin-layer and gas-liquid chromatography; and B) oxidation of ^{14}C-product and (−)-kaurene with osmium tetroxide to form the common derivative kaurane-16,17-diol. The enzyme system is heat labile and is dependent upon ATP and Mg^{2+} or Mn^{2+}, with Mn^{2+} being a more effective activator than Mg^{2+}. The reaction rate was proportional to enzyme concentration in reaction mixtures containing 0.45 to 1.8 mg protein N/ml, and was linear with time through 120 minutes in standard reaction mixtures. Enzyme preparations from immature seeds of tall and dwarf peas appeared to synthesize (−)-kaurene at the same rate. Synthesis of (−)-kaurene was readily inhibited by Amo-1618. (2-Chloroethyl)-trimethylammonium chloride (CCC) also inhibited (−)-kaurene synthesis; however, approximately 1000-fold higher concentrations of CCC were required to evoke the same percentages of inhibition as Amo-1618.

For many species of angiosperms, particularly various cultivated species, 2 or more varieties exist which are distinguishable entirely or at least partially by marked differences in rates of stem elongation and shoot heights at maturity. In the case of the garden pea (*Pisum sativum* L.), to cite an example of a species which has been investigated extensively, there are numerous cultivars, ranging from dwarf varieties that mature at heights of 30 cm or less to tall varieties that exceed a meter in shoot height at maturity (see 20). A common observation of related interest is that there are marked differences in the rates of stem elongation exhibited by etiolated and light-grown plants of the same variety (11, 13, 14, 15, 17, 18, 19, 32, 33), such differences often being of greater magnitude in the case of dwarf than in tall varieties. Thus, 2 related problems which have attracted considerable attention are: A) the biochemical basis for dwarfism, or more specifically, for differential rates of stem elongation among varieties of the same species when grown in a common environment; and B) the mechanism of the photoinhibition of stem elongation.

Solutions to both of these problems have been sought in investigations of possible correlative variations in endogenous auxins (30, 31, 32, 33) and gibberellins (11, 13, 14, 15, 17, 18, 19, 21, 27). The experimental approaches which have been used to date concerning possible qualitative or quantitative variations in endogenous gibberellin relationships have been of 2 major types: A) studies of comparative responses to applied gibberellins or gibberellin precursors (10, 11, 13, 14, 15, 17, 18, 19, 21); and B) extraction and bioassay of endogenous gibberellins (11, 13, 14, 15, 27).

A third kind of approach essential to the ultimate elucidation of comparative gibberellin relationships in seed plants is the investigation of pathways, rates, and mechanisms of regulation of gibberellin biosynthesis. Hence, development of cell-free systems with which to investigate gibberellin biosynthesis in seed plants is an important objective in current gibberellin research. Until recently, most of our knowledge concerning gibberellin biosynthesis stemmed from studies with *Fusarium moniliforme* (see 5). However, important progress toward the development of cell-free systems capable of gibberellin biosynthesis from seed plants was made recently when Graebe et al. (7) reported on the biosynthesis of (−)-kaurene, a known intermediate in gibberellin biosynthesis in *Fusarium* (5, 7), from mevalonate in cell-free homogenates of *Echinocystis macrocarpa* endosperm-nucellus. Conversion of mevalonic acid to several isoprenoid compounds, some of which are known intermediates in gibberellin biosynthesis, by an enzyme system from germinating pea seeds was reported by Pollard et al. (26). Robinson and West (28) subsequently de-

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scribed a system from germinating *Ricinus communis* seeds which is capable of producing 4 or more diterpenes including (−)-kaurene. Using a procedure modified from those of Graebe et al. (7) and Pollard et al. (26), we have developed, and here describe, a cell-free enzyme system from immature pea seeds in which a compound which we have identified as (−)-kaurene is formed enzymatically from ^{14}C-mevalonate.

Materials and Methods

Plant Material. Dwarf and Tall Telephone cultivars of *Pisum sativum* L. (W. Atlee Burpee Co., Riverside, Calif.) were grown in a greenhouse where the photoperiodic and temperature regime consisted of a 16-hour photoperiod at approximately 20° and an 8-hour nyctoperiod at approximately 17°. Seeds were excised and frozen when approximately half to two-thirds mature size, and the whole immature seeds were used in the preparation of extracts.

