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Properties of Kaurene Synthetase from *Marah macrocarpus* Endosperm: Evidence for the Participation of Separate but Interacting Enzymes[1]

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ABSTRACT

***Ent*-kaurene is synthesized from geranylgeranyl pyrophosphate in a two step sequence catalyzed by kaurene synthetase; the first step (*A* activity) involves the conversion of geranylgeranyl pyrophosphate into the intermediate *ent-trans* labda-8(17), 13-dien-15-yl pyrophosphate (copalyl pyrophosphate) which is further cyclized to *ent*-kaurene in the second step (*B* activity). The resolution of enzyme fractions which catalyze each step independent of the other has been accomplished for the first time by means of QAE Sephadex A-50 chromatography and polyacrylamide gel electrophoresis of kaurene synthetase preparations from endosperm tissue of immature seed of *Marah macrocarpus*. Molecular weights for the *A* and *B* enzymes were each estimated as approximately 82,000 by means of gel filtration chromatography and sedimentation velocity determinations.**

Experiments in which [^{14}C]geranylgeranyl pyrophosphate and [^{3}H]copalyl pyrophosphate were incubated simultaneously with kaurene synthetase preparations demonstrated that copalyl pyrophosphate derived from [^{14}C]geranylgeranyl pyrophosphate is more readily converted to kaurene than is exogenously added [^{3}H]copalyl pyrophosphate. This implies that copalyl pyrophosphate derived from the catalytic site of the *A* enzyme is preferentially channelled to the *B* enzyme catalytic site for conversion to *ent*-kaurene, rather than freely equilibrating with a pool of copalyl pyrophosphate in the medium. Experiments in which the rates of the overall *AB* and independent *B* activities of kaurene synthetase preparations were measured as a function of total protein concentration further suggest that overall *AB* activity is catalyzed by an *AB* enzyme complex which is in equilibrium with free *A* and *B* enzymes. A model is proposed for *M. macrocarpus* kaurene synthetase in which separate but interacting *A* and *B* enzymes must associate for the efficient production of *ent*-kaurene from geranylgeranyl pyrophosphate.

The diterpene hydrocarbon *ent*-kaurene[3], an intermediate in the pathway for the biosynthesis of gibberellins (6, 15, 17, 26), is synthesized from the acyclic precursor geranylgeranyl-PP in a two step reaction catalyzed by kaurene synthetase (Fig. 1). In the first step (*A* activity) the intermediate copalyl-PP is synthesized from geranylgeranyl-PP (16, 22). In the second stage of cyclization (*B* activity) copalyl-PP is converted to kaurene. Overall conversion of geranylgeranyl-PP into kaurene is referred to as *AB* activity.

Because geranylgeranyl-PP is a branch point metabolite and serves as a precursor of carotenoids (2, 3, 21), the phytyl chain of Chl and other pigments (25), and other diterpenes as well as kaurene (20), it is likely that kaurene synthetase activity is subject to regulation based on the organism's need for kaurene at a given time (17). However, the *in vivo* regulation of the activity is not well understood. The presence of *B* activity in some mature tissues in the absence of detectable *A* activity (23, 24, 27) may reflect some aspect of regulation, although the basis for this has not been established.

Overall *AB* activity can be detected in many immature plant tissues (4, 11, 13, 14, 19, 20, 23, 27). It has not been established previously whether one enzyme catalyzes the overall reaction or if two separate enzymes are required, one for the *A* step and another for the *B* step. Preparations active in the catalysis of *B* but not *AB* activity have been obtained from plant sources that possess overall *AB* activity (23); however, all previous attempts to isolate an *A* enzyme free of *B* activity have been unsuccessful (8, 11, 23). An enzyme that converts geranylgeranyl-PP into kaurene has been purified 170-fold from mycelia of the gibberellin-producing fungus, *Fusarium moniliforme* (8). Throughout this purification a constant ratio of *A* to *B* activity was observed. It was not possible to dissociate the complex of about 460,000 daltons and retain either *A* or *B* activity. Kaurene synthetase preparations from cell-free extracts of *Ricinus communis* seedlings were resolved by DEAE Sephadex A-25 chromatography into two regions with *B* activity and one broad region with *AB* activity (23). Partial purification of kaurene synthetase from cell-free preparations of the endosperm of immature *Marah macrocarpus* seeds did not result in resolution of the *A* and *B* catalytic activities; however, the ratio of the two activities did not remain constant throughout purification (11).

The present study was carried out to clarify further the relationship between the *A* and *B* activities of kaurene synthetase from a higher plant source because of its possible regulatory significance in gibberellin biosynthesis. Evidence will be presented which indicates that the synthesis of kaurene from geranylgeranyl-PP in endosperm tissue of *M. macrocarpus* is catalyzed by two separate but interacting enzymes.

MATERIALS AND METHODS

Plant Materials. *M. macrocarpus* fruit was picked in the Santa Monica Mountains in late March or early April. Immature seeds from the fruit were stored at −20 C prior to use.

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[3] Abbreviations: kaurene: *ent*-kaurene; geranylgeranyl-PP: all *trans*-geranylgeranyl pyrophosphate; copalyl-PP: *ent-trans*-labda-8(17),13-dien-15-yl pyrophosphate.

