Biosynthesis of Cyclic Diterpenes in Extracts from Seedlings of *Ricinus communis* L. II. Conversion of Geranylgeranyl Pyrophosphate into Diterpene Hydrocarbons and Partial Purification of the Cyclization Enzymes*

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ABSTRACT: Geranylgeranyl pyrophosphate serves as a precursor for the formation of the known cyclic diterpenes (+)-beyerene, (+)-sandaracopimaradiene, (−)-kaurene, and trachylobane and the cyclic diterpene casbene, for which a structure has been tentatively suggested (Robinson and West, 1970), in cell-free extracts from seedlings of the castor bean (*Ricinus communis* L.) Some purification of the enzymes responsible for these cyclizations was achieved by a combination of high-speed centrifugation, ammonium sulfate fractionation, and chromatography on carboxymethyl-Sephadex and diethylaminoethylcellulose columns. The latter column led to the separation of the activities responsible for the formation from geranylgeranyl pyrophosphate of beyerene and sandaracopimaradiene from one another and the partial separation of these activities from those for kaurene and trachylobane formation. The results of the purification studies suggested that separate enzymic components participated in the cyclization reactions leading from geranylgeranyl pyrophosphate to each of the diterpenes except for kaurene and trachylobane where no evidence for the separation of activities was obtained. Gel filtration experiments suggested molecular weights of the enzymes or enzyme complex of the order of 200,000. All of the cyclization activities were stimulated by the addition of either Mg^{2+} or Mn^{2+}, showed optimal activity in the pH range 7.0–7.2 and were inhibited by sulfhydryl-binding agents. The plant growth retardants Amo 1618 [2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate] and Phosfon (tributyl-2,4-dichlorobenzylphosphonium chloride) at 0.3 mM and CCC (β-chloroethyltrimethylammonium chloride) at 0.6 mM inhibit the formation of beyerene, sandaracopimaradiene, kaurene, and trachylobane from geranylgeranyl pyrophosphate; of these retardants only Phosfon significantly inhibited the formation of casbene. All of the cyclization activities were maximal in extracts prepared from 2.5- to 3-day-old seedlings germinated at 30° and appeared shortly before the enzymic activities which are thought to participate in the mobilization of the food reserves of the seed. Germination of the seeds at 25° resulted in extracts which were altered in the proportions of diterpene hydrocarbons formed from mevalonate with the almost complete absence of casbene synthesis as the most notable feature.

Enzyme preparations from germinated seeds of *Ricinus communis* convert mevalonic acid into at least five diterpene hydrocarbons. The preceding paper (Robinson and West, 1970) describes the identification of four of these as (−)-kaurene, (+)-beyerene, (+)-sandaracopimaradiene, and trachylobane and proposes a tentative structure for the fifth, a new diterpene hydrocarbon named casbene. The synthesis of kaurene from mevalonate has been demonstrated in cell-free preparations from immature seeds of *Echinocystis macrocarpa* (Graebe *et al.*, 1965), *Pisum sativum* (Anderson and Moore, 1967; Graebe, 1968), and *Cucurbita pepo* (Graebe, 1969) and from the fungus *Gibberella fujikuroi* (Shechter and West, 1969). The systems from *E. macrocarpa* and *G. fujikuroi* have also been shown to convert geranylgeranyl pyrophosphate into kaurene (Upper and West, 1967; Shechter and West, 1969). The present paper shows that geranylgeranyl pyrophosphate serves as a substrate for the production of all five diterpene hydrocarbons of the castor bean system and describes properties of the crude and partially purified enzymic activities for cyclization. A partial resolution of the enzymic activities for the formation of the various hydrocarbons was achieved.

Materials and Methods

Preparation of the Enzyme System. Crude enzyme systems were prepared from 2.5-day old seedlings as described previously (Robinson and West, 1970). Glycerol to a concentration of 2% was added to these and to partially purified preparations prior to storage at −20°. Under these conditions activity was preserved for several weeks.

Preparation of [^{14}C]Geranylgeranyl pyrophosphate. Geranylgeranyl pyrophosphate was biosynthesized from 2-[^{14}C]-mevalonic acid (New England Nuclear, 3.28 μCi/μmole) by enzyme preparations from *E. macrocarpa* seeds (Oster and West, 1968). The lyophilized product was dissolved in water before use to give a solution of 5 μM [^{14}C]geranylgeranyl

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pyrophosphate (13.1 μCi/μmole), 0.25 mM EDTA, and 6 mM Tris-chloride, pH 8.3.

Assay of Diterpene Hydrocarbon Formation from [^{14}C]-Geranylgeranyl Pyrophosphate in Enzyme Preparations. Either the crude, soluble extract or a purified preparation in dilute phosphate buffer, as described below, was used as the source of enzymes. Typical incubation mixtures were of the following composition: enzyme preparation, 0.01–0.10 ml; $MgCl_2$, 0.5 or 1.0 μmole; 2-mercaptoethanol, 5 μmoles; potassium phosphate (pH 6.7), 5.0 μmoles; [^{14}C]geranylgeranyl pyrophosphate, 0.45–0.50 nmole (13,100–14,500 dpm); and water to 0.50 ml. All components, except for the substrate, were mixed at 0° and then equilibrated for 3 min at 30°. Reactions were initiated by addition of the substrate and incubations were continued for periods of up to 30 min. Acetone (0.5 ml) was added to stop reaction and each mixture was extracted first with 2 ml of benzene and then with 1 ml of benzene plus 0.5 ml of acetone. The combined organic phases were finally washed with 1 ml of water. The benzene extract (0.1) was added to 10 ml of Bray's scintillation fluid [4 g of 2,5-diphenyloxazole, 0.2 g of *p*-bis-2′-(5′-phenyloxazolylbenzene, 60 g of naphthalene, 100 ml of methanol, and 20 ml of ethylene glycol made to 1 l. with *p*-dioxane (Bray, 1960)] and radioactivity was determined with a Packard 2008 liquid scintillation spectrometer at a carbon-14 counting efficiency of 80%.