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 homogenizer held in an ice bath. Homogenates were centrifuged at 40,000 × *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 1 hour at 30°, unless otherwise noted, and were stopped by boiling for 5 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 was then extracted with acetone (3 × 2 ml), and the combined acetone extracts were evaporated under reduced pressure using a rotary-film evaporator. Each residue was extracted with acetone (2 × 0.2 ml + 1 × 0.1 ml), and the entire 0.5 ml of extract was applied to a 5 × 20 cm glass plate coated with 250 μ of silica gel G. Thin-layer chromatograms were developed routinely in 100 % hexane, with the solvent front advancing 15 cm from the origin. After preparative isolation of kaurene, as described above, 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 (3) 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 8 % (w/w) $AgNO_3$ solution.

Radioassay Procedures. Thin-layer chromatograms were scanned for 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 aliquots of each homogenate supernatant were measured by a micro-Kjeldahl procedure (9).

Identification of ^{14}C-Product. The ^{14}C-product, which was suspected to be (−)-kaurene on the basis of preliminary extraction and thin-layer chromatographic behavior, was co-chromatographed with authentic (−)-kaurene using thin-layer and gas-liquid chromatography. Unlabeled (−)-kaurene was kindly supplied by Prof. L. H. Briggs, University of Auckland. Samples of (−)-kaurene and ^{14}C-product were applied at a common origin on thin-layer plates and were developed in 100 % hexane. An aliquot of unlabeled (−)-kaurene and ^{14}C-product in benzene was injected onto a column (6.35 mm × 1.22 m) packed with 10 % SE-52 on Gas-Chrom Q (80–100 mesh) (Applied Science Laboratories) and developed at a column temperature of 170° 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° and 170°, 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 scintillation 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 non-radioactive (−)-kaurene and of the ^{14}C-product were combined and subjected to oxidation with osmium tetroxide (4). 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 then 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 100% hexane, hexane:acetone (7:3, v/v), and 100% ethyl acetate. After development the plates were either scanned for radioactivity or sprayed lightly with 2 N sulfuric acid followed by heating at 100° for a few minutes, and then viewed under ultraviolet radiation. Prolonged heating charred the compounds so that inspection in ultraviolet light was not necessary.

Reagents. The 2-^{14}C-mevalonic acid 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 H_2O so that 50 μl contained approximately 0.5 μc. ATP was purchased from Sigma Chemical Company. The growth retardant 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methyl phenyl piperidine-1-carboxylate (Amo-1618) was purchased from Enomoto and Company, Redwood City, California. (2-Chloroethyl)-trimethylammonium chloride (CCC) was a gift of American Cyanamid Company. All other chemicals were of reagent grade and all organic solvents were redistilled.

Results and Discussion

Product Isolation and Preliminary Identification. Strip chart scans of thin-layer chromatograms of radioactive products isolated from reaction mixtures revealed 3 major areas of radioactivity (fig 1). On some chromatograms there was an indication that the peak of radioactivity nearest the origin represented 2 or more compounds. This finding is consistent with the results of Graebe et al. (7), who found both geranylgeraniol and (−)-kaurenol at the origin on chromatograms which were developed in 100% hexane. The second peak, which moves to an R_F of approximately 0.4, also represents at least 2 unidentified compounds. Apparently, the systems of Graebe et al. (7) and Dennis et al. (6) did not synthesize these compounds. The third peak, $R_F \sim 0.9$, which will be referred to as the ^{14}C-product or presumptive (−)-kaurene, migrated with authentic unlabeled (−)-kaurene when the 2 compounds were co-chromatographed on thin-layer plates coated with silica gel G and developed in 100% 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. (7) and Dennis et al. (6).

