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METABOLISM OF KAURENE IN CELL-FREE EXTRACTS OF IMMATURE PEA SEEDS*

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Abstract-Geranylgeraniol, (-)-kaurene, (-)-kaurenol, (-)-kaurenal and (-)-kaurenoic acid were tentatively identified as products synthesized from mevalonic acid-2-^{14}C in cell-free reaction mixtures containing 10,000 *g* supernatant of extracts of cotyledons of immature pea (*Pisum sativum* L. cv. Alaska) seeds. The identity of (−)-kaurenol was confirmed by co-crystallization of the radioactive product with authentic kaurenol to constant specific radioactivity. Repeated efforts to observe cell-free metabolism of exogenous (−)-kaurene in a variety of enzyme extracts consistently resulted in failure. Only when (-)-kaurene was synthesized *in situ* from mevalonic acid in reaction mixtures containing 10,000 *g* supernatant from cotyledon extracts could the metabolism of (−)-kaurene be observed. Thus, the procedure employed most extensively was to allow (-)-kaurene to be formed from mevalonic acid-2-^{14}C for 60 min, then to inhibit its further synthesis by the addition of AMO-1618, and finally to assay the carbon monoxide-sensitive oxidation of (−)-kaurene which occurred during a subsequent 60-min incubation. By this procedure it was demonstrated that the oxidation of (-)-kaurene which was formed *in situ* was enzymic, or at least heat labile; markedly sensitive to inhibition by carbon monoxide; and greater in extracts prepared with insoluble polyvinylpyrrolidone (PVP) than in extracts prepared without PVP. Possible reasons for the failure of exogenous (-)-kaurene to be oxidized are discussed.

INTRODUCTION

THE ROLE of the tetracyclic diterpene (-)-kaurene as an intermediate in the biosynthetic pathway for the gibberellins (GA's) was first established by Cross et *al.*[1] when they showed that kaurene-17-^{14}C was converted to GA_3 by the fungus *Gibberella fijikuroi* (Saw.) Wr. (*Fusarium moniliforme* Sheld., the asexual stage). It was subsequently reported that kaurenol,[2] kaurenal,[3] kaurenoic acid and 7β-hydroxykaurenoic acid[4] are converted to GA's by the fungus. Geranylgeranyl pyrophosphate and copalyl pyrophosphate also are intermediates in the GA pathway, since both compounds are readily converted to kaurene by extracts of *Gibberella fujikuroi*,[5] immature seeds of wild cucumber (*Echinocystis macrocarpa* Greene)[5,6] and seedling shoots of castor bean (*Ricinus communis* L.).[5,7]

Although all the aforementioned diterpenoid compounds are believed to be intermediates in GA biosynthesis in seed plants, as well as in *Gibberella jiijikuroi*, it is notable that no

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higher plant tissue or extract thereof actually has been reported to convert any of these compounds to a product which could be positively identified as a GA. However, the intermediary role of these compounds in GA biosynthesis is strongly supported by several lines of evidence: (a) the isolation of kaurene[8] and kaurenoic acid[9–13] from higher plant tissues; (b) the GA-like activity of these two diterpenes as well as kaurenol, 7β-hydroxykaurenoic acid and 6β, 7β-dihydroxykaurenoic acid in some higher plant systems;[14–18] (c) the development of a cell-free system from the endosperm-nucellus of immature seeds of *Echinocystis macrocarpa* (a tissue which is known to contain large quantities of identified GA's) which will incorporate mevalonic acid into geranylgeranyl pyrophosphate, kaurene, kaurenol, kaurenal, kaurenoic acid and 7β-hydroxykaurenoic acid in sequence and irreversibly;[3,4,19] (d) the incorporation of mevalonic acid into kaurenol in excised roots of sunflower (*Helianthus annuus* L.) seedlings;[20] and (e) the conversion of kaurenoic acid-^{14}C into GA-like compounds *in vivo* in leaves of red clover (*Trifolium pratense* L.)[21] and *in vitro* in cell-free extracts of *Brassica oleracea* leaves.[22]

Cell-free enzyme extracts capable of catalysing the metabolism of kaurene to other intermediates in GA biosynthesis have been prepared from only one seed plant source, the liquid endosperm of immature *Echinocystis macrocarpa* seeds.[2–4,23] Murphy and West[23] showed that the reactions converting kaurene to kaurenoic acid are catalysed by microsomal mixed function oxidase-type[24] enzymes. Cytochrome P-450, molecular oxygen and NADPH are required for the reactions.

Previously from the authors' laboratory it was reported that kaurene is synthesized from mevalonic acid in cell-free extracts of immature pea (*Pisum sativum* L.) seeds.[25,26] and that the enzymes catalysing kaurene biosynthesis are localized in the cotyledons.[27] Graebe[28] also reported on extensive investigations of the biosynthesis of kaurene, as well as squalene and phytoene, from mevalonic acid in a cell-free system from immature pea fruits and seeds. Thus the cellular tissues of immature pea cotyledons, like the anatomically dissimilar liquid, free-nuclear endosperm of immature *Echinocystis macrocarpa* and *Cucurbita pepo*[29] seeds, appear to be excellent materials with which to develop cell-free

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systems to be used in further biochemical investigations of GA biosynthesis in higher plants. The present paper reports the incorporation of mevalonic acid-2-^{14}C into (-)-kaurenol and other products tentatively identified as (-)-kaurenal and (−)-kaurenoic acid in a cell-free enzyme system developed from cotyledons of immature pea seeds.

