Biosynthesis of Gibberellins

II. ENZYMIC CYCLIZATION OF GERANYLGERANYL PYROPHOSPHATE TO KAURENE*

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SUMMARY

Geranylgeraniol-2-14C has been prepared by a modified Wittig reaction and converted to geranylgeranyl-2-14C pyrophosphate (17), which was purified by countercurrent distribution or chromatography on diethylaminoethyl cellulose.

An enzyme system from the endosperm of immature seeds of Echinocystis macrocarpa Greene catalyzed the cyclization of geranylgeranyl pyrophosphate to the tetracyclic diterpene kaurene. The enzymic activity, which is found in the 105,000 x g supernatant fraction, has been named kaurene synthase. It has a very sharp pH optimum at 6.6 and requires divalent metal ions, active in the order Mg++ > Co++ >> Zn++ > Mn++ > Cd++ 0 = Ca++, Cu++, Fe++. An assay for kaurene synthase is described, and a possible mechanism for cyclization of geranylgeranyl pyrophosphate to kaurene is discussed.

Geranylgeraniol, or a geranylgeranyl derivative, was suggested as the acyclic precursor of the bicyclic and tricyclic diterpenes by Ruzicka (2) in his exposition of the biogenetic isoprene rule. Wenkert (3) further rationalized the transformation of a pimaradiene to phyllocladene and other tetracyclic diterpenes through a nonclassical carbonium ion transition state. Cross et al. (4) first suggested the probable biogenetic relationship of the gibberellins and the diterpenes. Birch, Rickards, and Smith (5, 6) found that the positions which became labeled when gibberellic acid was biosynthesized by Fusarium moniliforme from 1-14C-acetate or 2-14C-mevalonate were consistent with the following sequence: acetate to mevalonate to geranylgeraniol to a phyllocladene-like tetracyclic diterpene to gibberellic acid. The stereochemistry of gibberellic acid resembles that of (-)-kaurene, a diterpene which differs from phyllocladene only by the stereochemistry at positions 5, 9, and 10 (7). Cross, Galt, and Hanson (8) have shown that kaurene is produced by F. moniliforme and is converted in the presence of cultures of this fungus to gibberellic acid.

Cell-free homogenates of wild cucumber (Echinocystis macrocarpa Greene) endosperm have been shown to convert mevalonate to geranylgeraniol, kaurene, kauren-19-ol, kauren-19-al, kauren-19-oic acid, and more oxidized derivatives (1, 9). Evidence presented in these papers and from other sources discussed in these papers supports a role for kaurene in gibberellin biosynthesis. Geranylgeraniol itself was not converted to gibberellic acid by P. moniliforme (1). It seems reasonable to propose that the pyrophosphate ester of geranylgeraniol is the actual precursor of kaurene by analogy with the involvement of farnesyl pyrophosphate in the biosynthesis of squalene and sterols. Geranylgeranyl pyrophosphate formation from mevalonate has been reported in extracts of Micrococcus lysodeikticus (10) and from farnesyl pyrophosphate and isopentenyl pyrophosphate in enzyme preparations from yeast (11) and plant and animal sources (12). Geranylgeranyl pyrophosphate is also produced from mevalonate in Echinocystis endosperm.2

Thus, gibberellin biosynthesis is presumed to occur by the following general pathway (in which GGPP stands for geranylgeranyl pyrophosphate and OPP is a pyrophosphate moiety).

2 M. O. Oster and C. A. West, to be published.
According to this view the free geranylgeraniol which is produced in cell-free homogenates of Echinocystis endosperm is the result of phosphatase action on the pyrophosphate ester. The work described in this paper was undertaken to test the hypothesis that geranylgeranyl pyrophosphate is the intermediate precursor of kaurene, a representative diterpene, and to obtain a cell-free enzyme system which would catalyze this reaction. The chemical synthesis of 2,4-C-geranylgeranyl pyrophosphate and its conversion to kaurene by an enzyme system from E. macrocarpa are described along with some properties of the crude enzyme system.

EXPERIMENTAL PROCEDURE

Preparation of Cell-free Extracts

Cell-free extracts of E. macrocarpa endosperm were prepared as previously described (1, 13). Three different enzyme preparations were used: crude homogenate which had been filtered through glass wool; the supernatant fraction from centrifugation of the crude homogenate at 37,000 x g for 30 min; and the supernatant fraction from centrifugation of the crude homogenate for 1 hour at 105,000 x g.