Separation of the five diterpene hydrocarbon products from one another and from an alcohol fraction, presumed to be geranylgeraniol formed by phosphatase action on the substrate, was achieved by chromatography on silica gel F-254, Merck, precoated analytical glass plates, 0.25 mm thick. Plates cut into 2 × 20 cm strips were immersed for 3 min in 3% silver nitrate in 95% ethanol to a depth such that 16.5 cm of each plate was impregnated with silver nitrate and 3.5 cm at one end was untreated. After the plates were air dried, the concentrated benzene extract of the reaction products was applied together with a mixture of the reference compounds isokaurene, isopimaradiene, kaurene, and kaurane (or tetradecane) as a single spot 1 cm from the end of the plate free of silver nitrate. The plates were developed in *n*-hexane–benzene (17:3). Reference compounds were visualized under ultraviolet light as yellowish spots on an orange-brown background after spraying the plates with a mixture of 0.075% 2′,7′-dichlorofluorescein plus 0.005% Rhodamine B in methanol. A diagram of a developed chromatogram is shown in Figure 1. The silica gel was divided into six areas for counting as follows: (i) the origin, containing the alcohol fraction; (ii) the boundary between the silver nitrate impregnated region and the plain silica gel, containing casbene which does not migrate further once it reaches the silver nitrate; (iii) the isokaurene region, containing radioactive beyerene; (iv) the isopimaradiene region, containing radioactive sandaracopimaradiene; (v) the kaurene region, containing radioactive kaurene; (vi) the kaurane region, containing radioactive trachylobane. The radioactivity content was determined by transferring the gel to 10 ml of scintillation fluid (4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis-2′-(5′-phenyloxazolyl) benzene/l. of toluene) and counting on the Packard 2008 instrument at a carbon-14 counting efficiency of 84%.

The fraction of the total radioactivity recovered from the plate for each zone was multiplied by the total radioactivity in disintegrations per minute calculated to be in the benzene extract from the radioassay of a portion of this extract prior to chromatography. This correction assumes that proportional losses occur in each fraction during manipulation.

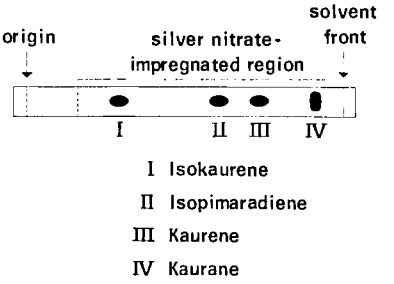

FIGURE 1: The appearance of a typical thin-layer chromatogram after development in *n*-hexane–benzene (17:3) showing the positions of the marker reference compounds.

For the most part enzymic activities are expressed in terms of enzyme units. One enzyme unit of hydrocarbon-forming activity is defined as the amount of enzyme catalyzing the conversion of 1 nmole of geranylgeranyl pyrophosphate into diterpene product in 1 hr. Where possible, measurements were made from the linear part of the curve of a reaction time course.

Ammonium Sulfate Fractionation of the Crude Enzyme Preparation. A standard 78,000*g* supernatant preparation (250 ml) was subjected to a stepwise fractionation by the slow addition at 0° of appropriate amounts of ammonium sulfate and sufficient K_3PO_4 to maintain the pH at approximately 7.0. The fractions precipitating, 0–20, 20–30, 30–40, 40–50, 50–60, and 60–100%, from saturation with ammonium sulfate (hereafter designated F_0^{20}, F_{20}^{30}, etc.) were collected by centrifugation. Each precipitate was redissolved in a minimal volume of 0.01 M potassium phosphate (pH 6.9) containing 0.01 M 2-mercaptoethanol and these solutions, together with a portion of the 100% saturation supernatant material, were dialyzed for 17 hr against four changes of the same buffer solution. The dialyzed solutions were clarified as necessary by centrifugation and suitable volumes were assayed for the conversion of geranylgeranyl pyrophosphate into diterpene hydrocarbons with incubation periods of 2, 4, 8, and 12 min.

Ion-Exchange Column Chromatography of Protein Fractions. A dialyzed F_{20}^{50} fraction prepared from 585 ml of a standard 78,000*g* supernatant fraction, dissolved in 0.01 M potassium phosphate (pH 6.9) and 0.01 M 2-mercaptoethanol, was used as the source of enzymes for chromatography. A small portion of this solution was made to 2% with glycerol and frozen for subsequent assay. The remainder of the solution was applied to a 2 × 14 cm column of carboxymethyl-Sephadex C-50 preequilibrated with 0.01 M potassium phosphate (pH 6.6), containing 0.01 M 2-mercaptoethanol. The column was eluted with the same buffer and 74 ml of eluate (pH 6.9) was collected. In exploratory experiments under the conditions employed here, it was found that the hydrocarbon-forming activities were not adsorbed by carboxymethyl-Sephadex, but a large proportion of the total protein was retained.

A small portion of the carboxymethyl-Sephadex eluate was stored in 2% glycerol for subsequent assay; the remainder was applied over a 7-hr period to a 1.3 × 30 cm column of

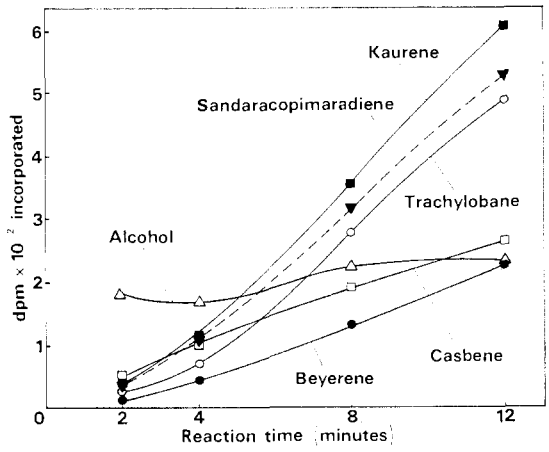

FIGURE 2: Time course of the formation of the diterpene hydrocarbons from geranylgeranyl pyrophosphate. Aliquots of a standard 78,000*g* supernatant preparation (0.02 ml) were incubated for the periods shown with 1 μmole of $MgCl_2$, 5 μmoles of K-PO_4 (pH 6.7), 5 μmoles of 2-mercaptoethanol, and 0.43 nmole of [^{14}C]geranylgeranyl pyrophosphate (12,500 dpm) in final volumes of 0.50 ml.