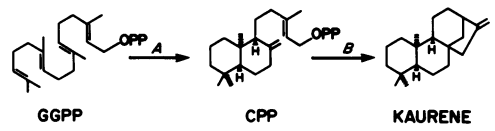

FIG. 1. The reactions catalyzed in the *A* and *B* enzymic steps of kaurene synthetase. The *A* step involves the conversion of geranylgeranyl-PP (GGPP) to copalyl-PP (CPP) and the *B* step involves the conversion of copalyl-PP to kaurene.

Radioactively Labeled Chemicals. [2-^{14}C]Geranylgeraniol (2 μCi μmol^{-1}) was prepared from *trans, trans*-farnesylacetone and methyl bromo[2-^{14}C]acetate by the method of Simcox (23). Farnesylacetone was a gift from G. Popjak and methyl bromo[2-^{14}C]acetate (10 μCi μmol^{-1}) was from Amersham/Searle. [1-^{3}H] Geranylgeraniol (60 μCi μmol^{-1}) and [1-^{3}H]copalol (33 μCi μmol^{-1}) were the generous gifts of Robert Coates of the University of Illinois. Pyrophosphate esters were synthesized from geranylgeraniol and copalol according to the method of Cramer and Böhm (5).

Other Materials. *Ent*-kaurene, a gift from Abbott Laboratories, was purified by silica gel column chromatography before use. Imidazole (Sigma) was recrystallized from ethyl acetate before use. Dye reagent concentrate for protein determinations by the method of Bradford (1) was obtained from Bio-Rad. All other reagents and enzymes from commercial sources were used without further purification.

Kaurene Synthetase Assays. Three different assay systems were used to measure kaurene synthetase activities. Conversion of geranylgeranyl-PP to copalyl-PP was measured by the *A* activity assay system. Incubation mixtures included 40 mM Tris or Tes (pH 7.2), 5 mM $MgCl_2$, and 2 μM [1-^{3}H]geranylgeranyl-PP (1.29×10^5 dpm) in a total volume of 0.5 or 1 ml. Incubations were for 10 to 30 min at 30 C after which the reactions were terminated by the addition of 100 μl of 0.4 M Na_2CO_3. *Escherichia coli* alkaline phosphatase (2 IU) was then added to hydrolyze pyrophosphate esters and the incubation was continued overnight. Conversion of geranylgeranyl-PP to kaurene was measured by the *AB* activity assay. Conditions were identical to the *A* activity assay except that the incubation mixture also included 10 mM K-phosphate. Conversion of copalyl-PP to kaurene was measured by the *B* activity assay. Incubations were identical to the *AB* activity assay except that 1.6 μM [1-^{3}H]copalyl-PP (5.6×10^4 dpm) served as the substrate. *AB* and *B* activity assays were performed for periods of 0.25 to 30 min at 30 C after which the reactions were terminated by the addition of 0.5 ml of ethanol.

Incubation mixtures were extracted with 3 successive 1-ml portions of petroleum ether (40–60 C)-benzene (10:1). The organic extracts were combined and concentrated under a stream of N_2. The concentrated extracts from *A* activity assays were applied to 2- × 20-cm analytical silica gel 60 plates impregnated with $AgNO_3$ and developed in petroleum ether (40–60 C)-ethyl acetate-isopropyl ether (1:1:1). Unlabeled geranylgeraniol, copalol, and kaurene were used as standards. The regions from the plates containing geranylgeraniol, copalol, and kaurene were scraped into scintillation vials and radioactivity was determined as described below. The concentrated extracts from *AB* and *B* activity assays were applied to 2- × 10-cm plain silica gel 60 plates and developed in petroleum ether. The kaurene region near the solvent front was scraped into a scintillation vial and radioactivity determined as described below.

The cocktail used for counting nonaqueous samples and regions scraped from silica gel plates consisted of 4 g of Omnifluor dissolved in 1 liter of a solution of toluene and dioxane (95:5). Ten ml of cocktail was added to each vial which was then precounted before the addition of the sample. Counting was carried out in either a Packard model 2002 or a Beckman model LS 100C scintillation spectrophotometer. Counting efficiencies ranged from 25 to 56% for tritium and from 60 to 98% for ^{14}C depending on the instrument and settings that were used. All counting rates have been corrected for background.

One unit of activity refers to the production of 1 pmol of product/min at 30 C. Kaurene was the only radioactive hydrocarbon detected in each of three different TLC systems following incubation with either substrate.

Enzyme Preparation. Seeds stored at −20 C were partially thawed, sliced length-wise into halves, and then the endosperm tissue was scooped out with a spatula. Endosperm tissue from seeds more than about one-third filled with cotyledonous tissue was normally not used. Pooled endosperm (30–150 ml) was diluted with 0.5 volume of a buffer consisting of 10 mM imidazole-HCl (pH 6.5 or 7.0), 20 mM 2-mercaptoethanol, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2% glycerol. The suspension was homogenized by hand in a 50-ml Thomas tissue grinder fitted with a Teflon pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 26,000*g* for 20 min. The pellet was discarded, ammonium sulfate was added to 45% of saturation, and the suspension was stirred for 20 to 30 min. The suspension was centrifuged 10 min at 12,000*g*; the resulting pellet was resuspended in a small volume of the homogenization buffer and then dialyzed overnight against 1 or 2 liters of the same buffer. This desalted enzyme solution catalyzed both *A* and *B* activities. If an enzyme preparation containing predominantly *B* activity was desired, the supernatant solution after removal of the 45% ammonium sulfate pellet was brought to 80% of saturation with ammonium sulfate, and a desalted enzyme solution prepared from the resulting pellet in a fashion similar to that described above. All of these operations were carried out either at 4 C or on ice. The enzyme solutions were either used directly or further purified as described below.