Chromatographic Procedures

Thin layer chromatography on 0.25-mm layers of Silica Gel G on glass plates was performed as described previously (1). Solvents were: System A, hexane; System B, benzene-ethyl acetate, 9:1; System C, hexane-ethyl acetate-methanol, 82:15:3; and System D, benzene. Compounds containing ethylenic double bonds were detected as yellow spots on a pink background after exposure of developed plates containing 0.05% sodium fluorescein to bromine vapor. Alternatively, most organic compounds were detected as yellow or brown spots after developed plates were exposed to iodine vapor. Thin layer chromatography with FeSO4 in H2SO4 followed by either distillation or passage over an alumina column (Woelm, basic, Activity Grade I). Butanol was redistilled before use. All other solvents and reagents were the best commercial grade available.

Bacterial alkaline phosphatase (28 units per mg) was obtained from Worthington. Methyl bromoacetate-2,4C was obtained from the Radiochemical Centre, Amersham, England, and from Calbiochem. Farnesylacetone was the generous gift of Dr. Otto Isler, Hoffman-La Roche. Reference samples of kaurene were the generous gifts of Dr. P. R. Jeffreys of the University of Western Australia, and of Dr. B. E. Cross of the Imperial Chemical Industries’ Akers Research Laboratories, Welwyn, England.

RESULTS

Synthesis of Geranylgeraniol

Methyl geranylgeranoate-2,4C was synthesized by a modified Wittig reaction, as described by Wadsworth and Emmons (15).
Methyl bromoacetate-2-14C (2 mmol, 13 μmol of 14C) was heated with 2 mmol of triethyl phosphate (redistilled, b.p. 52–55° at 16 mm) at 110° for 5 hours followed by 150° for 1 hour. The resulting oil was dissolved in 5 ml of rigorously dried 1,2-dimethoxyethane, and approximately 2.6 mmol of sodium hydride (50% suspension in oil) were added. The suspension was stirred until evolution of gas was no longer detected (about 15 min).

Farnesylacetone (2.15 mmol) was added in a small volume of 1,2-dimethoxyethane. The reaction mixture was stirred for 14 hours at room temperature and for 1 hour at 50°. The solution became yellow-brown, and an unidentified oil separated from solution during the course of the reaction. Water (20 ml) was added to the reaction mixture, and the aqueous suspension was extracted four times with 25-ml portions of diethyl ether. The combined organic extract contained approximately 50% of the 14C while 50% remained in the aqueous phase. Thin layer chromatography of a small portion of the organic extract in system D revealed three spots corresponding to farnesylacetone (Rf 0.24; no 14C), methyl geranylgeranoate (Rf 0.50; 77% of applied 14C), and methyl geranylgeranoate (Rf 0.61; 23% of applied 14C). The ether phase was dried over anhydrous sodium sulfate, and the residual oil, after removal of the diethyl ether under reduced pressure, was applied to a column (2 X 20 cm) of neutral alumina (Woelm, activity Grade II), which had been packed in petroleum ether (b.p. 50–70°). Elution of the column with petroleum ether gave incomplete resolution of the three components. Rechromatography of fractions enriched in methyl geranylgeranoate or methyl geranylgeranoate in the same system (a total of five columns were run) produced chromatographically homogeneous fractions in the following over-all yields (based on 7.7 μmol methyl geranylgeranoate-2-14C, 28.8%; unresolved cis-geranylgeranoate or methyl geranylnerole in the same system (a total of five columns were run) produced chromatographically methyl bromoacetate added) : methyl geranylgeranoate-2-14C, reduced pressure, was applied to a column (2 X 20 cm) of neutral alumina (Woelm, activity Grade II), which had been packed in petroleum ether (b.p. 50–70°). Elution of the column with petroleum ether gave incomplete resolution of the three components. Rechromatography of fractions enriched in methyl geranylgeranoate or methyl geranylgeranoate in the same system (a total of five columns were run) produced chromatographically homogeneous fractions in the following over-all yields (based on methyl bromoacetate added): methyl geranylgeranoate-2-14C, 7.7%; methyl geranylgeranoate 2-14C, 28.8%; unresolved cis-trans mixture, 3.7%; and unreduced farnesylacetone, 46.8% (of the 1.1-fold excess farnesylacetone added).