RESULTS AND DISCUSSION

Cell-free Metabolism of Exogenous Kaurene

Repeated attempts were made to observe enzymic oxidation of exogenous kaurene in 10,000 g supernatants and microsomal preparations from immature pea seeds under a wide variety of conditions. Experiments were performed using different concentrations of enzyme, substrate, NADPH, cations; different salt strengths; and using other additives such as NADP, NAD, NADH, ATP, dithionite, dithiothreitol, metabisulfite, ascorbate, and glycerol. All these attempts resulted in failure. In all these cases, some products different from kaurene were observed, but upon chromatographic examination, none of them appeared to be similar to the expected products, kaurenol, kaurenal, kaurenoic acid or other acids, and rarely did any of them accumulate to any extent above control activity. In two experiments, in which over 100,000 counts/min of kaurene was incubated with enzyme and dithionite, two products which behaved chromatographically like kaurenol and kaurenal accumulated to the extent of a few hundred counts/min, but this amount is considered to be of doubtful significance.

Preliminary experiments, not reported here in detail, on the differential centrifugation of reaction mixtures containing either exogenous kaurene-^{14}C or kaurene-^{14}C formed *in situ* revealed that all of the extractable kaurene in both cases was present in the microsomal fraction (i.e. the pellet resulting from centrifugation of 10,000 *g* supernatant at 100,000 *g* for 60 min). However while both exogenous kaurene and kaurene which had been synthesized *in situ* both pelleted with the microsomal fraction, it was consistently observed that exogenous kaurene did not undergo enzymic oxidation.

Carbon Monoxide Difference Spectra of Pea Seed Microsomes

The negative data on the metabolism of exogenous kaurene indicated that perhaps the techniques being used were not adequate for the isolation of active kaurene-oxidizing enzymes. The involvement of cytochrome P-450 in the expected reactions, as reported by Murphy and West,[23] and the known lability of the cytochrome in plant and animal systems[30,31] prompted an inspection of the carbon monoxide difference spectrum of pea seed microsomes to ascertain whether P-450 was present and in an active state. The spectra obtained in these experiments are shown in Fig. 1. The addition of NADPH and CO to the enzyme extract did not cause a peak to appear at 450 nm (Fig. 1). When dithionite was added to these enzyme preparations, peaks appeared promptly at 450 nm and 420 nm, indicating the presence of P-450. However, attempts to observe cell-free oxidation of exogenous kaurene even in the presence of dithionite also yielded negative results.

Whereas P-450 was not reducible by NADPH in the microsomal preparations, in subsequent experiments (Table 1, Fig. 2), enzymic oxidation of kaurene was observed in 10,000 g supernatants, indicating that P-450 apparently was participating in redox reactions

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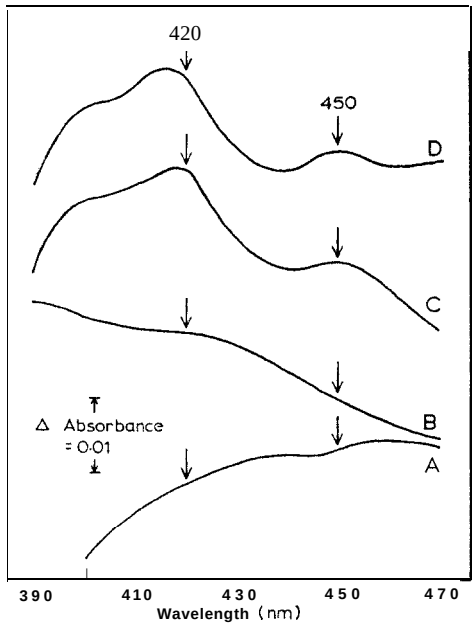

FIG. 1. CARBON MONOXIDE DIFFERENCE SPECTRA OF A MICROSOMAL FRACTION FROM IMMATURE PEA SEEDS.

Immature whole pea seeds were ground in KH_2PO_4–K_2HPO_4 buffer (0·1 M, pH 7.1) in a chilled mortar and pestle. The homogenate was centrifuged at 10,000 *g* for 15 min at 0–4° in a Sorvall RC2-B refrigerated centrifuge, and the supernatant centrifuged again at 100,000 g for 60 min in a Spinco Model L preparative ultracentrifuge. The pellet from this centrifugation was then resuspended in buffer containing 1 mM Mn^{2+} and 0·5 mM NADPH. The enzyme extract was distributed between two cuvettes and a baseline was established (A). Next, CO was bubbled through one cuvette, and the difference spectrum was observed between 390 nm and 470 nm (B). Thirdly, after this spectrum had been measured, a few crystals of dithionite were added to each cuvette and the spectrum was measured again (C). Finally, more CO was bubbled through the sample cuvette, and the spectrum was taken once more from 390 nm to 470 nm (D). All spectra were measured on a Cary Model 11 recording spectrophotometer.

in those extracts. Thus the failure of NADPH to reduce P-450 in the microsomal preparations is interpreted as indicating that an essential component of the microsomal fraction was either missing or inactive. Not excluded is the possibility that some component of the microsomal fraction was solubilized by the isolation procedure.

