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Properties of Kaurene Synthetase from *Marah macrocarpus*[1]

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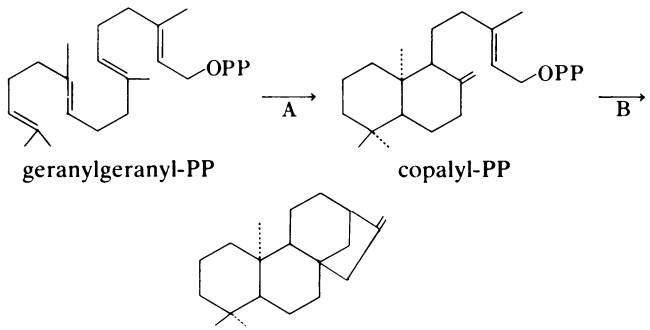
ABSTRACT

The kaurene synthetase from immature seeds of *Marah macrocarpus* (Greene) Greene was partially purified from cell-free homogenates of endosperm by a combination of QAE-Sephadex A-25 chromatography and hydroxyapatite chromatography and freed of contaminating phosphatase activity. The two catalytic activities associated with kaurene synthetase, the cyclization of geranylgeranyl-pyrophosphate to copalyl-pyrophosphate (activity A) and the cyclization of copalyl-pyrophosphate to *ent*-kaurene (activity B), were not even partially resolved from one another during these procedures. Both activities had identical elution profiles from a calibrated Sepharose 4B column corresponding to a molecular weight less than that of ovalbumin (45,000).

The A and B activities had pH optima of 7.3 and 6.9, respectively. Both activities required millimolar concentrations of the following divalent cations in the order: $Mg^{2+} > Mn^{2+} > Co^{2+}$. Activities A and B were both sensitive to inhibition by Hg^{2+}, Cu^{2+}, *p*-hydroxymercuribenzoate, and *N*-ethylmaleimide, but activity B was much more sensitive than activity A. The average value of K*m*′ (apparent K*m* in the absence of substrate inhibition) for geranylgeranyl-pyrophosphate was 1.6 μM. Values of 0.5 and 0.6 μM were obtained for K*m*′ and K*m*, respectively, for copalyl-pyrophosphate. The V*m*′ values for the two activities were similar: 12 and 9 pmol/minute · μg protein for activities A and B, respectively.

***N*,*N*-Dimethylaminoethyl-2,2-diphenylpentanoate (SKF-525A) and *N*,*N*-dimethylaminoethyl-2,2-diphenylpentyl ether (SKF-3301A), tributyl-2,4-dichlorobenzylphosphonium chloride (Phosfon D), tributyl-2,4-dichlorobenzylammonium chloride (Phosfon S), 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate (Amo-1618), 2-(*N*,*N*-dimethyl-*N*-heptylammonium bromide)-*p*-methan-1-ol (Q-58), and 2-(*N*,*N*-dimethyl-*N*-octylammonium bromide)-*p*-methan-1-ol (Q-64), at concentrations from 1 to 5 μM, were effective inhibitors of kaurene synthetase activity A. Acetylcholine chloride and 2-chloroethyl-trimethylammonium chloride were effective inhibitors of activity A only at concentrations of 5 mM or greater. Abscisic acid, indole-3-acetate, gibberellin A_1, gibberellin A_3, a mixture of gibberellins A_4 and A_7, gibberellin A_{13}, and *N*,*N*-dimethylaminosuccinamic acid (B995) were not inhibitory at any of the levels tested. None of these compounds was an effective inhibitor of activity B at concentrations less than 0.5 mM.**

The role of *ent*-kaurene[3] as an intermediate in gibberellin biosynthesis has been well established (7, 12, 32). Cell-free enzyme systems capable of catalyzing the biosynthesis of kaurene have been obtained from a number of higher plant sources (2, 4, 5, 11-13, 26, 33) and from the gibberellin-producing fungus *Fusarium moniliforme* Sheld (9, 28). The biosynthesis of kaurene from the acyclic precursor *trans*-geranylgeranyl-PP has been shown to occur in two steps (14, 28) as follows:



The name kaurene synthetase has been used to refer to the enzyme or enzymes catalyzing the over-all reaction that is the sum of the A and B cyclization steps (9).

The kaurene synthetase from *F. moniliforme* has been purified 170-fold and a number of its properties were examined (9). Relatively little information has been available about the properties of kaurene synthetases of higher plants. Several plant growth retardants were shown to inhibit kaurene synthetase activity in crude homogenates of the endosperm of immature *Marah macrocarpus*[4] seed (8); subsequent work established the A step of kaurene synthetase from this and other sources to be the one most sensitive to inhibition (28). The soluble nature and requirement for divalent metal ions were established for kaurene synthetases of *M. macrocarpus* (30) and *Ricinus communis* (27).

The objective of the work described in this report was to purify kaurene synthetase from *M. macrocarpus* endosperm to a stage free from interfering activities so that a more quantitative analysis of the kinetic properties of this activity from a higher plant source could be undertaken. In addition to the general properties of the enzyme, an analysis of the mode of interaction of growth regardants and the possible regulation by metabolic effectors at this branchpoint in isoprenoid metabolism were of

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[3] Abbreviations: kaurene refers to *ent*-kaurene ([−]-kaurene). Copalol refers to the *trans* isomer of *enantio*-labda-8(16); 13-dien-15-ol and geranylgeraniol refer to all-*trans*-isomer of 3,7,11,15-tetramethylhexadec-2,6,10,14-tetraen-1-ol; DTT: dithiothreitol; HMB: sodium-*p*-hydroxymercuribenzoate; NEM: *N*-ethylmaleimide; Amo-1618: 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate; and BCB: 2-bromoethyl trimethylammonium bromide. The trivial designations for the other plant growth retardants and related inhibitors are defined under "Materials and Methods."