Geranylgeraniol-2-14C was produced by lithium aluminum hydride reduction of methyl geranylgeranoate-2-14C. Methyl geranylgeranoate-2-14C (0.38 mmol) was dissolved in 5 ml of dry, peroxide-free diethyl ether and chilled to −80°. LiAlH4 (3.9 mmol) was added, and the solution was stirred for 50 min while the temperature was permitted to rise to 10°. Excess ethyl acetate was added to destroy unreacted LiAlH4. Saturated ammonium chloride in water (0.4 ml) was added, and the precipitate, after its removal by centrifugation, was thoroughly washed with diethyl ether. The combined ether extracts were dried over sodium sulfate, concentrated in a vacuum, and applied to a column of 40 g of neutral alumina (Woelm, Activity Grade II). Geranylgeraniol-2-14C (0.42 mmol; 73% for this step) was eluted from the column with benzene. The product was at least 99% geranylgeraniol as indicated by thin layer chromatography. The 4-(4′-nitrophenylnazo)benzoyl ester was prepared, chromatographed, and recrystallized to constant specific radioactivity (1, 16) after addition of unlabeled carrier geranylgeraniol. The specific activity of the recrystallized ester was within experimental error of the starting material, indicating the radiochemical purity of the product.

Geranylgeranyl Pyrophosphate

Geranylgeranyl pyrophosphate was synthesized by a modification of the method of Cramer and Böhm (17). A solution of geranylgeraniol-2-14C (330 μmol; 1.65 X 104 dpm) in less than 1 ml of benzene was taken up in about 6 ml of acetonitrile. Tri-chloroacetonitrile (700 μmol) was added, and the solution was stirred at room temperature (23°) for about 15 min. Triethyl ammonium dihydrogen phosphate (400 μmol 1.5 ml of acetonitrile) was added dropwise, and the reaction was stirred for 2 hours after addition was complete. An additional 700 μmol of trichloroacetonitrile were added, followed after 15 min of stirring by 400 μmol of di(triethylammonium) hydrogen phosphate in 1.5 ml of acetonitrile. Stirring was continued for 16 hours at about 23°.

Twelve milliliters of 0.12 M NH₄OH were added to the reaction mixture, which was then extracted three times with 20-ml portions of peroxide-free diethyl ether. The combined ether solutions were washed with 5 ml of water, and the water was added to the aqueous phase of the reaction mixture. The aqueous extract from the reaction mixture (1.91 X 105 dpm; 18.2%) was subjected to a 100-transfer countercurrent distribution between the two phases of a mixture of 1-butanol, isopropyl ether, concentrated NH₄OH, and water (15:5:1:19); 10 ml of each phase were used per tube. The countercurrent distribution machine was operated manually to assure sufficiently gentle mixing of phases to minimize the emulsions which otherwise occur in this system. An unidentified component (or components), presumably geranylgeranyl triphosphate, remained in the very early tubes (0 to 3 in this case). Geranylgeranyl pyrophosphate was found in a broad peak centered at tube 19. Geranylgeranyl phosphate was found as the major radioactive component in a broad peak centered at tube 64. Unidentified minor components, apparently less polar than geranylgeranyl phosphate, were found near the front (tubes 90 to 100 of most runs). The relative quantities of reaction products are roughly indicated in Fig. 1, although the proportions of the various reaction products varied somewhat from run to run, particularly the relative amount of the unidentified components with very high and very low partition coefficients. Those tubes containing geranylgeranyl pyrophosphate (tubes 8 to 34 in Fig. 1) were pooled; 3 moles of Tris (free base) per mole of geranylgeranyl

![Fig. 1. Countercurrent distribution of products of pyrophosphorylation of geranylgeraniol-2-14C. See the text for sample preparation (1.47 X 10⁵ cpm). The solvent system consisted of the two phases of a mixture of 1-butanol, isopropyl ether, concentrated NH₄OH, and water (15:5:1:19); 10 ml of each phase were used per tube.](http://www.jbc.org/)

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pyrophosphate were added, and the solution was concentrated in a vacuum on a rotary evaporator at 35°C or less.