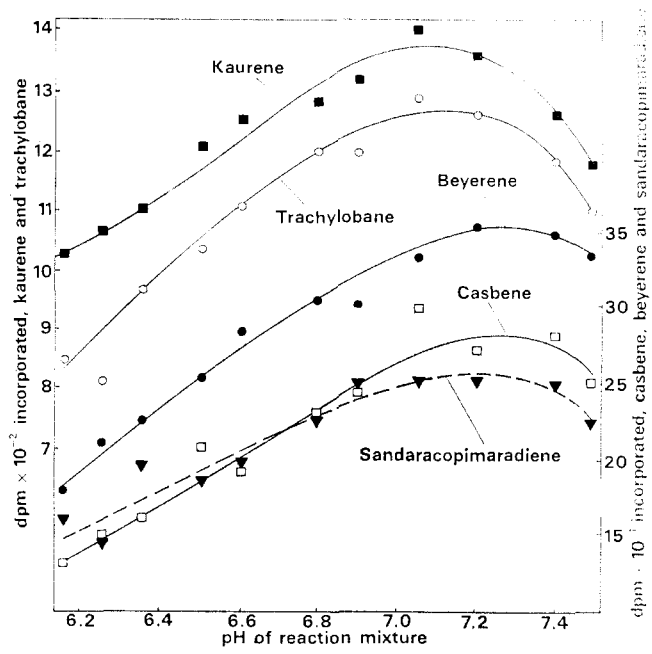

FIGURE 3: The dependence upon pH of the conversion of geranylgeranyl pyrophosphate into diterpene hydrocarbons. Aliquots of a standard 78,000*g* supernatant preparation (0.03 ml) were incubated with 1 μmole of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 5 μmoles of K-PO_4 at the indicated pH value, and 0.40 nmole of [^{14}C]geranylgeranylpyrophosphate (11,600 dpm) in final volumes of 0.50 ml, for 12 min.

Whatman microgranular DE-52 cellulose that had been preequilibrated with 0.01 M potassium phosphate (pH 6.9), containing 0.01 M 2-mercaptoethanol. The column was washed with the same buffer over a 4-hr period and was then developed with 600 ml of a linear gradient of 0.05–0.35 M KCl in 0.01 M potassium phosphate (pH 6.9), containing 0.01 M 2-mercaptoethanol. The flow rate was maintained at 6–8 ml/hr and 9-ml fractions were collected. The fractions were flushed with nitrogen and the tubes were stoppered and stored in ice prior to the assay for enzymic activity within 10 hr of collection. The assay was performed with single enzyme concentrations for a fixed incubation time.

Measurement of Protein Concentrations. All protein measurements were made according to the modification of the Folin–Ciocalteu method described by Layne (1957) and were based on standard curves determined concurrently with ovalbumin.

Results

Some of the characteristics of the metabolism of mevalonate in the castor bean extracts are summarized below. These observations were used as guide lines for the experiments with geranylgeranyl pyrophosphate as substrate. A peak of activity for the conversion of mevalonate into diterpene hydrocarbons was observed at 2.5–3-days germination at 30° falling off sharply at either side. The relative proportions of the hydrocarbons remained constant at the different stages of germination. Some variation in the proportions was observed between extracts prepared from seedlings grown at the alternative temperatures of 25 and 30°; the most obvious difference was the virtual absence of casbene formation in extracts from seedlings grown at the lower temperature.

Tris–bicarbonate buffer was normally used for the initial extraction of enzymes; phosphate buffer (pH 7.0), at 0.05 M, could be substituted but this tended to diminish the formation of casbene. The inclusion of 0.01 M phosphate in standard incubation mixtures resulted in a decrease in the incorporation of mevalonate into polar metabolites and an increase of 10–40% in the incorporation into each of the hydrocarbons. This effect was presumably the consequence of inhibition of endogenous phosphatase activity. Incorporation of radioactivity into hydrocarbons was increased threefold by the addition of Polyclar AT to the extration medium, and up to threefold by the addition of 1 mM $MgCl_2$ or 1 mM $MnCl_2$ to the crude enzyme extract. Sulfhydryl agents, such as dithiothreitol or 2-mercaptoethanol at concentrations of 0.01 M, were beneficial to hydrocarbon formation either when included in the extraction medium or subsequently added to the incubation mixtures. Maximal conversion into hydrocarbons occurred at a pH of 6.7 and at ATP concentrations of between 2 and 5 mM.

Substrate saturation with respect to hydrocarbon formation occurred at a level of 35 μM mevalonate. Maximal hydrocarbon formation was reached at 50 min, at which time the hydrocarbon-forming activity was equivalent to the conversion of 11.2 nmoles of mevalonate/hr per ml of enzyme preparation or 15 nmoles of mevalonate/hr per g fresh weight of seedlings. Similar rates were observed for the incorporation into polar materials.

The relative proportions of the hydrocarbons varied little in the different experiments (except in relation to germination temperature, as already noted); typically kaurene and sandaracopimaradiene each formed about 40% of the total C fraction and beyerene and trachylobane each about 10%. The amounts of casbene formed were more variable, but often were of the order of one-third of the total C fraction.

General Observations on Geranylgeranyl Pyrophosphate Metabolism. The same soluble enzyme preparations that catalyzed the metabolism of [^{14}C]mevalonate also converted

TABLE I: The Effects of the Growth Retardants Amo 1618, Phosfon, and β-Chloroethyltrimethylammonium Chloride on the Formation of the Diterpene Hydrocarbons from Geranylgeranyl Pyrophosphate.[a]

Retardant	Concn (mM)	Radioactivity (dpm)					
		Alcohol Fraction	Casbene	Beyerene	Sandaracopimaradiene	Kaurene	Trachylobane
None		2250	1690	1100	185	2200	3930
Amo 1618	0.3	1650	1290	1	0	0	0
Phosfon	0.3	990	9	4	2	4	0
β-Chloroethyltrimethylammonium chloride	0.6	2370	1500	195	33	375	655

[a] Portions (0.1-ml volumes) of a standard 105,000*g* supernatant preparation were incubated with 1 μmole of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 5 μmoles of K-PO_4 (pH 7.0), 0.43 nmole of [^{14}C]geranylgeranyl pyrophosphate (12,500 dpm), plus retardant as indicated, in final volumes of 0.50 ml. Incubations were for 2, 4, 8, and 12 min in order to measure time courses; only the values for 12 min are shown.