Preliminary experiments with gas-liquid chromatography indicated that the presumptive ^{14}C-kaurene was homogeneous. The labeled product exhibited the same retention time as 1 component of the unlabeled

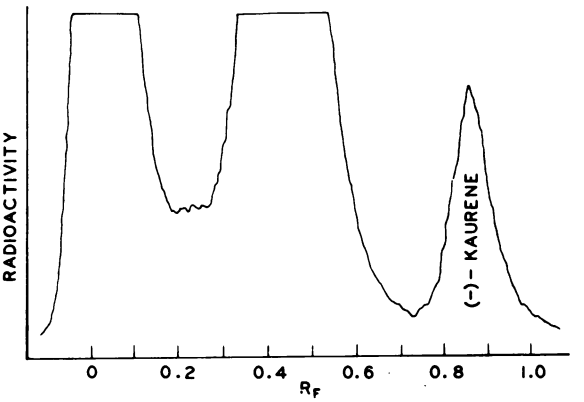

FIG. 1. Strip chart scan tracing of a typical thin-layer chromatogram illustrating resolution of products isolated from cell-free reaction mixtures after development in 100% hexane on silica gel G. The peak of radioactivity resolved at approximately R_F 0.9 coincided with the position of authentic unlabeled (—)-kaurene.

(−)-kaurene supplied by Prof. L. H. Briggs (fig 2). The discovery upon gas chromatography that the non-radioactive (−)-kaurene sample was resolvable into 2 compounds was not surprising in view of the fact (Prof. Briggs, personal communication) that the sample of (−)-kaurene was contaminated with isokaurene. That (−)-kaurene will isomerize to (−)-isokaurene and that these 2 isomers can be separated on silver nitrate-impregnated silica gel G plates and by gas-liquid chromatography has been reported previously (1).

In an attempt to verify the identification of the compounds in the authentic material as kaurene isomers and to determine which of the 2 was (−)-kaurene, portions of the sample were chromatographed on $AgNO_3$-impregnated silica gel G plates developed in hexane:methanol (99:1, v/v). The result of these investigations was the resolution of 2 distinct spots with quite different R_F values. The more mobile compound was identified as (−)-kaurene, and the less mobile compound was tentatively identified as isokaurene. Isokaurene would be expected to migrate more slowly than kaurene (Prof. D. J. Baisted, personal communication). The ^{14}C-product, when chromatographed on $AgNO_3$-impregnated silica gel G plates developed in hexane:methanol, migrated identically to (−)-kaurene.

Another sample of (−)-kaurene, which was generously supplied by Prof. C. A. West, University of California, Los Angeles, 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

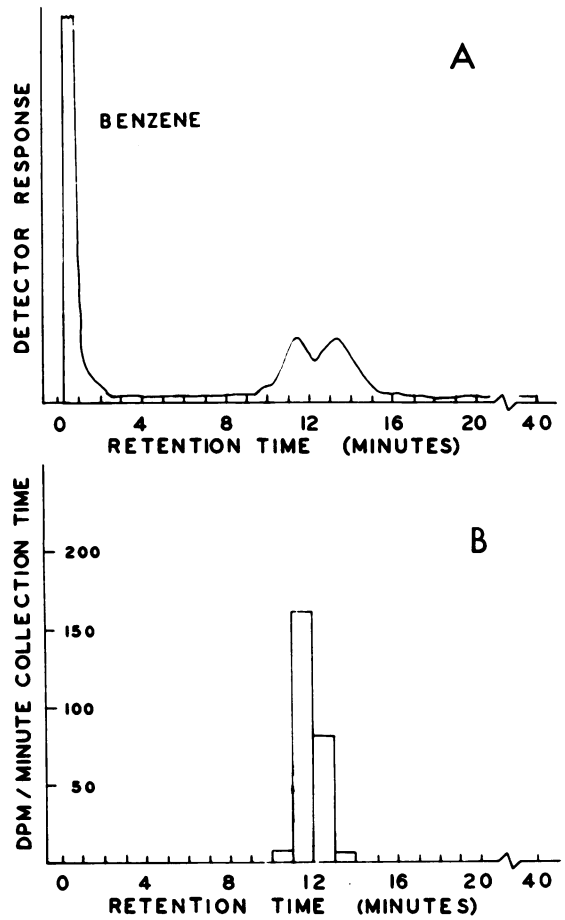

FIG. 2. A) gas chromatogram of a sample of unlabeled authentic material showing 2 components identified as (—)-kaurene (briefest retention time) and presumptive isokaurene. B) gas chromatographic data illustrating coincident retention time of ^{14}C-product with (—)-kaurene in A.