Cell-free Metabolism of Kaurene Formed in situ

In contrast to the results with exogenous kaurene, examination of several different enzyme preparations for cell-free metabolism of kaurene formed *in situ* by assay procedures II and III (see Experimental section) yielded positive results. Both assays make use of kaurene formed from mevalonic acid *in situ*, combined with the use of one or two inhibitors.

Assay procedure II is based upon the fact that the first reaction in the oxidative metabolism of kaurene in extracts of *Echinocystis macrocarpa* endosperm, the formation of kaurenol, is strongly inhibited by CO,[23] and the repeated observation that CO does not inhibit the synthesis of kaurene in extracts of pea seeds. The difference in kaurene accumulation between inhibited and noninhibited reaction mixtures containing the same enzyme

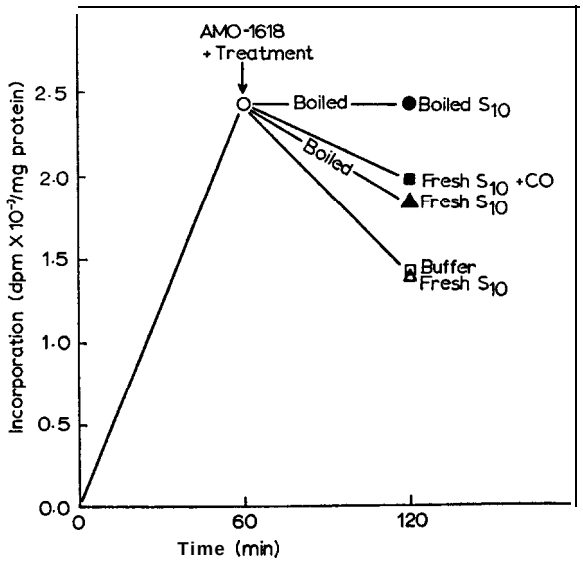

FIG. 2. OXIDATION OF KAURENE-^{14}C WHICH WAS SYNTHESIZED *in situ* IN CELL-FREE ENZYME EXTRACTS.

Routine reaction mixtures, prepared as described in Table 1, were incubated for 60 min at 30°, at which time AMO-1618 (10 μg/ml) was added to each reaction, and each of the several samples was treated in a different manner as described below:

(○) Reaction mixture boiled and incubated an additional 60 min, at which time buffer was added to make volume equal to other reactions.

(●) Reaction mixture boiled and 0·75 ml of boiled S_{10} enzyme added and incubated an additional 60 min.

(■) 0·75 ml of fresh S_{10} enzyme added and reaction mixture held under CO atmosphere for an additional 60 min incubation.

(▲) Reaction mixture boiled and 0·75 ml of fresh S_{10} enzyme added and incubated for an additional 60 min.

(A) 0·75 ml of fresh S_{10} enzyme added and reaction mixture incubated for an additional 60 min.

(□) 0·75 ml of buffer added and incubated an additional 60 min.

The amounts of kaurene-^{14}C which accumulated during the first 60 min incubation and which remained after the succeeding 60 min incubations were determined by TLC and liquid scintillation counting. In all cases, 'S_{10} enzyme' is supernatant resulting from the centrifugation of extract from isolated cotyledons at 10,000 *g* for 15 min at 0 to 4".

extract is considered to be a true indication of the amount of kaurene which is metabolized by CO-sensitive enzymes. The data presented in Table 1 are the results of two independent experiments on the effects of both CO and insoluble PVP on kaurene-oxidizing activity in enzyme extracts which were prepared from excised cotyledons. More kaurene accumulated in the presence of CO than in its absence, while the amounts of polar compounds which accumulated were reduced in the presence of the inhibitor. Enzyme extracts prepared in the presence of insoluble PVP showed greater differences in kaurene accumulation (between CO-inhibited and non-inhibited preparations) than enzyme extracts prepared in the absence of PVP (Table 1). This result with PVP is comparable to that of Frear *et al.*[31] who showed that inclusion of PVP was necessary in order to obtain active enzymes for the oxidative demethylation of substituted urea compounds in extracts of cotton seedling hypocotyls. This enzyme preparation is thought to also involve cytochrome P-450. In light of these results, all further experiments were conducted with enzyme extracts which were prepared from isolated cotyledons in the presence of insoluble PVP.