[^{14}C]geranylgeranyl pyrophosphate into casbene, beyerene, sandaracopimaradiene, kaurene, and trachylobane. As a general rule, kaurene and trachylobane (and less frequently sandaracopimaradiene) predominated among the products from geranylgeranyl pyrophosphate, whereas, sandaracopimaradiene and kaurene were invariably preferentially formed from mevalonate as substrate. No cofactor was required by the crude enzyme system, although $MgCl_2$ was slightly beneficial. With the more purified preparations (see later) addition of $MgCl_2$ (2×10^{-3} M) or $MnCl_2$ (2×10^{-4} M) was essential for the cyclizations to occur. [^{14}C]Geranylgeraniol did not give rise to any detectable hydrocarbon component when supplied with or without ATP to an enzyme preparation capable of converting geranylgeranyl pyrophosphate into hydrocarbons.

A time course for the formation of the diterpene hydrocarbons from geranylgeranyl pyrophosphate is shown in Figure 2. A distinct lag is observed in the production of beyerene, sandaracopimaradiene, kaurene, and trachylobane, whereas no apparent lag occurs in the formation of casbene. This particular preparation contains the following hydrocarbon-forming units per ml (as defined in Materials and Methods): casbene, 2.65; beyerene, 2.50; sandaracopimaradiene, 5.75; kaurene, 6.75; trachylobane, 5.70. The behavior of the alcohol fraction as depicted in Figure 2 was observed with crude enzyme extracts, but not with more purified preparations where alcohol formation follows a hyperbola. Under standard conditions, incorporation of geranylgeranyl pyrophosphate into all components ceased between 20 and 40 min, by which time 80–90% of the substrate radioactivity had been converted into extractable products.

A linear dependence of the production of the five diterpene hydrocarbons on enzyme concentration was observed with between 0.01 and 0.10 ml of a standard 78,000*g* supernatant preparation as the enzyme source under the usual incubation conditions (2 mM $MgCl_2$, 10 mM K-PO_4, pH 6.7, 10 mM 2-mercaptoethanol, and 0.86 μM [^{14}C]geranylgeranyl pyrophosphate for 10 min at 30°).

The optimal pH for the formation of kaurene and trachylobane was found to be close to 7 while the value for the formation of casbene, beyerene, and sandaracopimaradiene was slightly higher (Figure 3).

In view of other work in which a number of plant growth retardants had been shown to prevent cyclization of geranylgeranyl pyrophosphate to kaurene in *E. macrocarpa* (Dennis *et al.*, 1965) it was of interest to examine the effect of some of these compounds in the castor bean system. Table I illustrates the results of one experiment in which the retardants tested were Amo 1618 [2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate], obtained from Calbiochem, Phosfon (tributyl-2,4-dichlorobenzylphosphonium chloride), obtained from the Virginia–Carolina Chemical Corp., and β-chloroethyltrimethylammonium chloride, obtained from the American Cyanamid Co. All three retardants inhibited the formation of beyerene, sandaracopimaradiene, kaurene, and trachylobane, although β-chloroethyltrimethylammonium chloride was less effective than the other two. Phosfon alone inhibited the formation of casbene under the conditions used. Essentially the same results as these were observed when mevalonic acid served as the substrate.

Purification of the Enzymic Activities. The hydrocarbon-forming activities from a crude enzyme preparation, as assayed with geranylgeranyl pyrophosphate, eluted from a Sephadex G-200 column washed with 0.02 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol in 25% of the total free volume along with 20% of the material which reacted with the Folin–Ciocalteu reagent. The profiles of the activities were similar, and, except for the casbene-forming activity which eluted slightly later, the peaks occurred in one of two consecutive column fractions (after 10 ml of the total free volume of 34.5 ml had been collected). No appreciable separation of phosphatase activity (assessed by the incorporation of radioactivity into the alcohol fraction) from the hydrocarbon-forming activities was obtained in this experiment.

The distribution of units of enzymic activity obtained on fractionation of a crude enzyme extract with ammonium sulfate is shown in Table II. The time course for the formation of the diterpene hydrocarbons by the F_{30}^{40} fraction, that fraction with the highest activity, is shown in Figure 4. There

TABLE II: Distribution of the Diterpene Hydrocarbon-Forming Activities on Ammonium Sulfate Fractionation.[a]

Fraction	Protein (mg)	Volume Assayed (ml)[b]	Enzyme Units Per Fraction: Casbene	Beyerene	Sandaracopimaradiene	Kaurene	Trachylobane	Total Units	Units/mg of Protein
F_0^{20}	25	0.03 (1.8)	1.6	0.5	3.0	2.2	2.5	10	0.40
F_{20}^{30}	86	0.01 (0.46)	22.5	4.3	54.5	24.2	15.2	121	1.40
F_{30}^{40}	328	0.002 (0.07)	141	235	1145	788	691	3000	9.15
F_{40}^{50}	442	0.002 (0.07)	44	97	223	368	507	1238	2.80
F_{50}^{60}	445	0.01 (0.34)	0	0	0	0	0	0	
F_{60}^{100}	1636	0.05 (0.50)	0	0	0	0	0	0	
Supernatant	72	0.2 (0.13)	0	0	0	0	0	0	
Totals	3034		209	336	1426	1182	1216	4369	
Starting material	3420	0.05	469	460	1510	1415	1390	5240	1.55

[a] Appropriate aliquots of each fraction were incubated for 2, 4, 8, and 12 min with 1 μmole of $MgCl_2$, 5 μmoles of K-PO_4 (pH 6.7), 5 μmoles of 2-mercaptoethanol, and 0.45 nmole of [^{14}C]geranylgeranyl pyrophosphate (13,100 dpm) in volumes of 0.50 ml. Enzyme units were calculated as described in Materials and Methods. [b] The first figure is the volume of the ammonium sulfate fraction assayed; the second figure is the volume of the original 78,000*g* supernatant preparation to which this would be equivalent.

TABLE III: Purification of the Diterpene Hydrocarbon-Forming Activities.[a]

Stage in Purification	Protein (mg)	Enzyme Units per Total Sample: Casbene	Beyerene	Sandaracopimaradiene	Kaurene	Trachylobane	Total Units	Units/mg of Protein	Purification: -Fold	Yield (%)
78,000*g* supernatant preparation[b]	6700	1450	1040	3180	3800	3100	12570	1.9		(100)
F_{20}^{50} fraction[b]	4050	240	380	2010	2180	2145	6960	1.7		55
CM-Sephadex[b]	2010	185	288	1540	1575	1575	5165	2.6	1.4	41
DEAE-cellulose[c] (fractions 33–44)	260	44	263	403	1207	2920	4840	18.6	9.8	38

[a] Aliquots from each stage of purification (described in the text) were incubated with 1 μmole of $MgCl_2$, 5 μmoles of K-PO_4 (pH 6.7), 5 μmoles of 2-mercaptoethanol, and 0.47 nmole of [^{14}C]geranylgeranyl pyrophosphate (13,700 dpm) in final volumes of 0.50 ml. [b] Assayed by variable-time incubations. [c] Assayed by fixed-time incubations.

is some suggestion of differential fractionation of the individual hydrocarbon-forming activities; in particular, the activities for casbene and sandaracopimaradiene are proportionally greater in the F_{20}^{30} fraction and trachylobane activity is proportionally greatest in the F_{40}^{50} fraction.