derivatives of both ^{14}C-product and authentic 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 kaurane-16,17-diol was carried out according to the procedure of Briggs et al. (4). The oxidized derivatives were chromatographed on thin-layer plates developed in 100% 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 (fig 1). However, examination of chromatograms of the oxidized products from authentic unlabeled (−)-kaurene, which were developed in 100% ethyl acetate, revealed 2 spots after treatment with H_2SO_4, indicating the presence of more than 1 oxidized derivative. One

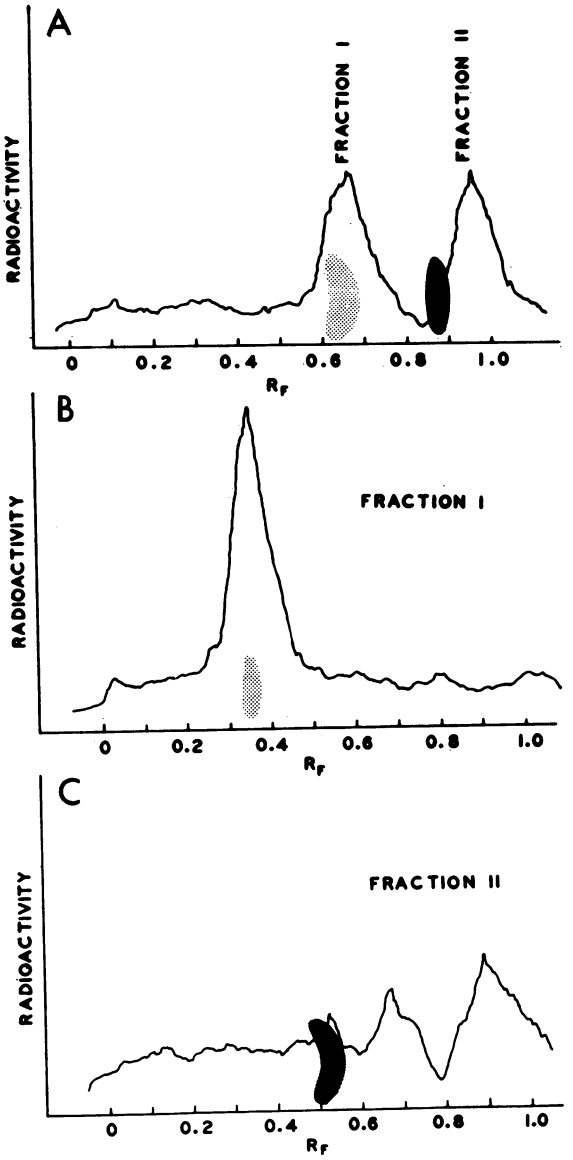

FIG. 3. A) strip chart scan tracing of a typical thin-layer chromatogram of derivatives formed by the oxidation of ^{14}C-product with osmium tetroxide after development in 100% ethyl acetate on silica gel G. The stippled and lined spots denote the locations of authentic, unlabeled kaurane-16,17-diol 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-diol-^{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 position 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.

substance was purple and moved to R_F 0.87, and the other substance was brown and moved to R_F 0.35. When the oxidized ^{14}C-product was chromatographed also in the same solvent, 2 peaks of radioactivity appeared on the strip chart scans (fig 3, A). 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 0.65, which coincided in position exactly with the co-chromatographed brown-colored compound made visible by H_2SO_4 treatment of the chromatograms (fig 3, B). When fraction II was eluted and re-chromatographed in hexane:acetone (7:3, v/v), 3 minor peaks of radioactivity were observed on the strip chart scans (fig 3, C). The compound from the authentic material which turned purple after treatment with H_2SO_4 coincided with 1 of these peaks, but the amount of radioactivity was very small. The peaks of radioactivity which appear in figure 3, C are presumed to represent products of side reactions resulting from 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 isokaurene.