[4] *Marah macrocarpus* (Greene) Greene has also been commonly referred to as *Echinocystis macrocarpa* Greene. Earlier papers from our laboratory utilized the latter name in reference to this organism.

particular interest. A preliminary account of some of these results has been published (32).

MATERIALS AND METHODS

PLANT GROWTH RETARDANTS AND RELATED COMPOUNDS

3'-Isopropyl-4'(trimethylammonium chloride)-6'-methylphenyl piperidine-1-carboxylate (Carvadan) was a gift of H. M. Cathey, United States Department of Agriculture, Beltsville, Md. Tributyl-2,4-dichlorobenzylphosphonium chloride (Phosfon D), technical grade, and tributyl-2,4-dichlorobenzylammonium chloride (Phosfon S) were gifts of the Research and Development Department of the Virginia-Carolina Chemical Corporation, Richmond, Va. *N,N*-Dimethylaminoethyl-2,2-diphenylpentanoate (SKF-525A), *N,N*-dimethylaminoethyl-2,2-diphenylpentyl ether (SKF-3301A), *tris*-(2-dimethylaminoethyl)-phosphate trihydrochloride (SKF-7732), and *tris*-(2-diethylaminoethyl)-phosphate trihydrochloride (SKF-7797), were gifts of Smith, Kline and French Laboratories, Philadelphia, Pa. 2-Chloroethyl trimethylammonium chloride (CCC) was obtained from the Agricultural Division, American Cyanamide Company. *N,N*-Dimethylaminosuccinamic acid (B995), technical grade, was obtained from the United States Rubber Company. 2-(*N,N*-Dimethyl-N-3,4-dichlorobenzylammonium chloride)-*p*-methan-1-ol (Q-53), 2-(*N,N*-dimethyl-N-heptylammonium bromide)-*p*-methan-1-ol (Q-58), and 2-(*N,N*-dimethyl-*N*-octylammonium bromide)-*p*-methan-1-ol (Q-64) were the generous gifts of W. F. Newhall, University of Florida. 2-(4-Chlorophenylthio)-triethylamine hydrochloride (Amchem) was obtained from Amchem Products Inc., Ambler, Pa. Delcosine was the generous gift of G. Waller, Oklahoma State University. Abscisic acid was a gift of J. Cornforth, Milstead Laboratory for Chemical Enzymology, Sittingbourne, England.

SUBSTRATES AND REFERENCE COMPOUNDS

Gibberellin A_{13} was a gift of R. H. B. Galt, Pharmaceutical Division of Imperial Chemical Industries, Ltd., Macclesfield, England. Kaurene was a gift of Abbott Laboratories, North Chicago, Ill. [5-^{3}H]-D,L-Mevalonic acid (0.380 and 6.25 Ci/mmol) and [2-^{14}C]-D,L-mevalonic acid (4.70 and 6.33 mCi/mmol), both as the *N,N'*-dibenzylethylenediamine salts, were obtained from New England Nuclear. Di-(triethylammonium) phosphate was prepared by the method of Popjak *et al.* (23) using 99% (crystalline) phosphoric acid (K and K Laboratories).

OTHER MATERIALS

Bacterial alkaline phosphatase (EC 3.1.3.1; 20 enzyme units/mg) was purchased from Worthington Biochemical Corp. Merck Silica Gel F-254 precoated glass plates in various thicknesses were obtained from Brinkmann Instruments. Hydroxyapatite was prepared by the method of Anacker and Stoy (1).

RADIOACTIVE GERANYLGERANYL-PP

[^{14}C]- and [^{3}H]Geranylgeranyl-PP were prepared by two methods.

Method I. [^{14}C]- and [^{3}H]Geranylgeranyl-PP were prepared biosynthetically from [2-^{14}C]- and [5-^{3}H]mevalonate by the procedure of Oster and West (21). The pooled bicarbonate extracts containing the [^{3}H]- or [^{14}C]geranylgeranyl-PP were adjusted to pH 8.5 and purified on a DEAE-Sephadex A-25 column (1 × 10 cm) developed with a linear gradient of $(C_2H_5)_3NH_2CO_3$, pH 8.5, obtained by mixing 300 ml of 0.75 M salt with 300 ml of 0.075 M salt. The pooled fractions containing the product were lyophilized to dryness, redissolved in 25 ml of 5 mM KOH, and concentrated to 0.1 ml under a stream of N_2. During the prolonged (20–40 hr) exposure to the stream of N_2, the remaining $(C_2H_5)_3NH_2CO_3$ was removed. The sample was adjusted to 10 μM in geranylgeranyl-PP by the addition of 5 mM KOH and stored at 4 C. Twenty-five per cent conversions of available [5-^{3}H]- or [2-^{14}C]mevalonic acid to [^{3}H]- or [^{14}C]geranylgeranyl-PP were routine. The preparations of [^{3}H]geranylgeranyl-PP used in this study had specific activities of either 84.2, 83.1, or 79.7 mCi/mmol; the [^{14}C]geranylgeranyl-PP had specific activities of either 18.8 or 25.3 mCi/mmol.