The results of the assay of DEAE-cellulose chromatography of an F_{20}^{50} fraction are shown in Figure 5. Only hydrocarbon-forming activities are shown; most of the protein and phosphatase activity were eluted prior to these activities. The formation of casbene is not shown in Figure 5 because the activity was very low. Such activity as was observed occurred in a broad peak centered in fractions 46 and 47. A notable feature of the experiment was the clear separation of the activities of beyerene and sandaracopimaradiene formation from one another and the partial separation of these activities from those of kaurene and trachylobane formation. In a separate experiment, the activities for kaurene and trachylobane formation recovered from DEAE-cellulose chromatography were rechromatographed over DEAE-Sephadex A-50. Similarly, no fractionation of the two activities was observed,

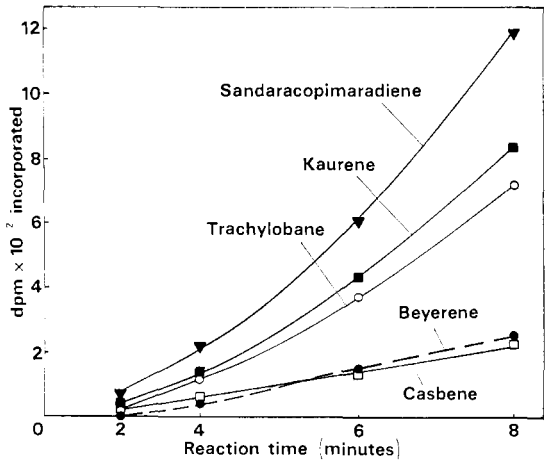

FIGURE 4: Time course of the conversion of geranylgeranyl pyrophosphate into the diterpene hydrocarbons by a fraction precipitated between 30 and 40% saturation with ammonium sulfate. The ammonium sulfate fraction was prepared as described under Materials and Methods from 250 ml of a standard 78,000*g* supernatant preparation. Aliquots of the dialyzed material, 0.002 ml (equivalent to 0.07 ml of the original crude enzyme preparation) was incubated for the periods shown with 1 μmole of $MgCl_2$, 5 μmoles of K-PO_4 (pH 6.7), 5 μmoles of 2-mercaptoethanol, and 0.45 nmole of [^{14}C]geranylgeranyl pyrophosphate (13,100 dpm) in final volumes of 0.50 ml.

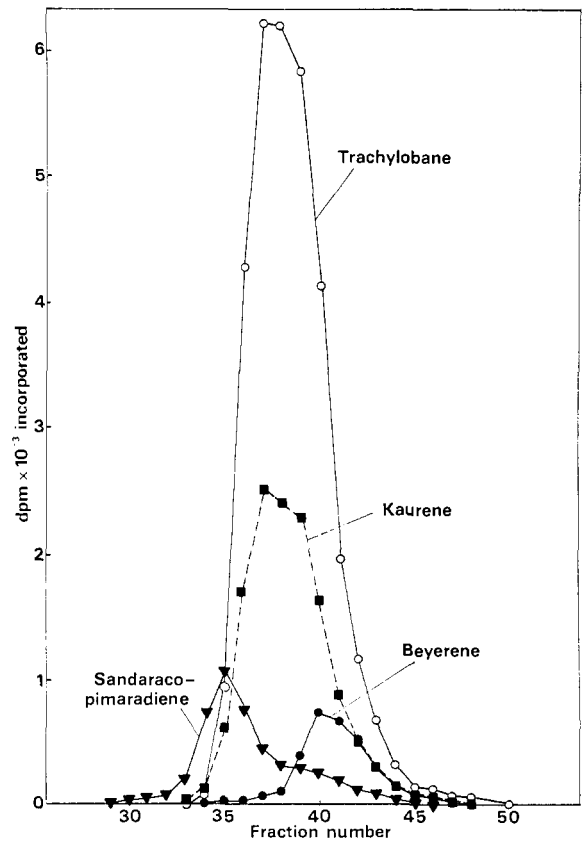

FIGURE 5: DEAE-cellulose column chromatography: elution profiles of the enzymic activities responsible for the formation of beyerene, sandaracopimaradiene, kaurene, and trachylobane as assayed with geranylgeranyl pyrophosphate as substrate. An F_{20}^{50} fraction was subjected to chromatography first on carboxymethyl-Sephadex C-50 and then on DEAE-cellulose. Details of the procedure are given under Materials and Methods. Aliquots of each column fraction (0.02 ml) were incubated for 10 min with 1 μmole of $MgCl_2$, 5 μmoles of K-PO_4 (pH 6.7), 5 μmoles of 2-mercaptoethanol, and 0.46 nmole of [^{14}C]geranylgeranyl pyrophosphate (13,700 dpm) in final volumes of 0.50 ml.

although additional phosphatase activity and 75% of the protein were separated from the hydrocarbon-forming activities.

Table III shows the activities expressed in enzyme units for the formation of the individual hydrocarbons during several stages of purification including DEAE-cellulose chromatography. Aliquots of the original 78,000*g* supernatant material, the F_{20}^{50} fraction, and the carboxmethyl-Sephadex eluate were assayed at several incubation times so that true maximal rates of hydrocarbon formation could be calculated. The activities for the DEAE-cellulose column eluate represent the sums of the separate assays of the individual column fractions 33 to 44. Single time incubations were used here and for the purposes of calculation it was assumed that reaction was linear from zero time. Because of the lag periods, however, (see Figures 2 and 4) this is not strictly true. Consequently, the calculated values for the enzyme units must be somewhat low and the overall purification would be correspondingly higher than the tenfold indicated.