**Acid and Phosphatase Hydrolysis of Synthetic Geranylgeranyl Pyrophosphate**

- **Pyrophosphatase**—An analysis of the products of acid hydrolysis or alkaline phosphatase hydrolysis of the pooled fractions containing suspected geranylgeranyl-2-14C pyrophosphate indicated that this product was present in reasonable purity. Treatment with m HCl at 100°C yielded 2 moles of P1 per mole of geranylgeranyl pyrophosphate (Table I). Treatment with m HCl for 10 min at 30°C caused release of 14C to a benzene-extractable form without releasing any P1. This is consistent with hydrolysis of the very acid-labile allylic pyrophosphate into a mixture of geranylgeraniol and geranyllinalool and inorganic pyrophosphate without hydrolysis of pyrophosphate to P1. Treatment of various geranylgeranyl pyrophosphate preparations with m HCl for 15 min at 50°C resulted in nearly quantitative release of 14C to a benzene-extractable form.

- Treatment of geranylgeranyl-2-14C pyrophosphate (55.5 nmoles) with 0.2 mg of bacterial alkaline phosphatase for 30 min in 0.1 M KHCO3 led to the release of 14C in a form extractable with benzene. Thin layer chromatography of this material in System C or B indicated that 82% of the 14C cochromatographed with geranylgeraniol. As expected, there was no indication of isomerization to geranylnerol (the cis isomer) or geranyllinalool (the isomeric, allylic tertiary alcohol) during these treatments. Small amounts of radioactivity, which may have been due to oxidized or hydrated geranylgeranyl derivatives either present in geranylgeranyl pyrophosphate or formed during phosphatase treatment, were found near the origin.

Geranylgeranyl pyrophosphate from a countercurrent distribution run sometimes contained unidentified impurities, presumably generated during the pyrophosphorylation reaction. These could be removed on a DEAE-cellulose column. The sample, 25.1 μmoles of 14C equivalent in 19 ml 2 m NaOH carbonate, was applied to a DEAE-cellulose column (1 x 10 cm) which had been equilibrated with m M Tris (free base), pH 8.9. The column was eluted with a linear gradient of 0.02 M KC1 to 0.2 M KC1 in 1 ml Tris, pH 8.9, in a total volume of 160 ml. Geranylgeranyl pyrophosphate (6.74 μmoles) was eluted when the KC1 concentration reached approximately 0.14 M. Fractions containing geranylgeranyl pyrophosphate from the column were pooled, diluted 4- to 5-fold with water, and applied to a column (0.5 x 4 cm) of DEAE-cellulose which had been equilibrated with m M Tris, pH 8.9. The column was then washed with about 50 ml of 2 mM Tris, pH 8.9, and eluted with 0.25 M KC1 in 2 mM Tris, pH 8.9. During elution, very small (about 0.5 ml) fractions were collected. Those containing geranylgeranyl pyrophosphate were pooled, and the pH was adjusted to 7 to 7.5 with HCl. In this way, concentrations of geranylgeranyl pyrophosphate of 0.3 to 0.4 mM were obtained.

**Phosphorylation of Geranylgeranyl Phosphate**—Geranylgeranyl monophosphate was converted to the pyrophosphate ester by the general procedure described by Michelson (18). The fractions which contained geranylgeranyl phosphate (Fractions 54 to 80 in Fig. 1) from a countercurrent distribution were pooled and converted to the anhydrous tri-n-butylammonium salt. This salt (22.2 μmoles) was converted to geranylgeranyl pyrophosphate via the intermediate formation of the P1-diphosphoryl-P geranyleranylpyrophosphate essentially as described by Michelson except that it was not possible to isolate the intermediate by precipitation with ether because of its solubility in this solvent. Instead, the intermediate was extracted into ether and, after removal of the solvent under vacuum, was treated with di(tri-n-butylammonium) hydrogen phosphate in pyridine. The product, on purification by countercurrent distribution as described above, yielded 4.2 μmoles of geranylgeranyl pyrophosphate (about 19% based on the monophosphate). Treatment of this product with acid or alkaline phosphatase yielded results similar to those with geranylgeranyl pyrophosphate obtained from pyrophosphorylation of geranylgeranil by the method of Cramer and Böhm.