After ammonium sulfate fractionation, *A* and *B* enzymic activities were further purified for some experiments by Sephadex A-50 or DEAE Sephadex A-25 chromatography. The conditions of chromatography for individual experiments are presented in the figure legends.

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed in pH 7.5, 5% or 7.5% polyacrylamide gels prepared according to the method of Gabriel (12), with slight modifications. The pH 7 running buffer consisted of 30 mM barbital, 8.3 mM Tris, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2% glycerol. Gels were routinely subjected to electrophoresis before application of protein samples since this was found to result in greatly increased levels of detectable kaurene synthetase activity in gel slices after electrophoresis. Stacking gels had a tendency to collapse as a result of this preelectrophoresis treatment and therefore were not used in these experiments. After a 20 to 30 min preelectrophoresis of gels at 3 mamp/tube, kaurene synthetase samples (up to 110 μg total protein), containing 10% sucrose and 0.01% bromophenol blue in a total volume of 20 to 70 μl, were gently layered onto the gels. Electrophoresis was then carried out at 2 to 4 C with a current flow of 3 to 5 mamp per tube. Gels to be assayed for kaurene synthetase activities were sliced into 3-mm segments which were individually placed in incubation tubes and assayed for *A*, *AB*, or *B* enzymic activities.

Molecular Weight Determinations. Molecular weights were determined by Sephadex G-100 gel filtration and sedimentation velocity analysis. Sephadex G-100 chromatography was performed on a calibrated 5-cm × 1.35-m column. The column was developed in an upwards direction at a flow rate of 70 to 80 ml/h. Chromatography was performed in a buffer consisting of 10 mM imidazole-HCl (pH 7.0), 20 mM 2-mercaptoethanol, 50 mM KCl, 50 mM $MgCl_2$, 0.1 mM EDTA, 2% glycerol, and 0.01% NaN_3. The column was calibrated using horse liver alcohol dehydrogenase (mol wt 83,000), BSA (mol wt 66,000), chicken ovalbumin

(mol wt 43,000), and horse heart Cyt *c* (mol wt 12,400) as protein mol wt markers.

For sedimentation velocity analysis 0.3 to 0.5 ml of a kaurene synthetase *A*, *AB*, or *B* enzyme preparation was layered on a linear 5 to 20% (w/v) sucrose gradient in the same pH 7 buffer used for Sephadex G-100 chromatography. Chicken ovalbumin (4 to 7.5 mg), horse liver alcohol dehydrogenase (0.75 to 3 mg) and pig heart fumarase (4 to 10 IU) were either included with the kaurene synthetase preparation or layered on separate gradients as mol wt markers. The gradients were then centrifuged in a Beckman SW41 rotor in a model L5-65 centrifuge at 4 C. The rotor speed and time of centrifugation for each experiment are given in the figure legends. After centrifugation, the gradients were collected from the bottom in 0.4- to 0.5-ml fractions. Relative distance sedimented was plotted *versus* log mol wt for each marker to give a calibration curve for determination of the unknown molecular weights (9).

Concentration of Protein Solutions. Solutions of kaurene synthetase were concentrated by ultrafiltration over Amicon XM50 or PM10 membranes.

RESULTS

Ammonium Sulfate Fractionation. The *B* activity from the 26,000*g* supernatant fraction of a cell-free homogenate could be partially resolved from *A* activity by ammonium sulfate fractionation. For example, in a typical experiment, 85% of the *A* activity and only 38% of the *B* activity were precipitated in the 0–45% ammonium sulfate fraction whereas 62% of the *B* activity and only 15% of the *A* activity were recovered from the 45–80% ammonium sulfate fraction. The recoveries of initial activity of both types were low if measured immediately after overnight dialysis at 4 C of the ammonium sulfate fractions; however, the activities slowly increased over a period of 24 to 36 h to the point that 70–100% of the initial *AB* activity and 60–80% of the initial *B* activity were recovered.

Ammonium sulfate fractionation did not result in any significant change in the specific activity for catalysis of either the *A* or *B* steps, but it did greatly increase the stability of both the *A* and *B* activities to storage.

QAE Sephadex A-50 Chromatography. To achieve the most effective purification, it seemed desirable to perform anion exchange chromatography at the lowest pH at which the enzymes for kaurene synthesis would bind to the gel and still retain activity. It was found that the enzymes which catalyze both the *A* and *B* steps were irreversibly denatured below pH 6, but at pH 6.5 both activities appeared to be reasonably stable. Therefore this pH was chosen.

A resuspended and dialyzed 45% ammonium sulfate fraction was prepared from 125 ml of *M. macrocarpus* endosperm. The sample, which catalyzed both *A* and *B* activities, was applied to a 400-ml bed-volume column of QAE Sephadex A-50 and chromatography was performed as described in the legend for Figure 2. Fractions containing *A* activity essentially free of *B* activity (125–135), *AB* activity (136–155) and *B* activity essentially free of *A* activity (156–185) were obtained from this column.