Additional Properties of the More Purified Enzyme System. Table IV shows the results of an experiment in which the effect on hydrocarbon formation of iodoacetamide, *p*-mercuribenzoate and *N*-ethylmaleimide was examined. The enzyme preparation was an F_{20}^{50} fraction that had been passed over carboxymethyl-Sephadex C-50 and then dialyzed against 0.01 M potassium phosphate (pH 6.9) for 13 hr. All three sulfhydryl agents effectively reduced or prevented the synthesis of all of the diterpene hydrocarbons.

The general use of glycerol in all enzyme preparations that were to be frozen for storage resulted from early attempts at storage of material that had been subjected to chromatography over DEAE-cellulose or DEAE-Sephadex. Freezing at −20° caused the complete loss of all five hydrocarbon-forming activities. Crude enzyme extracts were virtually unaffected by this treatment. Neither Tween 20 nor bovine serum albumin prevented inactivation; however, the presence of glycerol at between 1 and 20% allowed retention of all activities for at least 4 weeks at −20°. Over a similar length of time glycerol was partially effective at preserving the enzymic activities upon storage at 4°.

An experiment to test for the possibility of enzymic interconversion among the diterpene hydrocarbons was undertaken. Approximately 1 nmole of each of the five hydrocarbons (28,000–30,000 dpm) was incubated with 0.2 ml of a standard 78,000*g* supernatant preparation in the presence of 2×10^{-3} M $MgCl_2$, 0.01 M K-PO_4 (pH 6.7), and 0.01 M 2-mercaptoethanol for 10 and 30 min. Upon silver nitrate–silica gel thin-layer chromatography of the recovered radioactive material it was found that in each case more than 95% of the radioactivity migrated to the position expected for the unaltered substrate and the distribution of the remainder of the radioactivity was no different from that obtained with enzyme preparations incubated in the presence of 50% acetone as a control. The same enzyme preparation was capable of converting 22 nmoles

TABLE IV: The Effect of Sulfhydryl Inhibitors on the Conversion of Geranylgeranyl Pyrophosphate into the Diterpene Hydrocarbons.[a]

Sulfhydryl Agent or Inhibitor	Concentration (mM)	Radioactivity (dpm)					
		Alcohol Fraction	Casbene	Beyerene	Sandaracopimaradiene	Kaurene	Trachylobane
None		3820	86	58	1490	1450	1910
2-Mercaptoethanol	10	3930	76	68	1580	1500	1930
N-Ethylmaleimide	0.1	3960	58	66	1350	1190	1900
N-Ethylmaleimide	1.0	4780	21	18	228	230	510
N-Ethylmaleimide	10.0	5800	15	0	0	6	1
Iodoacetamide	10.0	4900	32	28	660	465	875
p-Chloromercuribenzoate	1.0	5830	1	0	0	14	0

[a] Incubations were for 12 min with 0.005 ml of the enzyme preparation [in 0.01 M $K\text{-}PO_4$ (pH 6.9)], 1 μmole of $MgCl_2$, 5 μmoles of $K\text{-}PO_4$ (pH 6.7), sulfhydryl agent or inhibitor, as indicated, and 0.43 nmole of [^{14}C]geranylgeranyl pyrophosphate (12,500 dpm) in final volumes of 0.50 ml.

of geranylgeranyl pyrophosphate/ml per hr into the hydrocarbons.

Discussion

The hydrocarbons formed from geranylgeranyl pyrophosphate are identical in chromatographic properties with the diterpene products of mevalonate metabolism in these same castor bean seedling extracts (Robinson and West, 1970). It seems most likely that the two sets of products are composed of the same substances even though in this case it was not possible to carry out the extensive physical measurements which were employed to identify the mevalonate-derived products. The differences seen between the proportions of the products formed from the two substrates are presumably due to differences in the incubation conditions with the two substrates. Evidence has been provided previously for the conversion of geranylgeranyl pyrophosphate to kaurene in cell-free extracts of the endosperm of *E. macrocarpa* seed (Upper and West, 1967) and the fungus *G. fujikuroi* (Shechter and West, 1969). Thus, the present work expands the number of diterpene hydrocarbons shown to be formed from geranylgeranyl pyrophosphate in support of the contention that geranylgeraniol or one of its derivatives should be a general precursor of cyclic diterpenes (Ruzicka *et al.*, 1953).

A mechanistic scheme to account for the biosynthesis of these diterpenes from geranylgeranyl pyrophosphate is presented in Figure 6. A proton-initiated cyclization at the double bond distal from the pyrophosphate group of geranylgeranyl pyrophosphate followed by the electronic shifts shown and the elimination of a proton (step 1) leads to the formation of the stabilized bicyclic intermediate copalyl pyrophosphate. The elimination of the pyrophosphate group of copalyl pyrophosphate coupled with an electronic shift from the exocyclic double bond as shown in step 2a yields the tricyclic carbonium ion pictured. In pathway a further cyclization to a tetracyclic ion (step 3), followed by the formation of a bridged carbonium ion (step 4) and bond scission to a rearranged tetracyclic carbonium ion (step 5), and finally elimination of a proton yields ultimately (−)-kaurene. In pathway b steps 2, 3, and 4 are identical with those pictured in pathway a. Elimination of a proton from the bridged carbonium ion in this case yields (−)-trachylobane. In pathway c the elimination of a proton from the tetracyclic carbonium ion formed by steps 2 and 3 identical with those in pathway a and b results in (+)-beyerene. In step 2 of pathway d an analogous cyclization coupled with pyrophosphate elimination gives the isomeric tricyclic carbonium ion from which the formation of (+)-sandaracopimaradiene results by proton elimination. The similarities in these proposed routes to the formation of the C group of diterpenes are evident.

A mechanistic scheme to account for the biosynthesis of the structure tentatively proposed for casbene is also presented in Figure 6. The elimination of the pyrophosphate coupled with attack of the electrons of the double bond distal from the pyrophosphate on the resulting carbonium ion generates a 14-membered carbocyclic ring. Formation of a cyclic carbonium ion from this intermediate followed by elimination of a proton yields the structure proposed for casbene. Note that no stabilized intermediates are proposed in this pathway.