Method II. [^{14}C]Geranylgeranyl-PP was biosynthesized from [2-^{14}C]mevalonate according to the procedure of Oster and West (21). After the normal incubation period in which the [^{14}C]geranylgeranyl-PP was produced, 100 E.U. of *Escherichia coli* alkaline phosphatase were added to the incubation mixture. The mixture was then maintained for 12 hr at 30 C under N_2. The reaction mixture was extracted three times with a mixture of benzene and acetone (3:1), and the [^{14}C]geranylgeraniol was purified according to the methods used by Fall and West (9) for the purification of [^{14}C]copalol. Thirty per cent conversion of the available [2-^{14}C]mevalonate to [^{14}C]geranylgeraniol was routinely observed. The purified [^{14}C]geranylgeraniol was pyrophosphorylated by the method of Cramer and Böhm (6) as modified by Popjak *et al.* (23). The reaction conditions and the procedure for extracting the [^{14}C]geranylgeranyl-PP were identical to those described by Fall and West (9) for [^{14}C]copalyl-PP preparation. The [^{14}C]geranylgeranyl-PP was purified on a DEAE-Sephadex A-25 column as described above in method I; 35% conversions of [^{14}C]geranylgeraniol to [^{14}C]geranylgeranyl-PP were routinely obtained.

RADIOACTIVE COPALYL-PP

[^{3}H]- or [^{14}C]Copalyl-PP prepared as described by Fall and West (9) was purified by chromatography on a DEAE-Sephadex A-25 column as described above for geranylgeranyl-PP. The preparations of [^{3}H]copalyl-PP used had specific activities of either 79.7, 83.1, or 84.2 mCi/mmol; the [^{14}C]copalyl-PP had a specific activity of 18.8 mCi/mmol.

PLANT MATERIAL

Immature fruit of *M. macrocarpus* was collected in the Santa Monica Mountains. The seeds were removed from the fruit, washed with water, dried, and frozen at −20 C within 24 hr of picking. Periods of storage of the seeds at −20 C varied from a few days to 1 year. The endosperm extract was prepared from seeds in which the embryo did not completely surround the endosperm. The endosperm was removed using a spatula, taking care to leave behind embryo and nucellus. The pooled endosperm fraction was gently homogenized in a glass homogenizer, filtered through glass wool to remove cell debris, and centrifuged at 26,000*g* for 30 min. The resulting supernatant fraction (S-26) contained from 1 to 2 mg protein/ml. The S-26 served as the crude enzyme source for the purification of kaurene synthetase. For some of the preliminary studies and the large scale enzyme purification procedures (350–900 ml), the S-26 was lyophilized and subsequently reconstituted in 5 mM K-phosphate, pH 7.4, to give the desired protein concentration. One g of lyophilized powder was equivalent to approximately 25 ml of S-26. Quantitative recovery of both kaurene synthetase activities was routinely observed following lyophilization.

RECOVERY AND ANALYSIS OF LIPID PRODUCTS

The general procedures for extraction of diterpene hydrocarbons and their analysis by TLC have been described previously (21, 27, 28). When kaurene was the only reaction product determined (assays A-1 and B described below), silica gel plates were developed in *n*-hexane to 6 cm; the front running kaurene

region was scraped into a toluene-based scintillation fluid. When kaurene, copalol, and geranylgeraniol were determined (assay A-2 described below), the thin layer system described by Shechter and West (28) was used. The details of this latter procedure are given by Fall and West (9).

ASSAYS FOR KAURENE SYNTHETASE WITH [^{3}H]- OR [^{14}C]GERANYLGERANYL-PP OR [^{3}H]- OR [^{14}C]COPALYL-PP AS SUBSTRATE

Crude and partially purified preparations of kaurene synthetase were diluted to achieve linear initial rates of reaction. To protect against possible loss of activity, PVP-40 (Mann Research Laboratories) was routinely included in the dilution medium (9). During the purification, enzyme fractions were diluted with 5 mM K-phosphate, pH 7.4, containing 10 mM 2-mercaptoethanol, 10% (w/v) glycerol, and 2.5 mg/ml PVP-40. In all incubations with enzyme which had been partially purified by a combination of QAE-Sephadex and hydroxyapatite chromatography, the dilution medium contained 10 mM TES, pH 7.5, and 2.5 mg/ml PVP-40.

The various assays used for kaurene synthetase were those described by Fall and West (9). Two different assays were used with geranylgeranyl-PP as the substrate: (*a*) an assay for kaurene formation, assay A-1; and (*b*) an assay for copalyl-PP plus kaurene formation, assay A-2. Assay A-2 involves termination of both kaurene synthetase activities A and B, followed by incubation of the reaction mixture with alkaline phosphatase. Assay A-1 was used during the enzyme purification procedure because phosphate buffer, which is known to inhibit alkaline phosphatase, was used in all stages of the purification. Assay A-1 was also used to detect activity in the effluent following chromatography of crude and partially purified kaurene synthetase on Sepharose 4B. When copalyl-PP was used as substrate, assay B was used. This assay involved measurement of kaurene production only (9). With assays A-1 and B, 1 unit of kaurene synthetase activity is defined as the production of 1 nmol kaurene/min. With assay A-2, 1 unit of kaurene synthetase is defined as the production of 1 nmol copalol plus kaurene/min.

PROTEIN DETERMINATIONS

The protein content of various enzyme fractions was determined by the method of Lowry *et al.* (18).