**Enzymic Conversion of Geranylgeranyl Pyrophosphate to Kaurene**

When geranylgeranyl-2-14C pyrophosphate was incubated with a homogenate of the liquid endosperm of *E. macrocarpa*, a 14C-labeled, nonpolar lipid was formed which cochromatographed with authentic kaurene (RF 0.92) in System A. When subjected to gas-liquid chromatography on 5% SE-30 on Chromosorb W (see Reference 1), the peak of radioactivity from the enzymically synthesized lipid and the mass peak from added carrier kaurene coincided. Careful chromatography of the extractable, radioactive lipids formed from geranylgeranyl-2-14C pyrophosphate in endosperm and several authentic diterpene hydrocarbon standards was performed on 3% AgNO3-Silica Gel G thin layer plates developed with hexane-benzene (85:15). This system is capable of resolving the following diterpene hydrocarbons: kaurane (RF 0.75), kaurene (RF 0.59), isopimaradiene (RF 0.47), and iso-kaurene ((-)-kaur-15-ene)/(RF 0.23). 24-C-Geranylgeranyl pyrophosphate (1.58 μmoles; 36 x 10 cp) was incubated with 0.5 ml of the 105,000 x g supernatant enzyme fraction from endosperm for 25 min, and the 24C-lipids formed were extracted as described in “Experimental Procedure.” A portion of this extract was chromatographed together with nonradioactive reference standards of kaurene, kaurene, isopimaradiene, and isokaurene. Two-millimeter portions across the kaurene region were scraped from the plate and assayed for radioactivity. The remainder of the plate was divided into approximately 1-cm zones, which were assayed similarly. A small amount (38 cp) of the approximately 6500 cp added to the plate was present at the origin. This was presumably due to geranylgeraniol released during the incubation. The majority of the radioactivity (5750 cp) was found to coincide precisely with the authentic kaurene, while only 55 cp were found distributed in all other portions of the plate. Thus, at least 98% of the radioactive material which is mobile under these conditions chromatographs as kaurene.

This same endosperm system also produced a single diterpene

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**Table I**

<table>
<thead>
<tr>
<th>Acid treatment</th>
<th>P1 released</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>10 min, 30°C, m HCl</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>10 min, 100°C, m HCl</td>
<td>143</td>
<td>143</td>
<td></td>
</tr>
</tbody>
</table>

* Assuming that 100% of the 14C is present as geranylgeranyl-2-14C pyrophosphate.
TABLE II

Properties of kaurene synthase in E. macrocarpa endosperm homogenate

Incubated for 30 min at 30° in 0.5 ml were the following: potassium phosphate, pH 7.0, 5 μmoles; geranylgeranyl-14C pyrophosphate (9,390 cpm), 4.2 × 10⁻⁴ μmole; enzyme solution, 0.05 ml; other additions as indicated; and water to 0.5 ml. The reaction was initiated by addition of substrate and terminated by addition of 0.5 ml of acetone. The substrate in this case was prepared biosynthetically in E. macrocarpa endosperm.2 Radioassay for kaurene-14C formed was performed as described in “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Endosperm fraction and addition</th>
<th>Kaurene formed</th>
<th>Radioactivity</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>37,000 X g supernatant</td>
<td></td>
<td>cpm</td>
<td>mmole/hr/0.05 ml enzyme</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3,247</td>
<td>0.146</td>
<td></td>
</tr>
<tr>
<td>Ascorbate, 1 μmole</td>
<td>3,062</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>2-Mercaptoethanol, 1 μmole</td>
<td>4,156</td>
<td>0.187</td>
<td></td>
</tr>
<tr>
<td>105,000 X g supernatant</td>
<td></td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4,381</td>
<td>0.196</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Boiled supernatant, 0.05 ml</td>
<td>3,054</td>
<td>0.137</td>
<td></td>
</tr>
<tr>
<td>105,000 X g pellet</td>
<td>1</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

a Boiled supernatant was used.
b Dialyzed supernatant was used.

Fig. 2. Metal activation of kaurene synthase. Incubated for 4 hours at 30° were potassium phosphate, pH 6.65, 40 μmoles per ml; geranylgeranyl-2-14C pyrophosphate, 0.02 μmole per ml; metal ion as shown; and 105,000 X g supernatant fraction which had been dialyzed for 18 hours against 20 volumes of 0.01 M potassium phosphate, pH 6.65. Kaurene-14C was determined as described under “Experimental Procedure.”