Investigations completed after the work reported in this paper resulted in the isolation of copalol ((−)-labda-8 (16), 13-dien-15-ol) and copalyl pyrophosphate from cell-free extracts of *G. fujikuroi* (Shechter and West, 1969). The pyrophosphate ester was further shown to be an intermediate in the formation of (−)-kaurene from geranylgeranyl pyrophosphate in *E. macrocarpa* and *G. fujiuroi* enzyme preparations and also in the formation of all of the C-group diterpenes (but not casbene) in *R. communis* extracts, as predicted in the schemes shown in Figure 6. Independently Hanson and White (1969) have demonstrated the incorporation of this labdadienol and its pyrophosphate ester into (−)-kaurene, the kaurenolides, and gibberellic acid in intact cultures of *G. fujikuroi*.

The observation of a time lag associated with the synthesis of the C-group hydrocarbons from geranylgeranyl pyrophosphate (Figures 2 and 4) can be attributed to the time required for the copalyl pyrophosphate concentration to build up to a sufficient extent to support higher rates of hydrocarbon synthesis. The absence of a stabilized intermediate in the

proposed scheme for the formation of casbene from geranylgeranyl pyrophosphate is consistent with the lack of an observed time lag in this case.

A stable tricyclic hydrocarbon has been suggested as a precursor of tetracyclic diterpenes such as kaurene (Wenkert, 1955). (+)-Sandaracopimaradiene has the wrong stereochemical arrangement of methyl and vinyl substituents at C-13 to serve as a precursor of the other C-group hydrocarbons. (−)-Pimaradiene, the hydrocarbon related to the tricyclic carbonium ion shown as the product of steps 2a, 2b, and 2c in Figure 6 by the loss of a proton, could on logical grounds, serve as such a precursor. However, this substance was not detected among the products formed from mevalonate in the castor bean extracts. Pimaradiene migrates very much like kaurene in the silver nitrate silica gel thin-layer chromatography system employed in the present work. However, the spectral and other evidence obtained with the C-3 component isolated from the large-scale biosynthesis with mevalonate was consistent with its identification as (−)-kaurene without the presence of significant amounts of any other hydrocarbon contaminant (Robinson and West, 1970). Thus, there is no evidence from the present work to support a role for (−)-pimaradiene as an intermediate in the synthesis of other polycyclic diterpene hydrocarbons.

Two groups of investigators have independently tested the possible role of a (−)-pimaradiene in gibberellic acid (and hence (−)-kaurene) biosynthesis in *G. fujikuroi* cultures. Cross and Stewart (1968) concluded that specifically labeled (−)-pimara-8(14),15-diene was not incorporated into gibberellic acid to a significant extent; whereas Hanson and White (1969) did find a low (0.024%), but apparently specific, incorporation of the same substrate into gibberellic acid. In view of the low incorporation of this substrate in comparison with kaurene under comparable conditions, the authors remained uncertain about the implication of these results for the role of this free pimaradiene as an obligate intermediate in the biosynthesis. Hanson and White (1969) also deduced from an analysis of the amounts and patterns of labeling of metabolites isolated after feeding (4*R*)-[4-^{3}H, 2-^{14}C]mevalonate and [2-$^{3}H_2$, 2-^{14}C]mevalonate to the fungus that (−)-pimara-8,15-diene and (−)-pimara-7,15-diene do not serve as (−)-kaurene and gibberellic acid precursors. Thus, these lines of research have also failed to produce compelling evidence for a pimaradiene as intermediate in kaurene biosynthesis.

It is also interesting to note that reincubation of each of the ^{14}C-labeled hydrocarbons of the C group with the castor bean extract did not lead to any detectable interconversion among them or to the formation of any new hydrocarbons. Although such experiments can not provide conclusive evidence, the results suggest that none of the four hydrocarbons is intermediate in the formation of any of the others. The nonenzymic isomerization of some of these hydrocarbons has been demonstrated. For example, HCl in chloroform has been shown to catalyze the isomerization of beyerene and trachylobane to mixtures of kaurene, isokaurene, atiserene, and isoatiserene (Appleton *et al.*, 1966; McAlees *et al.*, 1966); The fact that the enzymic synthesis of the C-group hydrocarbons occurs without any detectable formation of atiserene, isoatiserene or isokaurene illustrates that the factors which regulate the formation of products in the enzymic reactions are quite different from those in these nonenzymic isomerizations.

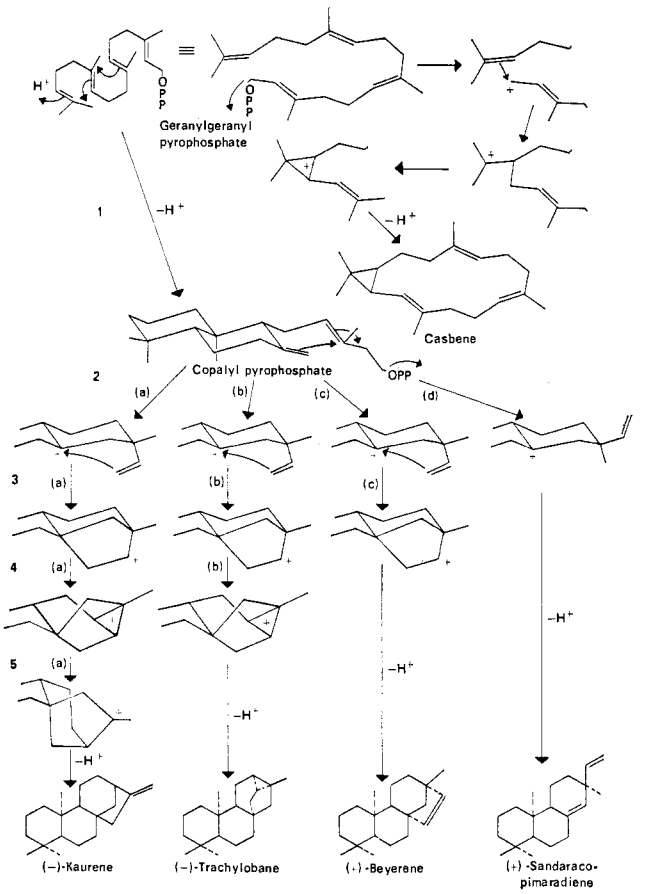

FIGURE 6: Schemes to account for the biosynthesis of the diterpene hydrocarbons from geranylgeranyl pyrophosphate.