PARTIAL PURIFICATION OF KAURENE SYNTHETASE

One hundred ten ml of S-26 were lyophilized and reconstituted at a protein concentration of 9 to 11 mg/ml in 0.05 M K-phosphate, pH 7.4. A 4% (w/v) solution of protamine sulfate, pH 6.5, was added to give 0.3 mg protamine/mg protein. The mixture was stirred at 4 C for 10 min and then centrifuged at 26,000*g*. The supernatant from protamine sulfate treatment was diluted 7-fold with 25 mM K-phosphate, pH 7.4, containing 10 mM 2-mercaptoethanol and 10% (w/v) glycerol and applied to a column of QAE-Sephadex A-25 which had been equilibrated with the same buffer (0.25 ml packed QAE-Sephadex/mg of endosperm protein). The column was washed with the initial buffer until the *A* at 280 nm in the effluent was less than 0.10, and was then developed with a linear gradient of between 0 and 0.30 M KCl in the same buffer. Kaurene synthetase activity eluted between 0.07 and 0.18 M KCl. The pool of kaurene synthetase activity from QAE-Sephadex was applied to a hydroxyapatite column which had been equilibrated with the 25 mM K-phosphate buffer, pH 7.4 (1.2 ml hydroxyapatite/mg protein). After application of the sample, the column was eluted with a linear gradient of between 0.025 and 0.33 M K-phosphate, pH 7.4. Kaurene synthetase activity eluted between 0.05 and 0.20 M K-phosphate. The pooled fractions containing kaurene synthetase activity were dialyzed for 18 hr *versus* 100 volumes of 25 mM K-phosphate, pH 7.4, containing 10 mM mercaptoethanol and 50% (w/v) glycerol. The dialyzed pool from hydroxyapatite (5–20 ml) was stored at −20 C.

RESULTS AND DISCUSSION

PURIFICATION OF KAURENE SYNTHETASE

Initial studies showed that *M. macrocarpus* kaurene synthetase activity was unstable in crude extracts and partially purified fractions, although the presence of 2-mercaptoethanol and 10% (w/v) sucrose improved stability somewhat. Application of the following purification procedures resulted in large losses of both A and B activities and therefore could not be used: pH precipitation at pH 5.5 or 6, fractionation at 0 or −20 C with ethanol or acetone, ammonium sulfate fractionation, high speed centrifugation, and Sephadex G-200 chromatography. The crude enzyme, after exhaustive dialysis of pH 6.5, did not bind to CM-cellulose or phosphocellulose; this treatment also did not purify kaurene synthetase by removing inactive proteins. The instability of kaurene synthetase made its purification difficult and, as a result, highly purified preparations could not be obtained. However, a procedure for partial purification was developed using protamine sulfate treatment, QAE-Sephadex A-25 chromatography and hydroxyapatite chromatography, as summarized in Table I. Most of the contaminating phosphatase activity was removed by this procedure, and the partially purified enzyme was suitable for kinetic experiments.

In larger scale purifications (450–900 ml of S-26), protamine sulfate was found to interfere with the binding of kaurene synthetase during subsequent chromatography steps; therefore, protamine sulfate treatment was not utilized in these larger scale purifications. Instead, the reconstituted S-26 was dialyzed for 18 hr *versus* 160 volumes of 5 mM K-phosphate, pH 7.4, containing 10 mM 2-mercaptoethanol and 10% (w/v) glycerol and applied directly to a column of QAE-Sephadex. The yield of partially purified kaurene synthetase activities was found to be drastically reduced to 1% or less of the initial activity when the amount of S-26 processed was greater than 450 ml (650 mg endosperm protein). The reason(s) for the greater instability of kaurene synthetase during these larger scale purifications is not known.

No evidence was obtained for even partial resolution of the A and B activities from one another during these purification steps. Samples of partially purified kaurene synthetase were only stable to storage at −20 C for about 3 months, and therefore, several different preparations of kaurene synthetase were used for the studies reported here. Despite the large variation in recovery of kaurene synthetase activities, the *Km'* (apparent *Km* in the absence of substrate inhibition) values of kaurene synthetase activities A and B for geranylgeranyl-PP and copalyl-PP in

TABLE I. Purification of Kaurene Synthetase of M. macrocarpus Greene.

Purification step	Protein	Total Units		Specific Activity	
		A	B	A	B
	mg	nmoles kaurene/min		units/mg protein	
Crude S-26	124	11.8	58.5	0.10	0.47
Protamine supernatant	94	14.9	51.8	0.16	0.55
QAE-Sephadex	21	10.2	15.9	0.49	0.76
Hydroxyapatite	4	5.8	20.3	1.45	5.08

The purification procedure described in Materials and Methods was followed. Assays A-1 (1.0 μM geranylgeranyl-PP as substrate) and B (1.1 μM copalyl-PP as substrate) were conducted as described in Materials and Methods.

different enzyme preparations were found to be similar. For the three partially purified preparations of kaurene synthetase used for these characterization studies, the *Km'* values for geranylgeranyl-PP were 1.4, 1.8, and 1.4 μM, and for copalyl-PP were 0.7, 0.5, and 0.6 μM.

PROPERTIES OF PARTIALLY PURIFIED KAURENE SYNTHETASE

Molecular Weight Estimations. The behavior of both crude and partially purified samples of kaurene synthetase were tested on a Sepharose 4B column prepared and calibrated as described by Fall and West (9). An essentially linear relationship between log Kav and log mol wt was observed for the standard reference proteins ovalbumin (mol wt 45,000), BSA (mol wt 134,000), catalase (mol wt 250,000) and bovine thyroglobulin (mol wt 670,000). Activities A and B of both crude and purified kaurene synthetase preparations ranging in protein concentration from 1.5 to 14.2 mg/ml showed identical elution profiles with a Kav consistently larger than that of ovalbumin (mol wt 45,000). This indicates that the mol wt of activities A and B are less than 45,000. A more precise estimation of the mol wt cannot be made with any degree of certainty from these data because of the limited capacity of Sepharose 4B to resolve proteins in this mol wt range.

pH Optima for Activities A and B. The pH optimum for the over-all conversion of geranylgeranyl-PP to kaurene was 7.3 in TES or tris buffers. Little activity was seen in imidazole buffers between pH 6 and pH 7.5. Little copalyl-PP accumulated (at most 10% of the amount of kaurene formed) at any pH in any of the buffers tested. TES buffer, pH 7.3, was used routinely for assays involving geranylgeranyl-PP as substrate, because TES has a pK_a of 7.5 and does not bind significant amounts of divalent cations (10).