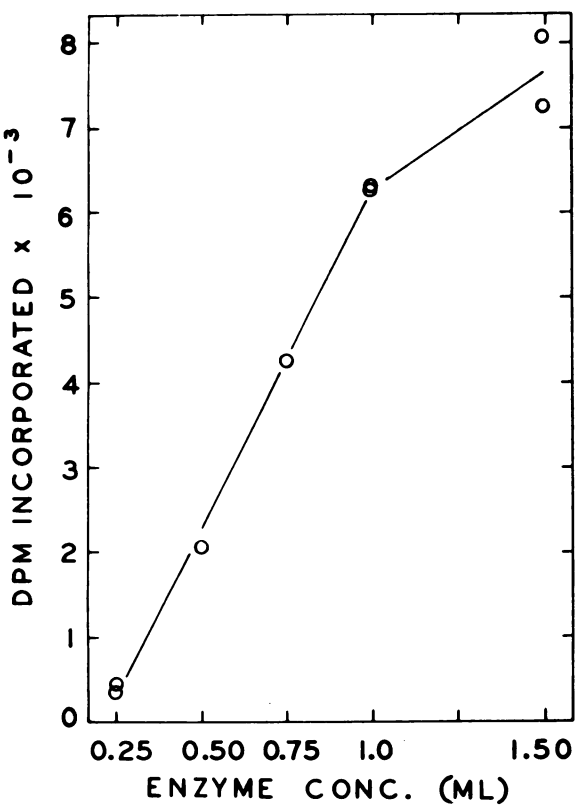

FIG. 4. Incorporation of mevalonate-^{14}C into (—)-kaurene-^{14}C at various enzyme concentrations. Each reaction mixture contained 101 mμmoles of Na 2-^{14}C-mevalonate (0.509 μc), 3 μmoles each of $MgCl_2$, $MnCl_2$ and ATP, 0.25 to 1.5 ml of enzyme extract and 0.1 M phosphate buffer (pH 7.4) in a total volume of 2.0 ml. Incubations were for 1 hour at 30°. The concentration of protein N was 3.6 mg/ml of enzyme extract. Each point represents radioactivity in ^{14}C-kaurene from 1 reaction mixture. Radioactivity measurements were by liquid scintillation counting.

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 authentic, unlabeled (−)-kaurene supplied by Prof. C. A. West. These studies also confirmed the identification of the unlabeled compound moving to R_F 0.65 and turning brown upon treatment with H_2SO_4 as kaurane-16,17-diol.

Partial Characterization of the Enzyme System. Reaction rate was found to be directly proportional to enzyme concentration in the range of 0.25 to 1.0 ml of enzyme extract per 2.0 ml total incubate volume (fig 4). Based on these results, each reaction mixture contained 0.75 ml of enzyme extract per 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 I). While the reaction mixtures routinely contained equimolar amounts of both Mg^{2+} and Mn^{2+}, Mn^{2+} was found to be a better activator than Mg^{2+}. This was a somewhat surprising result; however, Nandi and Porter (23) earlier 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 isopentyl and farnesyl pyrophosphates.

Reaction rate was linear with time up to 120 minutes, after an apparent initial lag time of approximately 15 minutes (fig 5). The rate of (−)-kaurene

Table I. *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, 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 2 replicates, except the complete value which was 1 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

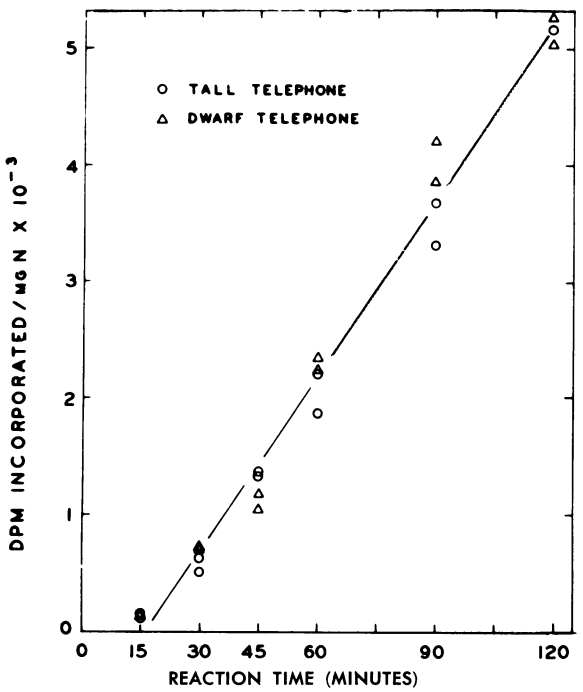

FIG. 5. Time-course of incorporation of mevalonate-^{14}C into (—)-kaurene-^{14}C in cell-free extracts of Dwarf Telephone and Tall Telephone pea seeds. Each point represents radioactivity in (—)-kaurene-^{14}C from 1 determination. There were 2 replicates for each time period for each variety. Each 2.0-ml reaction mixture contained 0.75 ml of enzyme extract, 3 μmoles each of $MgCl_2$, $MnCl_2$ and ATP, 98 mμmoles of Na 2-^{14}C-mevalonate (0.493 μc) and pH 7.4 phosphate buffer. Measurements of radioactivity were by liquid scintillation counting.