Since the occurrence of an independent *A* catalytic activity free of *B* activity had not been previously observed, the possibility was considered that it was formed during the homogenization or ammonium sulfate fractionation steps as a result of a modification of an enzyme which normally catalyzes overall *AB* activity. To test for this possibility, fractions from this first column which catalyzed overall *AB* activity (145–150) were pooled and rechromatographed on a second column of QAE Sephadex A-50 under identical conditions (Fig. 3). Fractions which catalyze *A* activity free of *B* activity were once again obtained even though the sample applied to the column contained an excess of *B* over *A* catalytic activity.

Fractions containing *A* essentially free of *B* activity, *AB* activity,

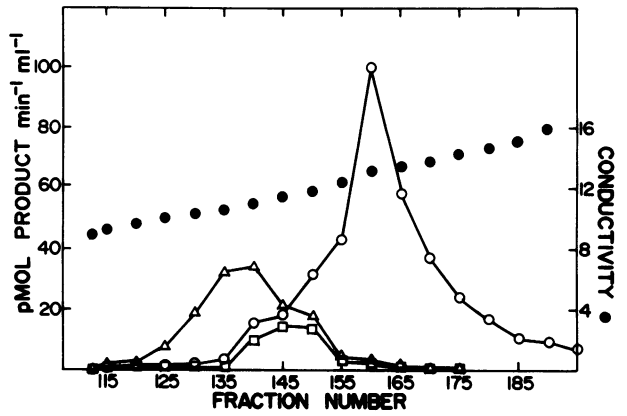

FIG. 2. QAE Sephadex chromatography. A 45% ammonium sulfate fraction from the endosperm of *M. macrocarpus* seeds containing 58 mg protein was applied to a 400-ml bed-volume column of QAE Sephadex A-50. The column was washed with 50 mM KCl in a pH 6.5 buffer consisting of 10 mM imidazole-HCl, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 2% glycerol, and 0.1 mM EDTA. After washing, the column was eluted with a 1.6 liter linear 0.05 to 0.5 M KCl gradient in the same buffer. Two-hundred μl aliquots were assayed for 10 min for *A* activity (△), *AB* activity (□), and *B* activity (○). Conductivity in mMhos is indicated by (●).

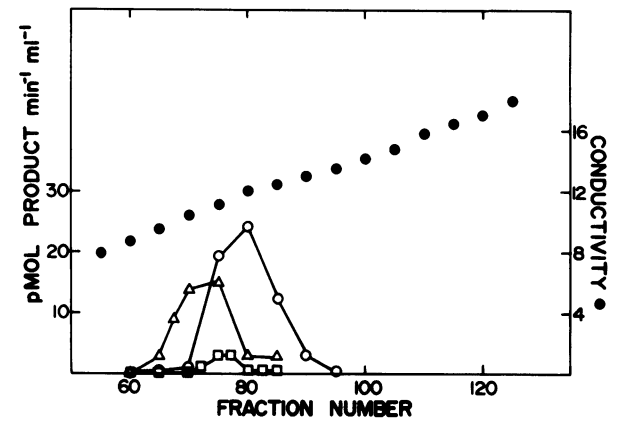

FIG. 3. QAE chromatography of an *AB* activity pool. Fractions 145 to 150 from the first QAE Sephadex column were pooled and applied to a 225-ml bed volume column of QAE Sephadex A-50. The column was eluted with a 1.2 liter linear 0.05 to 0.5 M KCl gradient in pH 6.5 buffer. Two-hundred μl aliquots were assayed for 30 min for *A* activity (△), *AB* activity (□), and *B* activity (○). Conductivity in mM hos is indicated by (●).

and *B* essentially free of *A* activity were also obtained from DEAE Sephadex A-25 sievorptive chromatography at pH 7.5 of a 45% ammonium sulfate fraction, and from QAE Sephadex A-50 chromatography of a 26,000*g* supernatant (results not shown).

Separation of *A* and *B* Enzymes by Disc Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed upon preparations which catalyzed overall *AB* activity. A sample from a 45% ammonium sulfate fraction was subjected to tube gel electrophoresis. Figure 4 shows *A*, *AB*, and *B* activities as a function of distance of migration into the gel. That the *B* enzyme migrates more rapidly towards the anode than the *A* enzyme is consistent with the observation that the *B* enzyme requires a higher concentration of chloride ion than the *A* enzyme for elution from anion exchange chromatography columns. Apparently the *B* enzyme is more negatively charged than the *A* enzyme in the vicinity of pH 7.

Coupling of *A* and *B* Enzymes. The product synthesized from geranylgeranyl-PP by *A* enzymic preparations was tentatively

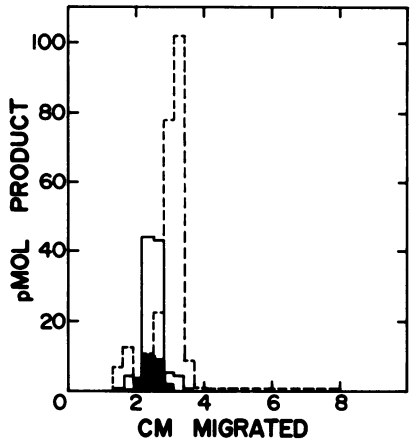

FIG. 4. Disc gel electrophoresis of a kaurene synthetase preparation. Seventy-four μg protein from a 45% ammonium sulfate fraction was subjected to electrophoresis at pH 7.5 in 5% polyacrylamide disc gels. Segments (3 mm) of the same gel were assayed for *A* activity (▬), *AB* activity (■), and *B* activity (----).