Activity B had comparable activity in TES, tris, and imidazole buffers and exhibited a distinct pH optimum at approximately pH 6.9 in imidazole, whereas relatively little dependence of activity B on pH was observed in either TES (between pH 6.2 and 7.2) or tris buffer (between pH 7.2 and 8.2). TES buffer, pH 6.9, was used routinely for assays of activity B.

Divalent Cation Specificities for Activities A and B. A sample of partially purified kaurene synthetase was diluted 5-fold with 10 mM TES, pH 7.5, containing 2.5 mg/ml PVP-40 and dialyzed for 15 hr at 4 C against 300-fold excess of 10 mM TES, pH 7.3, containing 0.1 mM DTT, and 10% (w/v) glycerol. Assays A-2 and B were conducted as described under "Materials and Methods" except that the source of added cations was varied. In the absence of added divalent cations, neither activity A nor activity B was significant. Kaurene synthesis from geranylgeranyl-PP (1 μM) (activity A) was greatest with added $MgCl_2$ at 1 mM or higher concentrations. $CoSO_4$ was slightly less effective, whereas $MnCl_2$ gave optimal activity at 0.1 mM and was inhibitory at higher concentrations. Little or no activity was observed in the presence of $Ca(NO_3)_2$, $ZnSo_4$, $Cu(SO_4)$, or $NiCl_2$.

With copalyl-PP (1.1 μM) as substrate (activity B), $MgCl_2$, $MnCl_2$, or $CoSO_4$ each stimulated kaurene synthesis. Maximal rates were seen with $MgCl_2$ at 10 mM concentrations. $MnCl_2$ and $CoSO_4$ gave lower rates at optimal concentrations of 0.1 mM and were progressively more inhibitory at higher concentrations. Low rates of kaurene synthesis were obtained in the presence of $NiCl_2$, and no kaurene formation was observed in the presence of $CuSO_4$, $ZnSO_4$ or $Ca(NO_3)_2$.

Since both activities A and B exhibited maximal rates of kaurene synthesis in the presence of millimolar levels of $MgCl_2$, saturation curves for $MgCl_2$ were determined with each activity as shown in Figure 1. When geranylgeranyl-PP was the substrate, low levels of copalyl-PP accumulated at or below 10 μM $MgCl_2$, with little or no kaurene formation. At 0.1 mM $MgCl_2$, approximately equivalent amounts of copalyl-PP and kaurene

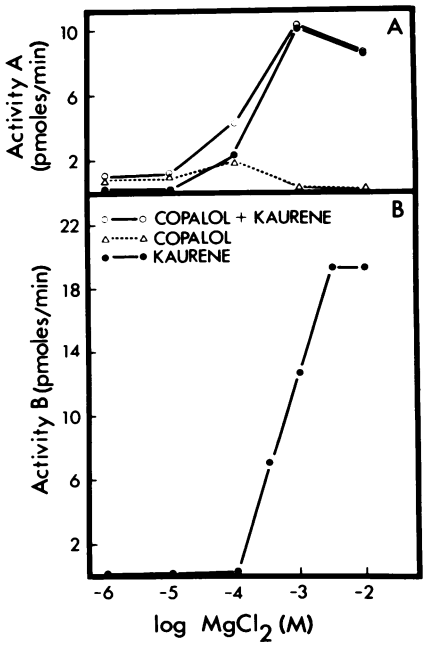

FIG. 1. Rate *versus* $MgCl_2$ concentration curves for activities A and B. A sample of partially purified kaurene synthetase was diluted 5-fold with 10 mM TES (pH 7.5) containing 2.5 mg/ml PVP-40, and dialyzed for 12 hr at 4 C against a 200-fold excess of 10 mM TES (pH 7.3) containing 0.1 mM DTT and 10% (w/v) glycerol. Assays A-2 (1 μM geranylgeranyl-PP as substrate) and B (1.1 μM copalyl-PP as substrate) were conducted as described under "Materials and Methods." Each assay mixture contained 34 μg of partially purified kaurene synthetase and $MgCl_2$ at the indicated concentrations.

were formed from geranylgeranyl-PP; however, added copalyl-PP conversion to kaurene was not detected at 0.1 mM Mg^{2+}. The formation of kaurene from geranylgeranyl-PP but not from added copalyl-PP at 0.1 mM Mg^{2+} suggests that the A and B activities are associated in a manner such that the copalyl moiety formed by reaction A binds to the site of B activity with greater facility than added copalyl-PP. At Mg^{2+} concentrations above 0.1 mM where B activity is stimulated, kaurene is the only product formed from geranylgeranyl-PP at detectable levels. These results indicate that a substantial pool of copalyl-PP may never accumulate from the action of this enzyme *in vivo*.

Effects of Thiols, EDTA, and Sulfhydryl Inhibitors. Kaurene synthetase activity in crude endosperm preparations was stabilized by added thiols; therefore, 10 mM 2-mercaptoethanol was routinely included in the medium throughout the purification of the enzyme. To evaluate the thiol requirements of activities A and B, a sample of partially purified kaurene synthetase was dialyzed in the absence of thiols and then assayed in the presence of varying levels of DTT. From 60 to 80% of the initial A activity was lost during dialysis in the absence of thiols. The residual A activity was significantly stimulated by 0.1 mM EDTA (3-fold stimulation), and to a lesser extent by 0.1 mM DTT (1.7-fold stimulation), suggesting that activity A might be sensitive to an inhibition by contaminating metal ions which can be only partially reversed by DTT or EDTA. Activity B was stable to dialysis in the absence of thiols, and was stimulated by a broad range of DTT concentrations (1 μM–10 mM). However, the stimulation by DTT was eliminated in the presence of 0.1 mM EDTA. It appears that activity B may be sensitive to an inhibition by contaminating metal ions which can be reversed by thiols or EDTA. In most subsequent assays of kaurene synthetase, 0.1 mM EDTA was included to protect against inhibition by metals. DTT, at 0.1 mM, was also included in subsequent assays of activities A and B because of the large losses of activity A which

were observed during dialysis in the absence of thiol compounds.