TABLE 1. KAURENE ACCUMULATION IN THE PRESENCE OF CARBON MONOXIDE IN EXTRACTS PREPARED WITH AND WITHOUT PVP

	Dpm incorporated/mg protein			
	Experiment 1		Experiment 2	
Fraction	Kaurene	Polar compounds	Kaurene	Polar compounds
S_{10} + CO	7380	11,600	4860	11,000
Difference S	+1710 5670	-1800 13,400	+1200 3650	-3000 14,000
S_{10} + PVP + CO	11,000	18,400	4600	13,700
S_{10} + PVP	7400	25,200	3100	21,000
Difference	+3700	-6800	+1500	--7300

The homogenates were centrifuged at 10,000 g for 15 min at 0–4°, and the resulting supernatants (S_{10}) were the enzyme extracts. In the preparation of particular extracts, wet PVP was mixed with the plant tissues before homogenization. Each reaction mixture contained 0·09 μmole of mevalonic acid-2-^{14}C (~0·5 μc), 0·75 ml of enzyme extract, 0·5 μmole of ATP, 3 μmoles each of Mn^{2+} and Mg^{2+} and 0·6 ml of phosphate buffer (pH 7.1) in a total volume of 1·6 ml. Incubations were for 75 min at 30°. In Experiment No. 1, 3 g of excised cotyledons from 50 freshly harvested seeds were homogenized with 1·5 g of wet PVP in 9 ml of phosphate buffer. In Experiment No. 2, 4·9 g of cotyledons were homogenized with 2·5 g of wet PVP in 11 ml of buffer. Products were isolated by TLC, and measurements of radioactivity were by liquid scintillation counting. Values are means based on duplicate samples.

The most conclusive positive evidence for enzymic oxidation of kaurene in cell-free extracts of pea seeds was obtained by assay procedure III, which makes use of AMO-1618, an inhibitor of the cyclization of geranylgeranyl pyrophosphate to copalyl pyrophosphate,[5] and CO. Kaurene is allowed to be synthesized endogenously in 10,000 g supernatant for 60 min, after which its further synthesis is blocked by AMO-1618. The use of CO in a subsequent 60-min incubation period permits measurement of enzymic oxidation of endogenous kaurene. The results of a representative experiment out of several which were performed are presented in Fig. 2.

By this method, it was demonstrated that: (a) kaurene which is formed *in situ* in the first 60 min does disappear during the subsequent 60 min of the incubation in the presence of the original enzyme; (b) the disappearance of kaurene during the second 60 min is dependent upon some heat labile substance(s); (c) adding fresh enzyme extract after 60 min does not stimulate the disappearance of kaurene; (d) the disappearance of kaurene during the second 60 min is at least partially sensitive to CO; and (e) heat denaturation of the original enzyme after the first 60 min causes a reduced level of kaurene disappearance during the second 60 min, even when additional fresh enzyme is added after the heat treatment. All five of these results were confirmed in at least one, and in some cases, four, independent experiments. In one experiment, not reported here in detail, the addition of boiled enzyme after 60 min did not significantly affect the subsequent disappearance of kaurene, as compared to added buffer or added fresh enzyme. Also included in these experiments were reactions in which kaurene was added exogenously. In these experiments, no CO-sensitive kaurene oxidation was observed above the control levels.

In four experiments utilizing assay procedure III, the difference in kaurene disappearance between the 60-min incubation and the 120-min incubation with added enzyme ranged 25-66 per cent of the kaurene present at 60 min (Fig. 2). The differences in kaurene disappearance in these experiments were not correlated with the amount of kaurene which had formed in 60 min, but did occur to the largest extent in the enzyme extracts from the oldest seeds used, and to the smallest extent in extracts from the youngest peas used. This result suggests that the capacity for kaurene oxidation may vary with stage of seed development, as has been previously reported for kaurene synthesis.[26]

The result with additional enzyme added after 60 min, as compared to buffer added at this time, indicated that the additional enzyme did not contribute to additional disappearance of kaurene (Fig. 2). This would seem to indicate that the enzymes originally present were present in saturating concentration. This is particularly interesting since, in one case, the enzyme concentration was increased while in the other case the enzyme was diluted. Perhaps the kaurene which was formed at 60 min was already attached to the complex of oxidizing enzymes before the addition of more enzyme or buffer.

The inhibition of kaurene disappearance by CO (Fig. 2) varied from 70 to 120 % in three experiments. This variability probably was due to variable effectiveness of the CO treatments. Murphy and West[23] have shown that an atmosphere of 100 % CO completely inhibits the conversion of kaurene to kaurenol in extracts of *Echinocystis macrocarpa*, but 90 % CO inhibits only about 75%. By the gassing technique which was utilized (see Experimental section), it cannot be said with certainty that the atmosphere in these reaction mixtures was 100% CO. It should also be noted that a variable amount of time (up to 20 min) elapsed between the addition of AMO-1618 to all reaction mixtures and the administration of carbon monoxide gas, and that light was not rigorously excluded.

The result obtained by heat denaturing the original enzyme and then adding fresh enzyme (Fig. 2) is very interesting. In two experiments, the amounts of disappearance of kaurene in these preparations were 28 % and 37 %, respectively. The disappearance of kaurene did not appear to be dependent upon the amount of kaurene present at 60 min. It was clear that these preparations catalysed much less kaurene disappearance than in the two parallel preparations in which the original enzymes were not thermally denatured (44 % and 66 %, respectively). A possible explanation for this is that the kaurene formed in the original extract was bound to protein, and that denaturation of the protein rendered the kaurene inaccessible to the fresh enzyme. This interpretation is compatible with the concept that there may be a requirement for some type of intimate association between the kaurene-synthesizing enzymes and the kaurene-oxidizing enzymes in this system. No proof of this phenomenon was obtained, but if it were true, this would also account for the failure to observe kaurene oxidation in these preparations when the substrate was added exogenously. It is interesting to note that Scallen *et al.*[32] reported on a 'carrier' substance, considered to be of microsomal origin, in the 105,000 g supernatant of rat liver homogenates which is required for the conversion of squalene to sterol. This substance is reported to be heat labile, and required in several microsomal oxidations in cholesterol biosynthesis.