Table I. *Coupling of A and B Enzymic Fractions Purified from M. macrocarpus Endosperm*

A and *B* enzyme preparations were obtained as described in the text. One-hundred-fifty μl (16 units, 4.5 μg protein) of the *A* preparation and 150 μl (9.4 units, 5.7 μg protein) of the *B* preparation were assayed for *AB* activity separately and when combined. Incubations were for 10 min. Each value is an average of at least 5 separate determinations.

A Enzyme	*B* Enzyme	Kaurene Synthesized
μl		*pmol* ± *SD*
150		0.8 ± 0.2
	150	0.4 ± 0.4
150	150	14 ± 2

identified as copalyl-PP by TLC. Experiments were performed to confirm the identification by determining whether this product could be utilized for kaurene synthesis by the *B* enzyme. *A* enzyme preparations essentially free of *B* activity and *B* enzyme preparations essentially free of *A* activity were obtained from QAE Sephadex A-50 chromatography of 45 and 80% ammonium sulfate fractions, respectively. These *A* and *B* enzyme preparations were assayed for their capacity for kaurene synthesis from geranylgeranyl-PP both separately and in combination. The results (Table I) show that the product synthesized by the *A* enzyme can be utilized for kaurene synthesis by the *B* enzyme, and therefore the identity of this product is confirmed as copalyl-PP.

A similar experiment was performed in which an *A* enzyme preparation was combined with a 50% ammonium sulfate fraction obtained from a spinach leaf chloroplast stromal preparation according to a procedure of Simcox (23). Such a preparation catalyzes only *B* activity. The combination of the *M. macrocarpus* *A* enzyme with the spinach leaf chloroplast stromal preparation is active in the synthesis of kaurene from geranylgeranyl-PP, whereas neither of these preparations alone were active in this conversion (Table II).

Determinations of Molecular Weights. Molecular weights were determined by Sephadex G-100 gel filtration. Preparations possessing *A*, *AB*, and *B* activity were analyzed separately by this procedure. The *A* enzyme preparation was obtained after QAE Sephadex A-50 chromatography of a 45% ammonium sulfate fraction. A 45% ammonium sulfate fraction served as the sample with *AB* activity. The preparation with only *B* activity was obtained after QAE Sephadex A-50 chromatography of a 45 to 80% ammonium sulfate fraction. The elution profiles for the proteins which catalyze *A*, *AB*, and *B* activities are shown in Figure 5. All

Table II. *Coupling of an A Enzyme Fraction from M. macrocarpus Endosperm with a B Enzyme Preparation from Spinach Leaf Chloroplasts*

An *A* enzyme preparation was obtained as described in "Materials and Methods". A *B* enzyme preparation was obtained after ammonium sulfate fractionation of a spinach leaf chloroplast stromal preparation using the procedure of Simcox (23). One hundred-eighty μl (19 units) of the *A* preparation was combined with 150 μl (6.8 units) of the spinach fraction and assayed for overall *AB* activity. Each value is an average of three determinations.

A Enzyme *M. macrocarpus* Endosperm	*B* Enzyme Spinach Chloroplast	Kaurene Synthesized
μl		*pmol* ± *SD*
180		1.1 ± 0.4
	150	0.4 ± 0.5
180	150	15.5 ± 1

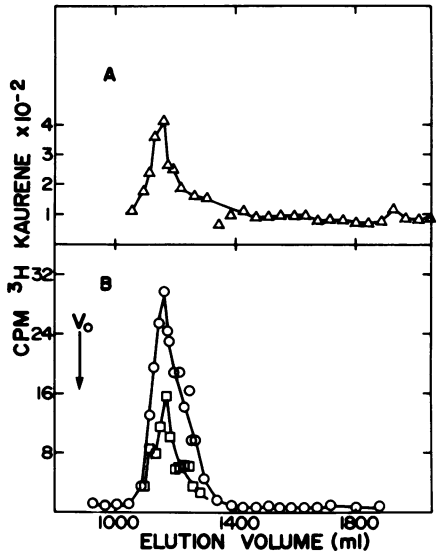

FIG. 5. Sephadex G-100 Chromatography of *A*, *AB*, and *B* enzymic preparations. Preparations active in the catalysis of *A*, *AB*, and *B* activity were obtained and chromatographed as described in the text. (A) *A* activity (Δ) free of *B* activity was measured by a coupling assay. Two-hundred μl from each column fraction to be assayed was combined with 200 μl of a *B* preparation, obtained as described in "Materials and Methods." Assays for overall *AB* activity were then performed for 30 min. Control incubations in which the exogenous *B* preparation was not added did not catalyze kaurene synthesis from geranylgeranyl-PP. (B) *AB* activity (□) and *B* activity (○) were assayed as described in "Materials and Methods."

three activities appear to be catalyzed by species with mol wt in the range of 83,000 ± 3,000.

Sedimentation velocity measurements were employed as a second method for the estimation of the molecular weights of the *A* and *B* enzymes. It was hoped that this method might reveal small differences in the molecular weights of the *A* and *B* enzymes more sensitively than Sephadex G-100 gel filtration.