The effect of sulfhydryl inhibitors on activities A and B was also tested. A sample of kaurene synthetase was dialyzed in the absence of thiols; approximately 60% of activity A was lost during this treatment. The dialyzed enzyme was assayed for activities A and B in the presence of 0.1 mM EDTA in order to overcome any inhibitory effects of metal ion contaminants plus 10, 1, or 0.1 mM concentrations of the inhibitors HMB, NEM, $HgCl_2$, and $CuSO_4$. Although both activities were susceptible to inhibition by all of these agents, activity B was more sensitive than activity A. The relative rates for activity A in the presence of 1 mM inhibitor plus 0.1 mM EDTA compared with a rate of 100 for 0.1 mM EDTA alone were: NEM, 57; HMB, 32; $HgCl_2$, 25; $CuSO_4$, 12. The results for activity B under the same conditions were NEM, 46; HMB, 0; $HgCl_2$, 0; $CuSO_4$, 0. These results suggest that free sulfhydryls are important for activity in both stages of cyclization.

Substrate Saturation Experiments. Figure 2 shows a double reciprocal plot of initial rate as a function of geranylgeranyl-PP concentration for activity A. Apparent substrate inhibition is observed at geranylgeranyl-PP concentrations above 1 μM. Extrapolation of the rate values at the lower concentrations of geranylgeranyl-PP gave a value of 1.4 μM for *Km'* (the apparent *Km* in the absence of substrate inhibition). The *Vm'* obtained by this extrapolation was 12 pmol/min · μg protein. The same inhibition at higher substrate concentrations was evident, and approximately the same *Km'* was observed whether the source of geranylgeranyl-PP was direct isolation of biosynthesized material or chemical pyrophosphorylation of biosynthesized geranylgeraniol. Substrate prepared by both procedures showed a single major peak of radioactivity at R_F 0.3 plus variable amounts of a smaller radioactive peak at R_F 0.5 when chromatographed on silica gel thin layer plates developed with 1-propanol-NH_3-1% EDTA in water (6:3:1). The major peak is geranylgeranyl-PP and the minor peak is believed to be due to geranylgeranyl-P formed by hydrolysis during storage. The latter peak never exceeded 10% of the major peak and was normally less than that. It seems most likely that the inhibitions seen at substrate concentrations above 1 μM are due to inhibition by the substrate itself; however, the possibility that an impurity present in both types of preparations was responsible can not be ruled out.

Figure 3 shows a reciprocal plot of initial rate *versus* copalyl-PP concentration for activity B. Inhibition is observed at copalyl-PP concentrations above 0.2 μM. Extrapolation of the rate

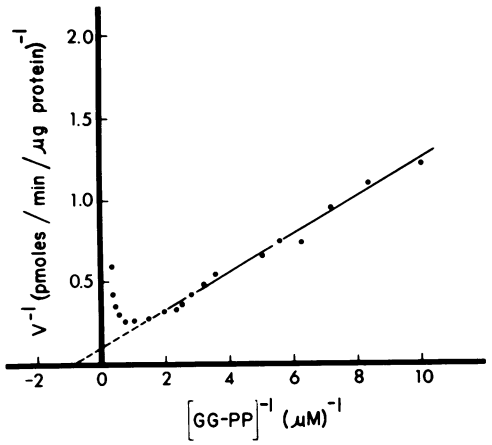

FIG. 2. Lineweaver-Burk plot of the effect of geranylgeranyl-PP concentration on the rate of activity A. Assay A-2 was conducted as described under "Materials and Methods." Each assay mixture contained 0.1 M TES (pH 7.3), DTT, and EDTA, each at 0.1 mM, 0.5 mM $MgCl_2$, 6.8 μg enzyme protein, and [^{14}C]geranylgeranyl-PP as indicated. The [^{14}C]geranylgeranyl-PP was prepared by method II, as described in "Materials and Methods."

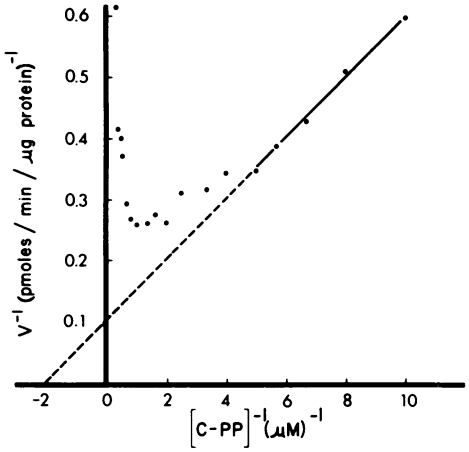

FIG. 3. Lineweaver-Burk plot of the effect of copalyl-PP concentration on the rate of activity B. Assay B was conducted as described under "Materials and Methods." Each assay mixture contained 0.10 M TES (pH 6.9), DTT, and EDTA, each at 0.1 mM, 5 mM $MgCl_2$, 2.3 μg enzyme protein, and [^{3}H]copalyl-PP as indicated.

values at the lower substrate concentrations gave a value for *Km'* of 0.49 μM. The value of *Vm'* estimated by this extrapolation was 9 pmol/min · μg protein. During these studies, two preparations of copalyl-PP were obtained which did not exhibit apparent substrate inhibition; a *Km* value of 0.59 μM comparable to the *Km'* cited above was obtained for these preparations. Since some preparations of copalyl-PP did not exhibit inhibition at higher concentrations, it is concluded that the inhibitory properties of other preparations were due to the presence of a contaminant. All preparations showed a behavior similar to that described above for geranylgeranyl-PP when chromatographed on silica gel thin layer plates developed with 1-propanol-NH_3-1% EDTA in water (6:3:1). The *Km* values found for these two substrates were of the order of magnitude expected for intermediates participating in the biosynthesis of plant growth hormones and are very similar to those found for the kaurene synthetase of *F. moniliforme* (9).