Identification of Products of Cell-free Mevalonic Acid Metabolism

The products of mevalonic acid metabolism in large scale reaction mixtures (30-100 ml) containing 10,000 g supernatant as the enzyme extract were extracted from neutral buffer

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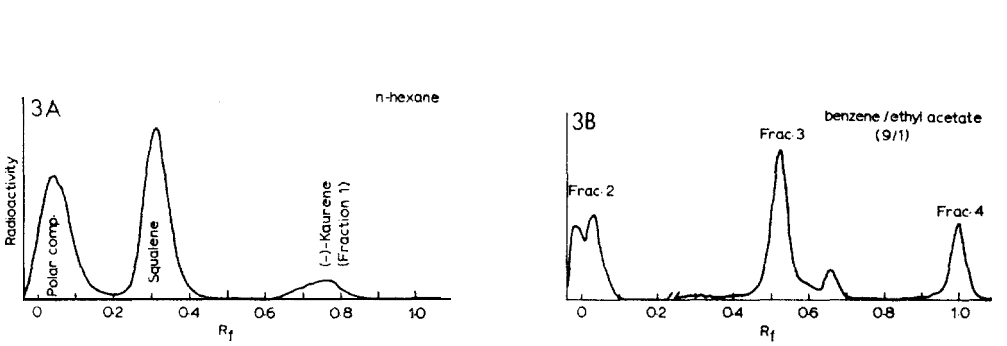

FIG. 3. PRIMARY THIN-LAYER CHROMATOGRAPHIC SEPARATIONS OF PRODUCTS OF MEVALONIC ACID-%'% METABOLISM IN CELL-FREE ENZYME EXTRACTS.

A, Radiochromatogram scan of lipid-soluble products of mevalonic acid-2-W metabolism after chromatography in hexane. B, Radiochromatogram scan of polar compounds after elution from thin-layer chromatogram in A and rechromatography in benzene-ethyl acetate (9: 1). The fractions in these tracings are numbered according to the system of Graebe et *al.*[2]

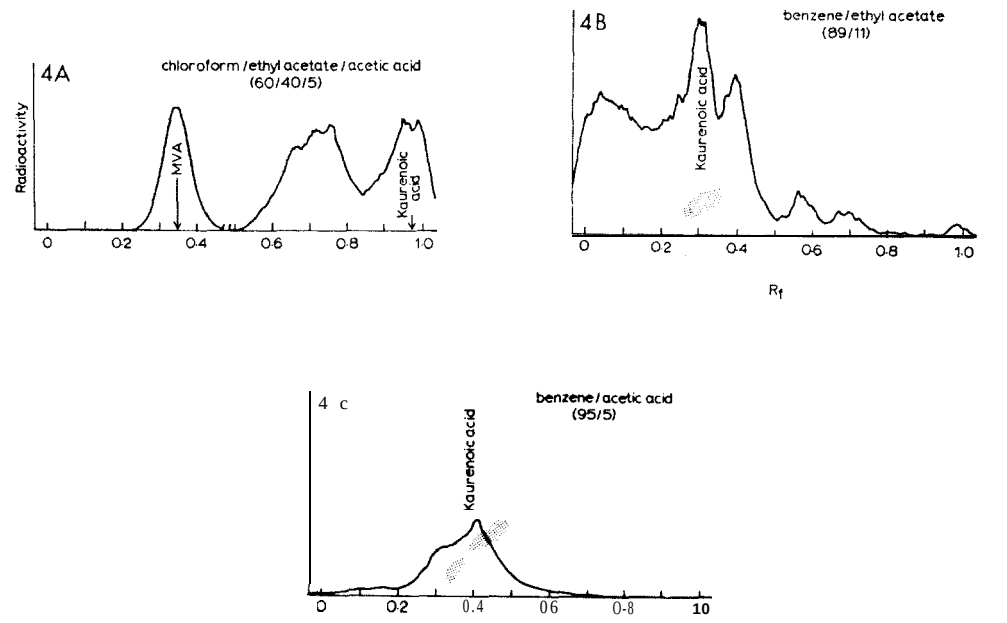

FIG. 4. ISOLATION OF PRESUMPTIVE KAURENOIC ACW FROM FRACTION 2.

A, Fraction 2 (see Fig. 3B) was eluted from the gel and rechromatographed in chloroform-ethyl acetate-acetic acid (60:40:5). B, Material corresponding to the position of kaurenoic acid was eluted from the chromatogram (Fig. 4A) and rechromatographed in benzene-ethyl acetate (89: 11). C, Material corresponding to the position of kaurenoic acid in Fig. 4B was eluted from the gel and rechromatographed in benzene-acetic acid (95: 5) with authentic karuenoic acid. The stippled spots represent the positions of samples of authentic (-)-kaurenoic acid.

with acetone-benzene (3: 1), and from acidified buffer (pH 2·5) with ethyl acetate, and subjected to TLC analysis.