A 45% ammonium sulfate fraction prepared from 100 ml endosperm was subjected to QAE Sephadex A-50 chromatography and the fractions containing *A* enzyme essentially free of *B* activity were pooled. Fifteen ml of this pool was concentrated to 6 ml by ultrafiltration, layered on a sucrose gradient, and subjected to sedimentation (Fig. 6). A *B* enzyme preparation was also layered on and sedimented in a sucrose gradient in the same manner (Fig. 7). The mol wt for the *A* and *B* enzymes as determined from sedimentation velocity were 75,000 ± 6,000 and 81,000 ± 6,000, respectively. These values are in reasonable agreement with the

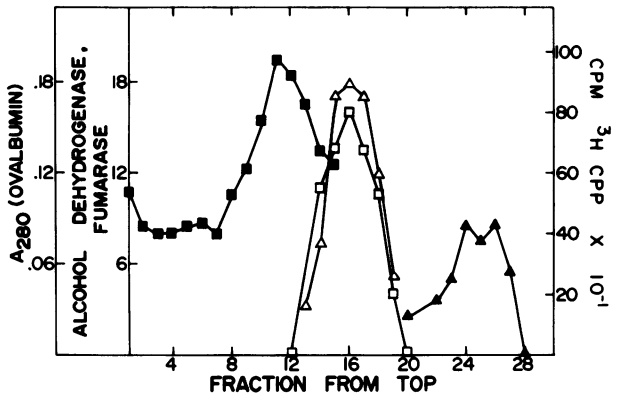

FIG. 6. Sedimentation of an *A* enzyme preparation. Four-tenths ml of an *A* preparation free of *B* activity which contained pig heart fumarase, horse liver alcohol dehydrogenase, and chicken ovalbumin as mol wt marker proteins was layered on a 5–20% linear sucrose density gradient and centrifuged 26.5 h at 40,000 rpm in a Beckman SW41 rotor. Fumarase activity (▲) and alcohol dehydrogenase activity (□) are expressed in arbitrary units. Ovalbumin (■) was determined by measuring the *A* at 280 nm. Kaurene synthetase *A* activity (Δ) was determined as described in "Materials and Methods." Sedimentation is towards the right.

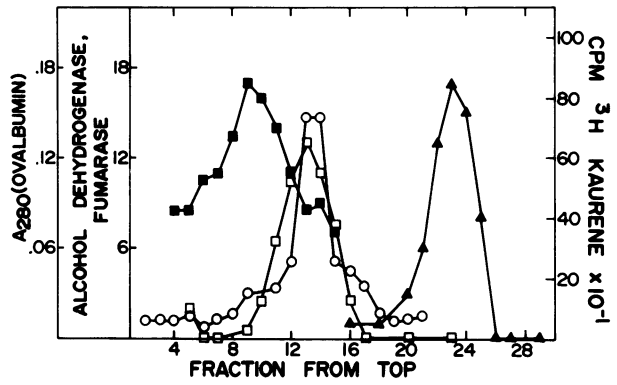

FIG. 7. Sedimentation of *B* enzyme. Five-tenths ml of a *B* preparation was layered on a 5–20% linear sucrose density gradient and centrifuged 66 h at 27,000 rpm and 4 C in a Beckman SW41 rotor. Mol wt markers were fumarase (▲), alcohol dehydrogenase (□), and ovalbumin (■). Kaurene synthetase *B* activity (○) was assayed as described in "Materials and Methods."

values of 83,000 ± 3,000 determined for each by Sephadex G-100 gel filtration.

The possibility that a native *AB* enzyme might consist of an *A* subunit covalently bound to a *B* subunit through disulfide linkage was investigated by determining molecular weights for *A* and *B* enzymes in a 45% ammonium sulfate fraction prepared in the absence of thiol reducing agents. The 45% ammonium sulfate fraction was prepared as described in "Materials and Methods" except for the omission of 2-mercaptoethanol in the buffer used for homogenization and dialysis. The *A* and *B* enzymes in this preparation were analyzed by sedimentation velocity in the absence of thiol reducing agents for determination of mol wt. The mol wt for the *A* and *B* enzymes were similar to those determined in the presence of a thiol reagent, and therefore it is unlikely that these two enzymes are normally linked to one another through disulfide bonds in the absence of such reducing agents.

Channelling in Substrate Utilization. Frost and West (11) observed that incubations of kaurene synthetase from *M. macrocarpus* endosperm with geranylgeranyl-PP under conditions of low divalent metal ion concentration led to the synthesis of some kaurene, whereas exogenous copalyl-PP was not converted to kaurene under these conditions. These results suggested that copalyl-PP produced *in situ* from geranylgeranyl-PP is preferentially channelled for kaurene production. Since the *A* and *B* steps of kaurene synthesis in *M. macrocarpus* endosperm appear to be catalyzed by two distinct enzymes, channelling of copalyl-PP would imply that the separate *A* and *B* enzymes must associate during kaurene synthesis.

Fall and West (8) proposed a model to predict the relative amounts of kaurene that would be synthesized from labeled geranylgeranyl-PP and labeled copalyl-PP as a function of time if the copalyl-PP derived from geranylgeranyl-PP were to equilibrate completely with an exogenously supplied pool of copalyl-PP before its conversion to kaurene. The following assumptions and conditions are inherent in this model: (a) The *A* and *B* steps are sequential first order reactions, (b) the system is at a steady state and therefore the concentration of copalyl-PP remains constant throughout the incubation, and (c) initially, geranylgeranyl-PP is ^{14}C-labeled and copalyl-PP is labeled with ^{3}H. The following equation derived by Fall and West (8) can be used for the determination of the ratio of [^{14}C]kaurene to [^{3}H]kaurene that would be expected at any time after reaction is initiated if complete equilibration of endogenously produced copalyl-PP and the exogenous pool of copalyl-PP were to occur under these conditions:

$$\frac{[^{14}C]\text{kaurene}}{[^{3}H]\text{kaurene}} = \frac{Vt}{[\text{copalyl-PP}]\left(1\text{-exp}\dfrac{Vt}{[\text{copalyl-PP}]}\right)} - 1$$

where *V* is the reaction velocity for overall kaurene synthesis and *t* is the reaction time.