Effects of Potential Inhibitors of Kaurene Synthetase. The general properties of plant growth retardants and their effects on gibberellin biosynthesis have been reviewed by Lang (17). Several of the known plant growth retardants have been shown to be effective inhibitors of kaurene biosynthesis from either mevalonate or geranylgeranyl-PP in crude cell-free homogenates from higher plants (2, 8, 11, 28). A more quantitative assessment of the effectiveness of these retardants was possible with the sample of partially purified kaurene synthetase from *M. macrocarpus*. With the availability of copalyl-PP, it was also of interest to retest these compounds to determine their relative effectiveness in the two stages of cyclization. In addition to the plant growth retardants and structurally related compounds, several plant metabolites were also tested for possible regulatory effects on kaurene synthetase.

The results of the screening of potential inhibitors of activity A are shown in Table II. The compounds are listed in order of decreasing inhibition. Most effective were the steroid synthesis inhibitor SKF-525A, Phosfon D and Phosfon S. These inhibitors plus Amo-1618, the steroid synthesis inhibitor SKF-3301A, and the limonene derivatives Q-64 and Q-58, also known plant growth retardants (22), were effective inhibitors of activity A at 1 μM.

Most of the compounds tested for inhibitory effects on activity A were also tested for their ability to inhibit activity B. The results are summarized, in order of decreasing inhibition, in Table III. None of the compounds tested appeared to be very potent inhibitors of activity B. Amchem (2-[4-chlorophenyl-

TABLE II. The Effect of Potential Inhibitors of Activity A

Assay A-2 was conducted as described in Materials and Methods, with geranyl-geranyl-PP at a concentration of 1.0 μM. Dashes indicate concentrations of potential inhibitor which were not tested.

Inhibitor	Percent Inhibition of Activity at Inhibitor Concentration (M)				
	$5x10^{-7}$	$1x10^{-6}$	$5x10^{-6}$	$5x10^{-5}$	$5x10^{-4}$
SKF-525A	56	69	100	100	100
Phosfon D	20	44	100	100	100
Phosfon S	24	-	88	100	100
Q-64	17	-	84	100	100
Amo-1618	16	47	65	100	100
SKF-3301A	13	29	64	100	100
Q-58	0	30	60	100	100
Deoxycholate	5	-	35	75	100
Amchem	-	-	21	100	100
Carvadan	-	-	20	84	100
Delcosine	-	-	11	39	78
Q-53	-	-	0	10	79
Nicotine	-	-	0	11	65
CCC	-	-	0	0	4
Acetylcholine Chloride	-	-	0	0	0
BCB	-	-	0	0	0
B995	-	-	3	4	1
SKF-7997	-	-	0	0	0
SKF-7732	-	-	3	0	3
Indole Acetate	-	-	0	0	5
GA_1	-	-	0	0	2
GA_3	-	-	0	0	11
GA_4/GA_7	-	-	0	0	0
GA_{13}	-	-	0	0	12
Choline Chloride	-	-	11	0	1
ABA	-	-	0	0	0

TABLE III. The Effect of Potential Inhibitors of Activity B.

Assay B was conducted as described in Materials and Methods, with copalyl-PP at a concentration of 0.65 μM. Dashes indicate concentrations of potential inhibitor which were not tested.

Inhibitor	Percent Inhibition of Activity at Inhibitor Concentration (M)			
	$5x10^{-6}$	$5x10^{-5}$	$5x10^{-4}$	$5x10^{-3}$
Amchem	26	53	70	-
Deoxycholate	11	28	76	100
SKF-525A	8	28	63	-
SKF-3301A	2	38	52	-
Q-53	7	10	35	-
Amo-1618	11	12	-	-
Phosfon D	2	8	34	-
Acetylcholine Chloride	12	6	9	9
ABA	0	13	24	-
Phosfon S	0	0	18	-
Indole Acetate	0	5	0	-
GA_1	0	0	0	-
GA_3	0	0	12	-
GA_4/GA_7	0	0	7	-
GA_{13}	0	11	5	-
Q-64	6	0	0	-
Q-58	0	7	0	-
Carvadan	0	0	4	-
Delcosine	0	0	0	-
Nicotine	0	0	0	-
CCC	0	0	0	-
BCB	0	0	0	-
B995	2	0	0	0

thio]-triethylamine hydrochloride), which has been shown to cause the accumulation of lycopene in several plants (3), was the most effective; at a concentration of 50 μM it inhibited both activity A (Table II) and activity B. Deoxycholate, one of the more effective inhibitors of activity B, still required about a 10-fold higher concentration to achieve an inhibition equivalent to that given by this substance with activity A.

The inhibitory effects of Phosfon D, Phosfon S, and Amo-1618 on activity A of the partially purified *M. macrocarpus* kaurene synthetase were consistent with previous studies using

crude cell-free homogenates of endosperm (8). Several attempts were made to determine the pattern of inhibition of activity A by Phosfon D and Amo-1618, but the results of these determinations did not fit any simple inhibition pattern due to the scatter obtained in the low rate data.