The polar products isolated from these extracts after chromatography in hexane and benzene–ethyl acetate (9 : 1) (Fig. 3) were divided into three fractions which are designated according to the system of Graebe *et al.*:[2] Fraction 2, the acids and other very polar materials, moving between R_f *O-O* and 0·3; Fraction 3, the alcohols, appearing from R_f 0·3 to 0·7; and Fraction 4, the least polar compounds, appearing from R_f 0·7 to 1·0. Fraction 1, kaurene, was removed after chromatography in hexane. The gel containing each of the fractions 2-4 was scraped from the plates, eluted with acetone and rechromatographed in solvent systems suitable for the separation and tentative identification of products. From Fractions 2, 3 and 4 products tentatively identified as kaurenoic acid, kaurenol and kaurenal, respectively, were isolated (Figs. 4–6). An unidentified product also was observed which was more polar than kaurenoic acid, but less polar than mevalonic acid.

The identification of kaurenol-^{14}C was confirmed by co-crystallization of the radioactive compound with authentic kaurenol to constant specific radioactivity. The radioactive material which corresponded to the R_f of authentic kaurenol on the final TLC (Fig. 5C) was mixed with approximately 60 mg of authentic kaurenol and recrystallized twice from methanol-water, and three times from methanol. At least 60 % of the radioactivity in the original mixture was retained with the crystals of authentic kaurenol (Table 2).

TABLE 2. CO-CRYSTALLIZATION OF PRESUMPTNEAND AUTHENTIC KALJRENOL

Recrystallization	Solvent	Net counts/min/mg
Original mixture		619
First	methanol-water (1: 1)	492
Second	methanol-water (1: 1)	527
Third	methanol	438
Fourth	methanol	425
Fifth	methanol	412

Radioactive material from large-scale, cell-free reaction mixtures which behaved chromatographically like authentic kaurenol was partially purified by successive TLC in four solvent systems (Figs. 3-5): hexane, benzene-ethyl acetate (9: 1), hexane-ethyl acetate–propanol (82: 15: 3), and hexane-ethyl acetate-isopropyl ether (2: 1: 1), and was finally rechromatographed in benzene-ethyl acetate (9: 1). The radioactivity still remaining at an identical R_f with authentic kaurenol on the last chromatogram (Fig. 5C) was eluted and a small portion taken for radioactivity determination. The bulk of this radioactive material (approximately 40,000 counts/min) was mixed with 60·2 mg of authentic kaurenol and recrystallized as shown. Numbers represent the mean counts/min/mg of duplicate samples of crystals.

The identification of kaurenol and the tentative identification of other oxidized kaurene derivatives is not surprising, since these products have been identified as products of mevalonic acid and kaurene metabolism in cell-free extracts of *Ectiinocystis macrocarpa* endosperm.[3] However, these products were obtained in the present case with extracts from immature pea seeds, only with mevalonic acid as substrate, and not with exogenous kaurene. The possibility that kaurene is subject to oxidation by microsomal enzymes in cell-free extracts of pea seeds only when the substrate is bound to a heat-labile moiety which is