Fig. 3. pH dependence of kaurene synthase. Incubated at 30° in 0.5 ml were potassium phosphate at the pH values indicated, 16 μmoles; ascorbate, 3 μmoles; geranylgeranyl-2-14C pyrophosphate (5,500 cpm), 0.01 μmole; and 0.32 ml of 37,000 X g supernatant. Kaurene-14C was determined as described under “Experimental Procedure.”

hydrocarbon from 2-C- mevalonate and ATP which cocrystallized with authentic kaurene without a significant change in specific radioactivity after several successive recrystallizations (1). This fact, coupled with the above evidence, leads to the conclusion that geranylgeranyl pyrophosphate is converted to kaurene by an enzyme (or enzymes) present in E. macrocarpa endosperm. Furthermore, kaurene appears to be the major, or only, cyclic diterpene hydrocarbon produced under these conditions.

When geranylgeraniol-2-14C was added to the incubation system in place of geranylgeranyl-2-14C pyrophosphate, no detectable kaurene formation occurred.

Properties of the crude enzyme system are shown in Table II. After centrifugation of the crude homogenate for 1 hour at 105,000 X g, kaurene synthase activity remained in the supernatant fraction (Table II) (13). This facilitated study of the system since the enzymes which oxidize kaurene to (-)-kaurene-19-ol (9) and most of the phosphatase activity which competes for geranylgeranyl pyrophosphate are found in the 105,000 X g pellet.

Kaurene formation in the 37,000 X g supernatant fraction was slightly stimulated by the presence of either 1 mM ascorbate or 1 mM 2-mercaptoethanol. The reaction proceeded equally well under either aerobic or anaerobic conditions.

Dialysis of the homogenate stopped all detectable kaurene formation; addition of supernatant fraction from boiled enzyme partially restored activity, suggesting a cofactor requirement. Addition of 1 mM EDTA inhibited the reaction, suggesting that the cofactor might be a metal ion. Fig. 2 shows that MgCl₂ (1 to 5 mM) and CoCl₂ (0.2 to 0.3 mM) restore activity to dialyzed enzyme. The maximum activity obtained with Co++ is approximately two-thirds of that obtained with Mg++. Addition of both 0.3 mM Co++ and 1.0 mM Mg++ led to kaurene formation equivalent to that in the presence of 0.3 mM Co++ alone. ZnCl₂
and, to a much lesser extent, MnCl₂ also activated the enzyme slightly. At 0.4 mM CdCl₂, the only concentration tried, there was somewhat less than 10% of the activity observed with 1 mM Mg²⁺. At the single concentrations tried, FeCl₂ (0.8 mM), CuCl₂ (0.02 mM), and CaCl₂ (12 mM) allowed no formation of kaurene when incubated with dialyzed 105,000 × g supernatant fraction and geranylgeranyl pyrophosphate.

Kaurene synthase exhibits a sharp pH optimum at pH 6.55, as shown in Fig. 3.

**Assay for Kaurene Synthase**

An assay was devised which gave a linear response of kaurene formed (up to 3 nmoles per 30 min of incubation) with respect to enzyme added (Fig. 4), as described under “Experimental Procedure.” However, as shown in Fig. 4, the line usually failed to go through the origin. Thus at least two levels of each enzyme solution to be assayed were tested, and the amount of activity was calculated from the slope of the line, not from the amount of kaurene formation which occurred in a single tube. One unit of kaurene synthetase activity is defined as the amount of enzyme which will catalyze formation of 1 n mole of kaurene in 1 hour in this system.

Attempts to purify the enzyme, and to determine whether more than one enzyme is involved in conversion of geranylgeranyl pyrophosphate to kaurene, have been largely unsuccessful. Preliminary experiments with DEAE-Sephadex A-50 and Sephadex-G-100 have achieved 6-fold and 2-fold purifications, respectively, from the 105,000 × g supernatant fraction, with good yields and no apparent separation into more than one ac-
tivity. Combination of fractions from these columns did not cause an increase in the activity over the sum of the individual fractions added. Although these experiments need to be extended, there is no evidence at present that more than one enzyme is involved in the cyclization of geranylgeranyl pyrophosphate to kaurene.

**DISCUSSION**

The data presented above describe an enzyme system in the higher plant *Echinocystis macrocarpa* Greene, which catalyzes cyclization of geranylgeranyl pyrophosphate to kaurene (Reaction 2), presumably with the release of inorganic pyrophosphate. This, taken together with (a) the known formation of kaurene from mevalonate in this tissue (1), (b) the known conversion of kaurene to gibberellins by *F. moniliforme* (8), and (c) the known formation of geranylgeranyl pyrophosphate from mevalonate in *Echinocystis* endosperm and elsewhere (10), is considered evidence for the pathway of gibberellin biosynthesis represented by Reactions 1 to 3. The widespread distribution of gibberellins in higher plants would suggest that the reactions in this pathway, including kaurene synthesis, are present in all higher plants.