synthesis was apparently identical for the enzyme systems isolated from both dwarf and tall peas (fig 5). Hence, if a difference in the rate of gibberellin 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. Of course, genetic factors associated with differences in gibberellin metabolism might be inoperative in immature embryos but become active later in ontogeny. Both of these possibilities are under current investigation.

Effects of Growth Retardants on (−)-Kaurene Biosynthesis. The effects of 2 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* (12), developing pea seeds (2) and in *Echinocystis macrocarpa* endosperm-nucellus (6). This retardant specifically inhibits the enzymic cyclization of *trans*-geranylgeranyl pyrophosphate to form (−)-kaurene (6). Ruddat (29) has further shown that Amo-1618 inhibits the biosynthesis of steviol in *Stevia rebaudiana*. Results of the present investigation (fig 6) revealed that Amo-1618 is a potent inhibitor of (−)-kaurene biosynthesis in preparations of immature pea seeds.

An unexpected result of the present work was that CCC also inhibited (−)-kaurene production (fig 6). CCC was a very much less effective inhibitor than Amo-1618, with approximately 1000-fold higher concentrations of CCC than Amo-1618 being required to evoke similar percentages of inhibition. CCC has been reported to inhibit gibberellin biosynthesis in *Fusarium* (8, 12, 24) and to reduce the gibberellin content of *Pharbitis* seeds harvested from previously treated parent plants (34). However, CCC failed to inhibit kaurene synthesis significantly in *Echinocystis* endosperm-nucellus (6); therefore, it has been proposed (6, 8) that CCC acts at a site in the biosynthetic pathway beyond (−)-kaurene. The results here reported would suggest that either: A) there is a difference in sensitivity of *Pisum* and *Echinocystis* enzyme systems to CCC; or B) 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. (6). 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 here described would support the idea that perhaps CCC acts at more than 1 site in the pathway

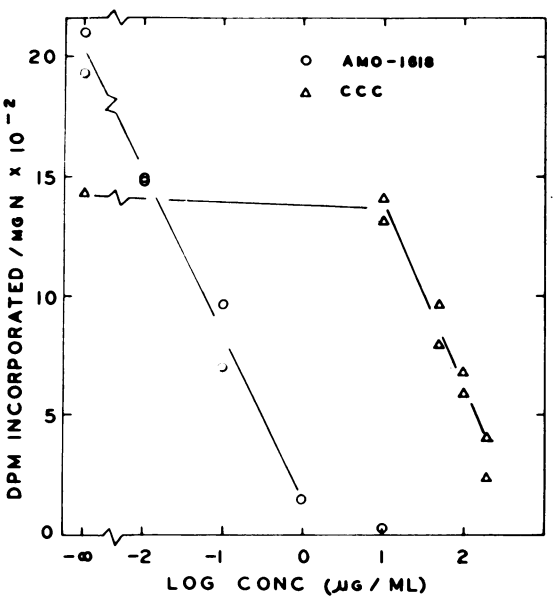

FIG. 6. Effects of Amo-1618 and CCC on incorporation of mevalonate-^{14}C into (—)-kaurene-^{14}C in standard cell-free reaction mixtures. Reaction mixtures were prepared as described in legend for figure 5 and were incubated for 1 hour at 30°. Each point represents radioactivity in (—)-kaurene-^{14}C from one determination; there were 2 replicates for each determination. Enzyme activity was somewhat lower in reaction mixtures containing no growth retardant in the experiment with CCC than in the experiment with Amo-1618. Measurements of radioactivity were by liquid scintillation counting.

leading to gibberellin production. Furthermore, there is limited evidence for an interference by CCC with indole compound metabolism (16, 22, 25). Perhaps the mode of action of CCC is more complex than has yet been fully realized.

Acknowledgments

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