Rechromatogaphy of Fraction 3

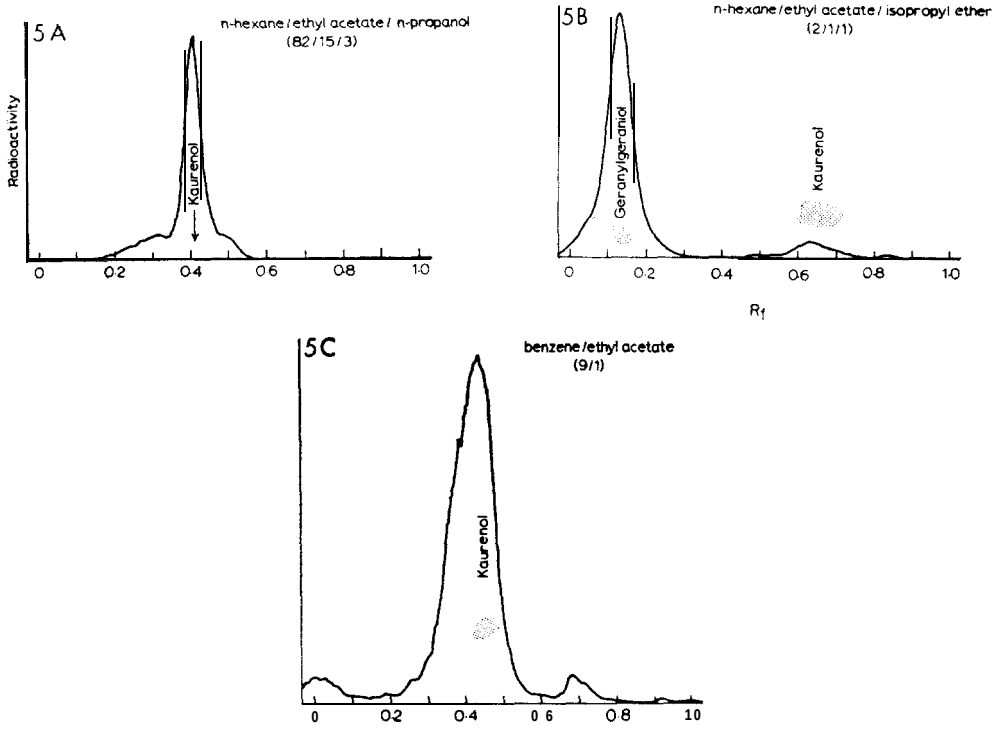

FIG. 5. ISOLATION OF PRESUMPTIVE KAURENOL FROM FRACTION 3.
A, Fraction 3 (see Fig. 3B) was rechromatographed in hexane-ethyl acetate-propanol (82:15:3). B, The radioactivity corresponding to the position of kaurenol in Fig. 5A was rechromatographed in hexane-ethyl acetate-isopropyl ether (2: 1: 1) on silver nitrate-impregnated silica gel G. C, The radioactive material corresponding to kaurenol in Fig. 5B was then rechromatographed in benzene–ethyl acetate (9: 1). The stippled spots denote the positions of authentic geranylgeraniol and (−)-kaurenol in 5B and (-)-kaurenol in 5C.

functionally associated with the soluble enzymes which catalyse kaurene biosynthesis is the subject of continuing investigations.

EXPERIMENTAL

Plant material. 'Alaska' peas (***Pisum sativum*** L.; W. Atlee Burpee Company, Riverside California) were cultured as described previously.[27] Enzyme extracts were prepared either from whole immature seeds or from excised cotyledons which were harvested on the 12th to the 15th day after anthesis. The material was either used when freshly harvested, or was frozen and stored in liquid nitrogen until used, as noted in the descriptions of individual experiments in the Results and Discussion section.

Source and purity of reagents. AMO-1618 was purchased from Enomoto Company, Redwood City, California. Squalene was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, and geraniol and farnesol from International Chemical and Nuclear Corporation, City of Industry, California. Polyvinylpyrrolidone (PVP) (insoluble Polyclar-AT) was purchased from General Aniline Film Corporation, Graselli, New Jersey. Samples of (-)-kaurene were generously supplied by Dr. L. H. Briggs, University of Auckland, New Zealand, and Dr. Charles A. West, University of California, Los Angeles, California. Reference samples of (±)-kaurenol and ($)-kaurenoic acid were the generous gifts of Dr. Kenji Mori.

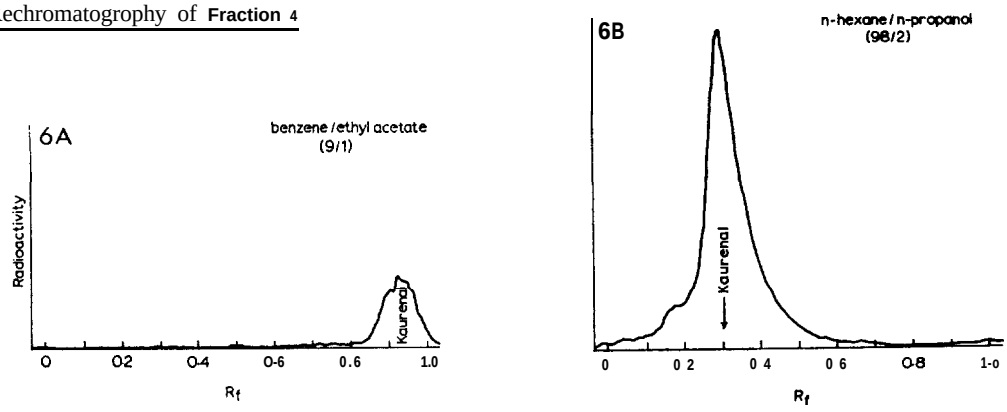

FIG. 6. ISOLATION OF PRESUMPTIVE KAURENAL FROM FRACTION 4.

A, Fraction 4 was eluted from the TLC illustrated in Fig. 3B and rechromatographed in the same solvent system. B, The gel containing the radioactivity in Fig. 6A was eluted and rechromatographed in hexane–propanol (98: 2). The arrow in B denotes the R_f of kaurenal which was obtained in earlier experiments with authentic material and is the approximate published R_f value for kaurenal.

Department of Agricultural Chemistry, University of Tokyo, Bunkyo-Ku, 113, Japan. Samples of (−)-kaurenol and (-)-kaurenoic acid were gifts from Drs. P. R. Jeffries and E. L. Ghisalberti, Department of Organic Chemistry, University of Western Australia, Nedlands, W.A. A sample of 2-*cis*–*trans*,6-*trans*,10-*trans*-geranylgeraniol was kindly supplied by Hoffmann-La Roche and Company, Basel, Switzerland. Other reagents and the sources from which they were obtained were listed previously.*'

Preparation of enzyme extracts. Enzyme extracts were prepared from seeds or excised cotyledons as described previously.[27] In some cases, the tissues were ground in the presence of insoluble PVP or other additives such as glycerol or $Na_2S_2O_5$. PVP was purified by boiling in 3 N HCl and then filtering and washing until neutral with H_2O. The PVP was then rinsed twice with phosphate buffer, pH 7·1, and finally centrifuged for 15 min at the same centrifugal force as the enzyme to be prepared. The wet PVP was then mixed with the plant tissue (0·5 or 1·0 g PVP: g tissue) before grinding. Unless otherwise stated, these crude homogenates were centrifuged at 10,000 g for 15 min at O-4" in a Sorvall RC2-B refrigerated centrifuge, and the resultant supernatant was used as a source of enzymes. Occasionally the enzyme was prepared from frozen tissue and then the liquid enzyme preparation was again frozen in liquid N_2 for use in other experiments.