If channelling of copalyl-PP from the *A* to the *B* enzyme were to occur the observed ratio of [^{14}C]- to [^{3}H]kaurene would be greater than that predicted by this complete equilibration model. The approach used by Fall and West (8) was taken in this present research to test directly for channelling of copalyl-PP in kaurene synthetase preparations from the endosperm of *M. macrocarpus*.

A 45% ammonium sulfate fraction was prepared and diluted with buffer to a protein concentration of 0.8 mg/ml. Assumption (b) for the model was approximated when substrate concentrations of 3.8 μM geranylgeranyl-PP and 0.41 μM copalyl-PP were present. Kaurene synthesis from either substrate incubated alone was linear for at least 6 min. [2-^{14}C]Geranylgeranyl-PP (2 μCi μmol^{-1}) and [1-^{3}H]copalyl-PP (28.6 μCi μmol^{-1}) were used as substrates in dual isotope experiments at the indicated concentrations. One-ml incubations were started by the addition of 20 μl of the diluted 45% ammonium sulfate fraction, and the reaction was allowed to continue for 5 min before the amounts of [^{14}C] and [^{3}H]kaurene were determined. The experimental parameters, the observed ratio of [^{14}C]- to [^{3}H]kaurene, and the expected ratio of [^{14}C] to [^{3}H]-kaurene if no channelling of copalyl-PP were to occur as calculated for the complete equilibration model under the present experimental conditions are shown in Table III.

The experimentally determined ratio of [^{14}C] to [^{3}H]kaurene is 13 times greater than that predicted if no channelling had occurred. The experiment was repeated with variable incubation times and the experimentally determined ratio of [^{14}C]- to [^{3}H]-kaurene was always at least ten-fold greater than that expected if complete equilibration of copalyl-PP had occurred.

Rates of Kaurene Synthesis as a Function of Protein Concentrations. The results of the previous experiment imply that the *A* and *B* enzymes must associate during the synthesis of kaurene from geranylgeranyl-PP. For such a situation it would be expected that the overall rate of kaurene synthesis from geranylgeranyl-PP would be much greater for associated than dissociated *A* and *B* enzymes, and therefore the concentration of the *AB* enzyme complex would be rate determining. The formation of the *AB* complex and hence the rate of the reaction would show a greater

Table III. *Channelling in Substrate Utilization*

The experimental parameters V, t and [copalyl pyrophosphate] are given. The experimentally determined ratio of [^{14}C]- to [^{3}H]kaurene which was observed is given for the comparison with that calculated by the equation of Fall and West (8) for these experimental parameters and the model in which no channelling of copalyl-PP occurs during kaurene synthesis. (See text for the equation and a discussion of the reference model). The ratios are averages of 4 experiments.

V	t	Copalyl-PP Concentration	^{14}C/^{3}H Kaurene ± SD	
			Found	Calculated
nmol/min·ml	<*min*>	*nmol/ml*	*ratio*	
0.0072	5	0.41	0.57 ± 0.04	0.044 ± 0.007

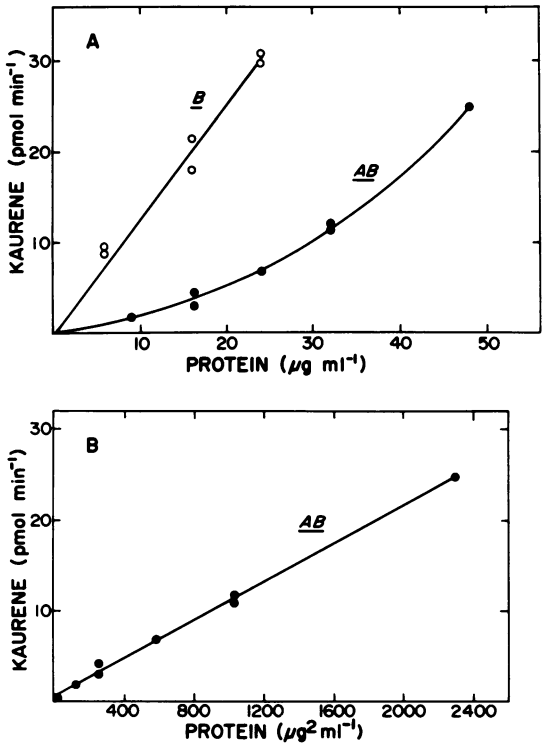

FIG. 8. Rates of kaurene synthesis as a function of protein concentration. (A) Each point was determined from the slope of the least squares line obtained from measurements of pmol kaurene synthesized as a function of time. Incubation times ranged from 1-6 min. Each point was determined from at least four separate incubations. The enzyme preparation used for the incubations was a 45% ammonium sulfate fraction. *AB* activity (●), *B* activity (○). (B) A least squares line is fitted to a plot of *AB* activity *versus* the square of the protein concentration.

than first order dependence on total concentration of enzyme when the concentrations of *A* and *B* are simultaneously varied. For example, for an A_1B_1 complex the change of rate with respect to total enzyme concentration would approach second order in regions where the concentrations of *A* and *B* are low relative to the dissociation constant for the A_1B_1 complex.