Cyclization of geranylgeranyl pyrophosphate to kaurene may proceed via a proton-initiated cyclization (2), followed by a Wagner-Meerwein rearrangement (3), as shown schematically in Fig. 5. This sequence predicts one stabilized intermediate, labeled I in Fig. 5, suggesting a two-step cyclization which may be catalyzed by two separate proteins. The very sharp pH-activity curve (Fig. 3) suggests the distinct possibility that more than one protein is involved, although ionization of geranylgeranyl pyrophosphate, which probably has a pH somewhere in the region of the enzymic pH optimum, may contribute to this sharpness. Alternatively, the entire cyclization reaction can be visualized as occurring on a single protein surface. In line with this possibility, it should be noted that no resolution into two fractions which gave greater activity on recombination than the sum of activities of the separate fractions was achieved during partial purification of the protein. Thus, whether one or more than one protein is involved in the catalysis of this reaction remains an unresolved question.

Mechanisms similar to that depicted by Fig. 5 probably occur during cyclization of acyclic precursors to form most of the di- and triterpenes. Formation of the A and B rings of lanosterol probably proceeds by a closely analogous mechanism (19, 20), with cyclization initiated by attack of a species equivalent to an OH\(^+\) ion, generated from molecular oxygen or, as recently suggested, by preliminary formation of the 2,3-epoxide of squalene followed by cyclization (21, 22). Subsequent steps, forming the C and D rings of lanosterol, differ from the scheme shown in Fig. 5. A more significant difference is that cyclization of squalene to lanosterol probably proceeds without stabilization of any of the carbonium ions formed along the way, while cyclization of geranylgeranyl pyrophosphate to kaurene may be better rationalized via a two-step cyclization and the discrete intermediate, I.

It is highly probable that geranylgeranyl pyrophosphate is the precursor of most C\(_{29}\) and C\(_{30}\) isoprenoid compounds. It can be visualized as the immediate precursor of other diterpenes by reactions which differ from that depicted in Fig. 5 only in the stereochemistry of attack at those carbon atoms which will become positions 5, 9, and 10 of the product diterpene, and in the stages after C ring closure. Enzymically prepared geranylgeranyl pyrophosphate is a substrate for phytoene (23) and lycopersene (24) synthesis. The phytyl side chain of chlorophyll also may arise from geranylgeranyl pyrophosphate. This suggests that kaurene synthase catalyzes the first step (Reaction 2) unique to kaurene-derived diterpene and gibberellin biosynthesis. It might be expected, in line with this reasoning, that the regulation of the activity of this enzyme will be of importance in the control of gibberellin formation. On the other hand, if gibberellin synthesis occurs in specialized cells or cell compartments in which the synthesis of other types of isoprenoid compounds does not occur, kaurene synthase would not be expected to be a regulatory enzyme.

We have found that a group of plant growth retardants, which previously had been implicated in blocking gibberellin biosynthesis, inhibit the reaction catalyzed by kaurene synthase (13). Attempts to examine the kinetics of these inhibitions, or even to determine kinetic constants for the enzyme itself, have been complicated by the probable presence of an inhibitor in the substrate or the reaction medium (see Fig. 4) and by the presence in the enzyme preparation of phosphatase activity which hydrolyzes geranylgeranyl pyrophosphate to geranylglycerol. Careful kinetic examination of this enzyme must therefore await adequate evidence that this is (or is not) a single enzyme, and further purification of both the enzyme and the substrate.

Even very simple phenomena, such as the pH dependence curve and the metal ion activation curves presented above, are complicated by the fact that the plot of enzyme with respect to product formed, although linear, does not go through the origin. Since the pH and metal ion dependence experiments were performed with single levels of enzyme, with product formed rather than reaction rate plotted as the dependent variable, the actual shapes of these curves are slightly in error, especially in the regions of very low activity. However, the positions of the optima under these conditions should not change.

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Biosynthesis of Gibberellins: II. ENZYMIC CYCLIZATION OF GERANYLGERANYL PYROPHOSPHATE TO KAURENE
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