The purification of the hydrocarbon-forming enzymic activities resulted in a partial resolution of the activities responsible for the formation of the individual hydrocarbons. The most convincing evidence was provided by DEAE-cellulose chromatography as shown in Figure 5 and Table III. It can be seen that the fractions able to catalyze the formation of (+)-sandaracopimaradiene and (+)-beyerene from geranylgeranyl pyrophosphate were largely separated from one another and were partially resolved from the fractions maximally active in kaurene and trachylobane synthesis. No clear evidence for the resolution of the enzymic activities responsible for the formation of these latter two hydrocarbons was obtained in these investigations. The recent discovery of copalyl pyrophosphate as a stabilized intermediate in these reactions makes it important to repeat the fractionations using this as the assay substrate for hydrocarbon-forming activities. It seems most probable that separate enzymes catalyze the formation of copalyl pyrophosphate and its further cyclization. If this is the case, then assays with geranylgeranyl pyrophosphate as substrate will show hydrocarbon-forming activity only in fractions where the two enzymes are present. This conceivably could account for the low recovery of activity observed for (+)-sandaracopimaradiene formation in the fractions from the DEAE-cellulose column. The peak of activity for this reaction was seen at the leading edge of the

total hydrocarbon-forming activity. If fractions 30–35 were very low in copalyl pyrophosphate-synthesizing activity, the overall rate of (+)-sandaracopimaradiene synthesis might have been greatly reduced.

Although it is not illustrated in Figure 5, the casbene synthase showed a peak of activity which emerged from the column even later than the (+)-beyerene-forming activity. However, the recovery of casbene synthase from this purification step and the total units remaining at this stage were so low that this result must be regarded with some reservation. At the same time it should be pointed out that both ammonium sulfate fractionation and Sephadex G-200 gel filtration gave some small resolution of casbene-synthesizing activity from that for the C-group hydrocarbons. Also Amo 1618 and CCC were seen to inhibit the conversion of geranylgeranyl pyrophosphate into the C-group hydrocarbons but did not have an appreciable effect on casbene synthesis. The overall impression from these observations is that casbene synthase is separate from the other activities.

Although other interpretations are possible, the simplest explanation of these findings is to assume that at least one unique enzymic component is involved in the synthesis of each of the diterpene hydrocarbons except for kaurene and trachylobane where no resolution of the synthetic activities was observed and thus no conclusions can be drawn. This consideration led to the presentation of separate pathways to each of the hydrocarbons as shown in Figure 6 in spite of the fact that common ionic intermediates are envisioned in several of them.

The position of elution of the hydrocarbon-forming activities from the Sephadex G-200 gel filtration column suggests a molecular weight of the order of 200,000 or more for these enzymes or enzyme complexes. No attempt was made to more accurately estimate the molecular sizes. The inhibitory actions of *N*-ethylmaleimide, iodoacetamide, and *p*-mercuribenzoate toward the formation of all of the hydrocarbons from geranylgeranyl pyrophosphate suggests a requirement for one or more free sulfhydryl groups in these reactions. The pH optima of 7.0–7.2 are slightly higher than the optimum of 6.65 found for kaurene synthase of *E. macrocarpa* (Upper and West, 1967). Another difference between these two systems was detected in that either Mg^{2+} of Mn^{2+} ions support diterpene hydrocarbon synthesis from geranylgeranyl pyrophosphate in *R. communis* extracts while only magnesium ions were effective with the *E. macrocarpa* kaurene synthase.

The production of a family of cyclic diterpene hydrocarbons in the extracts of young *R. communis* seedlings contrasts with the results obtained with other cell-free systems examined to date. Only kaurene has been detected as a diterpene hydrocarbon product in preparations from endosperm of immature *E. macrocarpa* seed (Upper and West, 1967) and immature *Cucurbito pepo* seed (Graebe, 1969), from immature pea seed (Anderson and Moore, 1967; Graebe, 1968), and from the fungus *G. fujikuroi* (Shechter and West, 1969). In view of these results it was of interest to examine the products formed from mevalonate and geranylgeranyl pyrophosphate in extracts of immature *R. communis* seed. The hydrocarbon product of these incubations was chromatographically identical with kaurene and no evidence of the multiplicity of products formed in extracts of young seedlings was seen. Thus, in all instances of immature seed examined to date, including *R. communis*, kaurene appears to be the only diterpene hydrocarbon product formed.

A specific physiological role can be proposed with any degree of certainty only for kaurene among the various diterpene hydrocarbons produced in the seedling extracts. Several lines of evidence have established kaurene as an intermediate in the biosynthesis of gibberellins (Cross *et al.*, 1964; Phinney *et al.*, 1964). It seems likely that the kaurene formed in young seedlings would serve this role. Although, as summarized in the discussion of the preceding paper (Robinson and West, 1970), the other diterpene hydrocarbons are related to known natural products, no physiological role has been suggested for any of these substances. However, several considerations lead us to propose that these other diterpenes, or products derived from them, are of importance in the development of the seedling as opposed to being metabolic by-products. (1) The pattern of production as a function of the age of the seedling from which extracts are prepared is similar for these hydrocarbons and for kaurene; the peak of production (2–3 days after germination) precedes by a short time the peaks noted for the production of other enzymic activities in seedling extracts such as those of lipases (St. Angelo and Altschul, 1964), malic synthase and isocitrate lyase (Canvin and Beevers, 1961), and fatty acid β-oxidation enzymes (Yamada and Stumpf, 1965). (2) The relative amounts of each of the hydrocarbons produced are similar. (3) Separate enzymes appear to participate in the formation of most or all of these substances. (4) Casbene appears to inhibit the endogenous growth of the dwarf-5 mutant of *Zea mays* (see Table III in Robinson and West, 1970). (5) The balance of the hydrocarbon products formed is dependent on the temperature of germination of the seedlings.

Amo 1618 and Phosfon inhibited kaurene synthesis in *R. communis* extracts as was shown to be the case in *E. macrocarpa* endosperm (Dennis *et al.*, 1965) and *G. fujikuroi* extracts (Shechter and West, 1969). β-Chloroethyltrimethylammonium chloride was also an effective inhibitor of this step in *R. communis* as in *G. fujikuroi* extracts, whereas this retardant was not active in blocking kaurene synthesis in *E. macrocarpa* endosperm. These results suggest that the differing sensitivities of plants to various growth retardants may be the result in some cases of differing susceptibilities of the cyclization enzymes to inhibition by these agents. The results seen with growth retardants in the present work clearly indicate that these substances can influence the production of diterpene hydrocarbons other than kaurene. Thus, one must be cautious in assuming that the physiological effects of the growth retardants can be attributed in all cases to the influence they exert on kaurene production and hence gibberellin formation.

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