The growth retardants CCC and B995, at concentrations as high as 1 mM, did not exhibit a significant inhibitory effect on either the A or B activities. Thus, these results are in contrast to the effects of Phosfon D, Phosfon S, and Amo-1618. The lack of significant inhibition by these two retardants was noted earlier with crude cell-free extracts of *M. macrocarpus* endosperm (8). If these substances directly influence the biosynthesis of gibberellins, both these data and earlier work with purified *F. moniliforme* kaurene synthetase (9) would suggest that it is at an enzymic step other than kaurene synthetase.

Acetylcholine was tested as a possible inhibitor of kaurene synthetase for the following reasons. The plant growth retardant Amo-1618 and several synthetic quaternary ammonium derivatives of limonene exhibited a good correlation between their activities as plant growth retardants and pseudocholinesterase inhibitors (19, 20). Experiments by Riov and Jaffe (16, 24, 25) led them to suggest that acetylcholine might serve as an endogenous plant growth retardant. It was proposed that Amo-1618 might exert its effect on plants by inhibiting acetyl cholinesterase, thereby increasing the level of acetylcholine which, in turn, acts as the endogenous inhibitor of plant growth. However, acetylcholine was found to have little effect on activity B of kaurene synthetase, and caused significant inhibition of activity A only at a concentration of 5 mM or greater. Thus, it does not appear likely that acetylcholine is acting as a natural inhibitor of the *M. macrocarpus* kaurene synthetase. However, it is possible that acetylcholine might require other components not present in this test system for its action on kaurene synthetase, or it might be inhibiting some other step in the gibberellin biosynthetic pathway.

Several other compounds were tested as possible natural regulators of kaurene synthetase. None of the gibberellins tested as possible feedback inhibitors (GA_1, GA_3, GA_4 + GA_7, GA_{13}) showed any inhibitory effects. This is not surprising since one might expect the production of a hormone to be governed by factors other than its own concentration level. It is conceivable that the concentration of other types of hormones might contribute more to the regulation of gibberellin biosynthesis. However, neither ABA nor IAA in the concentration range from 5 to 500 μM exerted a significant inhibitory effect on kaurene synthetase activity.

Nicotine was tested because it has been shown to inhibit the cyclization of carotenes in crude extracts of mycobacteria (15), a process mechanistically similar to the initial stages of geranylgeranyl-PP cyclization. Nicotine did not exhibit any effect on activity B at the concentrations tested, and caused inhibition of activity A only at the relatively high concentration of 0.5 mM. Similar results were found for the tetracyclic diterpenoid alkaloid delcosine, which has some structural features in common with (−)-kaurene. This compound had been previously shown to inhibit the growth of decapitated pea seedlings (31).

Thus, there is no support from this work for the speculation that there are low mol wt natural inhibitors of kaurene synthetase. It might be noted that recent research has revealed the presence in extracts of some plant systems of readily detectable kaurene synthetase B activity when there is little or no detectable A activity (29, 33). This phenomenon may be related to the *in vivo* regulation of kaurene synthetase activity; at present there is no evidence that small molecules are involved in the limitation of expression of A activity.

GENERAL DISCUSSION

It is not clear from these results whether the catalytic sites responsible for the *M. macrocarpus* kaurene synthetase A and B activities are the same, are at separate locations in the same protein, or reside on separate proteins. It was not possible to resolve even partially the A and B activities from one another by any of the purification procedures attempted. However, distinct differences in pH optima, divalent cation requirements, sensitivity to sulfhydryl inhibitors, and inhibition by plant growth retardants were seen for the A and B activities. Whatever the relationship of these sites, they seem to be situated such that utilization of copalyl-PP generated as a product of the A activity is facilitated in comparison with added copalyl-PP. These results indicate that substantial pools of copalyl-PP may never accumulate under physiological conditions.

Both similarities and differences are evident in the characteristics of the partially purified kaurene synthetases from *M. macrocarpus* and *F. moniliforme* (9). Many of the kinetic properties of the enzymes from the two sources were similar. Both enzymes utilized at their B sites copalyl-PP generated at their A sites in preference to exogenous copalyl-PP. The *F. moniliforme* enzyme exhibited a constant ratio of A to B activity throughout a 170-fold purification. The mol wt of both the crude and purified enzyme was estimated to be 460,000 ± 30,000 from sucrose density gradient centrifugation and Sepharose 4B gel filtration chromatography. Kaurene synthetase A and B activities of the purified enzyme were associated with a major protein band after polyacrylamide gel electrophoresis at pH 8.9; many protein bands (but no kaurene synthetase activity) were detected on gels run at pH 10.2 or in the presence of SDS. In contrast, highly purified preparations of the *M. macrocarpus* enzyme could not be obtained due to instability of activities A and B. Also, the ratio of A to B activities were not constant or reproducible during the partial purification achieved; and the mol wt estimated by gel filtration chromatography was less than 45,000, or more than an order of magnitude less than that of the *F. moniliforme* kaurene synthetase.

The general pattern of inhibitions of the A activities of kaurene synthetases from both sources by growth retardants and related substances were similar; the steroid synthesis inhibitors SKF-525A and SKF-3301A were exceptions in that they were relatively more effective with the *M. macrocarpus* enzyme. In contrast, responses of the B activities to several substances were somewhat different. Q-64, Q-58, Q-53, SKF-525A, and SKF-3301A were effective inhibitors of the *F. moniliforme* B activity in the range of 50 to 500 μM. Of these, only SKF-525A and SKF-3301A gave rise to any significant inhibition of the *M. macrocarpus* B activity, and these only at relatively higher concentrations. Thus, the growth retardants and related substances are even more selective in their inhibition of the A activity of the higher plant kaurene synthetase.

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