Preparation of substrate quantities of kaurene- ^{14}**C.** Substrate quantities of kaurene-^{14}C were prepared biosynthetically in large-scale, cell-free extracts of immature pea seeds. The methods and materials for these incubations were as described previously.[26] The specific activities of two batches of kaurene-^{14}C were 2·8 and 3·6 μc/μmole.

Incubation with kaurene as substrate. Two methods were used to present substrate quantities of kaurene to the enzyme extracts. By one method, exogenous kaurene-^{14}C obtained from large-scale incubations was added directly to reaction mixtures; by the other method, kaurene was allowed to form *in situ* from mevalonic acid-2-^{14}C. In the former case, a sample of kaurene-^{14}C (10–40 x 10^3 dpm; 1·5–6 nmoles) was placed in the incubation tube and evaporated to dryness under a stream of N_2. Then 10 μl of either acetone, EtOH or 0·1% Tween 20 (sorbitan polyoxyethylene monolaurate) in acetone/buffer (1/2) were added to each reaction tube before the other reactants (2 mM Mn^{2+}, 2 mM Mg^{2+}, 0·5 mM NADPH, and enzyme) were added. In the latter case, kaurene was allowed to form *in situ* from mevalonic acid in routine reaction mixtures (2 mM Mn^{2+}, 2 mM Mg^{2+}, 0·3 mM ATP, 0·05 mM MVA, and enzyme) and after 60 min, AMO-1618 was added to stop the formation of kaurene.

Assays for kaurene oxidation in cell-free extracts. Kaurene oxidation was assayed by three different methods. When exogenous kaurene was added to reaction mixtures, oxidation was measured both by disappearance of kaurene and by the appearance of products, according to assay procedure number I described below. When kaurene was allowed to form *in situ,* only the disappearance of kaurene was assayed according to procedure number II or number III.

Assay procedure number I is identical to a procedure described by Dennis and West[3], according to which acetone-benzene extracts are chromatographed first in hexane, with the solvent front advancing 15 cm from the origin, and then after marking each chromatogram 10 cm from the origin, the chromatograms are rechromatographed in benzene-EtOAc (9:1). In utilizing this procedure, the reactions were

stopped by adding 3 ml of acetone, and the aqueous acetone solution was then extracted two or three times with 1 ml of benzene. After initial extraction, the aqueous phase was acidified with 0·4 N HCl to pH 2·5 and extracted twice. with 1 ml EtOAc. The combined organic extracts were evaporated to dryness; then the residue was extracted with acetone and analysed for kaurene and products of kaurene oxidation. Typically, a small sample of authentic kaurenol and/or kaurenoic acid was co-chromatographed with each sample.

Assay procedure number II is simply to incubate routine reaction mixtures for kaurene biosynthesis for 75 min at 30" in the presence and absence of CO. Product isolation and measurement of radioactivity incorporated into kaurene are as described previously.[26,27] The activity of the kaurene oxidizing enzymes is estimated by the difference between the amount of kaurene which accumulates in CO-inhibited preparations minus that accumulated in the normal preparations. Treatment with CO was accomplished by gassing the reaction tubes for 30 sec, sealing with serum caps and sparging for an additional minute using syringe needles.

Assay number III depends not only upon the inhibition of the first reaction in kaurene metabolism by CO, but also upon the inhibition of kaurene synthesis by the inhibitor AMO-1618. After kaurene biosynthesis has proceeded for 60 min in a routine reaction mixture, as described in the preceding section, AMO-1618 is added to a final concentration of 10 μg/ml of reaction. At this time, some of the reaction mixtures are boiled to stop all enzymic reactions, while more enzyme or buffer is added to others which are allowed to incubate for an additional 60 min. Addition of a saturating atmosphere of CO to some of the reaction mixtures then gives an additional control. At the end of the 120-min incubation, ail reactions are stopped by boiling, and each mixture is analysed for kaurene.[26,27]

TCL analysis of the products of mevalonic acid metabolism. On two occasions the products of large-scale incubations (30–100 ml) with mevalonic acid-2-^{14}C as substrate, were subjected to analysis by co-chromatography with authentic compounds in several TLC systems. Ail chromatography was done using silica gel G (250-750 μ thick) on glass plates 5 x 20 cm. In each case the material being tested was spotted in the center of the origin, with authentic samples of one or two compounds spotted on either side of the extract. All applications of samples were made under N_2. The chromatograms were developed 15 cm and then scanned for radioactivity. The gel containing the radioactive compounds was scraped into a centrifuge tube, while the remaining gel was sprayed with 10% H_2SO_4 in EtOH and heated to 100" for 10 min to detect the authentic compounds. The radioactive material corresponding to these samples was then eluted from the gel with acetone and spotted on another plate for further chromatographic analysis.

Radioassays and other procedures. All measurements of radioactivity and protein determinations were made as previously described.[27]

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