Rates of kaurene synthesis from both geranylgeranyl-PP and copalyl-PP were measured as a function of total protein concentration as an experimental test of this model (Fig. 8a). The rate dependence of overall *AB* activity shows a higher than first order dependence over the protein concentration range investigated in the experiment, while *B* activity appears to be directly proportional to protein concentration. When *AB* activity is plotted *versus* the square of the protein concentration a linear relationship is seen (Fig. 8b). This indicates a second order dependence of *AB* activity on protein concentration over the range of concentrations investigated.

DISCUSSION

The partial separations of *A* and *B* activities by anion exchange chromatography and polyacrylamide gel electrophoresis are most readily explained if the two catalytic activities reside in separate enzymes. The results of the rechromatography experiment (Fig. 3) indicate that the *AB* activity present in some fractions eluted from the first column (Fig. 2) was due to incomplete resolution of *A* and *B* enzymes and not to the presence of one enzyme which catalyzes overall *AB* activity. Partial resolutions of enzymes for the *A* and *B* activities were also evident from the results of ammonium sulfate fractionation and when a different anion exchange resin was used for chromatography. Finally, it should be noted that the catalysis of overall *AB* activity by a single enzyme would be inconsistent with its observed second order dependence on protein concentration.

In earlier work, Frost (10) did not find substantial evidence for the resolution of *A* and *B* activities during purifications of kaurene synthetase from this same source. However, his results did show that the peak of synthetic activity for the conversion of mevalonate to kaurene eluted slightly ahead of a peak of kaurene synthetase *B* activity during QAE Sephadex A-25 chromatography of a lyophilized 26,000*g* supernatant preparation, although the two activities overlapped substantially. The peak fractions for *AB* activity also eluted slightly ahead of the peak fractions for *B* activity. *A* activity alone was not measured directly in that study. In retrospect, those earlier results indicated that an *A* enzyme was being partially resolved from a *B* enzyme by this chromatographic procedure, although that conclusion was not reached at the time.

The close similarity in the apparent mol wt for the *A* and *B* enzymes suggests the possibility of some evolutionary relationship between them. Based on the different mechanistic requirements for the *A* and *B* steps (18) and the selective inhibition of the *A* step by some plant growth retardants and related substances (11), it seems most likely that different polypeptides are involved in the two steps. However, it is conceivable that separate molecular species, one for catalysis of the *A* step and another for catalysis of the *B* step, could arise from different *in vivo* covalent modifications of a single polypeptide. It would be interesting to explore these possibilities by examining the structural properties of the two proteins.

The apparent mol wt of the *AB* activity when both *A* and *B* enzymes were present together during gel filtration or velocity sedimentation experiments was not significantly different from that of the *A* or *B* enzyme alone. No evidence was seen for an active species of higher mol wt corresponding to a dimer or oligomer made up of *A* and *B* monomers. Either the concentrations of *A* and *B* enzymes during these procedures were too low for significant association to occur, or alternatively, association may occur only in the presence of one of the substrates. Attempts were made to trap the *A* and *B* enzymes in an active state as an *AB* complex by cross-linking with bifunctional reagents, but these experiments were not successful.

Frost and West (11) earlier reported a mol wt of <40,000 for the kaurene synthetase from this source. The basis for this discrepancy is not known. The two methods based on different principles employed in the present work give good agreement with one another. Therefore, we believe that the mol wt values reported in this paper are more reliable estimates.

The results of this present research are most consistent with a model for *M. macrocarpus* kaurene synthetase in which the *A* and *B* activities are catalyzed by two distinct enzymes which associate during the synthesis of kaurene from geranylgeranyl-PP. A situation analogous to this occurs in the oxidation of histidinol to histidine by the action of histidinol dehydrogenase in *Salmonella*

typhimurium (7). The model is consistent with the inability to detect significant levels of copalyl-PP when geranylgeranyl-PP is incubated under the usual assay conditions (11). Channelling has now been reported to occur in a number of enzyme complexes including the undissociated kaurene synthetase *AB* complex from *F. moniliforme* (8). Most of these cases involve either multifunctional proteins or multienzyme complexes so the present case involving two apparently monofunctional associating enzymes is unusual. Channeling may be most important as a means of keeping the concentrations of intermediates (such as copalyl-PP in this case) low to prevent side-reactions and to conserve cellular solvent capacity.

If the occurrence of separate, but associating, enzymes for the catalysis of *A* and *B* activities as described here for *M. macrocarpus* kaurene synthetase is general in higher plants, it would appear to have some important implications for the regulation of biosynthesis of not only kaurene and the gibberellins, but also other cyclic diterpenes. For example, it is known that copalyl-PP is a precursor of *ent*-trachylobane, sandaracopimaradiene and *ent*-beyerene in addition to *ent*-kaurene in germinating castor bean seedlings (19, 20). It is possible that the partitioning of copalyl-PP between various cyclic diterpene products is governed by the degree of association of a common *A* enzyme with each of a group of *B* enzymes specific for the production of a particular cyclic diterpene. Also, the apparent absence of overall *AB* activity for kaurene synthesis in some plant tissues may best be explained by an inactivation or degradation of the *A* enzyme during maturation. Elucidation of the mechanisms operating in the regulation of the activity of the *A* enzyme and the partitioning of its product between various metabolic fates *in vivo* will require further investigation.

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