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BIOSYNTHESIS OF *ENT*-KAURENE IN CELL-FREE EXTRACTS OF *PISUM SATIVUM* SHOOT TIPS*

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Key Word Index—*Pisum sativum*; Leguminosae; biosynthesis; diterpenes; *ent*-kaurene; gibberellins; mevalonic acid-2-^{14}C; squalene.

Abstract—Soluble enzyme preparations from pea shoot tips incorporated mevalonic acid-2-^{14}C into *ent*-kaurene-^{14}C, squalene-^{14}C and other products. The assay for either *ent*-kaurene or squalene is quite direct; both products can be obtained apparently free of radioactive contaminants by TLC on silica gel G in hexane. The enzyme system is dependent upon added ATP and Mn^{2+} or Mg^{2+}, with Mn^{2+} being a more effective activator than Mg^{2+} under the experimental conditions. Reduced pyridine nucleotide had no effect on *ent*-kaurene production but stimulated squalene synthesis. The accumulation of both *ent*-kaurene and squalene was stimulated by dithiothreitol and carbon monoxide and was reduced by the addition of particulate cell components. AMO-1618 inhibited *ent*-kaurene production and had no effect on the synthesis of squalene. Enzyme extracts from shoot tips are much less active in *ent*-kaurene synthesis than extracts from the cotyledons of immature seeds on either a fresh weight or protein basis.

INTRODUCTION

MOST OF the investigations of gibberellin biosynthesis in seed plants have utilized cell-free enzyme systems prepared from developing seeds and fruits of *Echinocystis macrocarpa* Greene (wild cucumber),[1-8] *Pisum sativum* L. (pea)[9-14] and *Cucurbita pepo* L. (pumpkin),[15,16] all of which are abundant sources of gibberellins. In each of these systems, *ent*-kaurene (hereafter called kaurene) appears to be a key intermediate in the gibberellin biosynthetic pathway: kaurene is the final product of a series of reactions involving soluble enzymes;[2,10,12,15] it is the first reactant in a series of oxidative steps involving particulate enzymes;[3-6,13,14,16] its synthesis is inhibited by a number of growth regulators;[3-5,9,10,17]

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and there is a good correlation between the kaurene-synthesizing activity of enzyme extracts from developing pea seeds and the levels of gibberellins extractable from those seeds.[11]

While these cell-free enzyme preparations from developing seeds and fruits yield valuable information about the biosynthetic pathway, the development of similar systems from vegetative plant parts is essential to an ultimate understanding of gibberellin relations in plants and the influence of environmental factors on this pathway. Graebe,[10] while studying mainly young pea fruits, reported the synthesis of kaurene, squalene and phytoene in cell-free extracts of various parts of pea plants, including comparatively very small amounts of kaurene and large amounts of squalene and phytoene in vegetative shoots. Robinson and West[18,19] developed and described in detail a system from cell-free extracts of *Ricinus communis* L. (castor bean) seedlings which was capable of incorporating mevalonic acid-2-^{14}C (MVA-^{14}C) and geranylgeranyl pyrophosphate-^{14}C into five similar diterpene hydrocarbons including kaurene-^{14}C. In addition, Stoddart[20] has reported on the conversion of *ent*-kaurenoic acid into gibberellins by chloroplast preparations from leaves of *Brassica oleraces* var. *acephala* (Canson kale).

The present communication corroborates and extends the earlier paper by Graebe[10] and reports the incorporation of MVA-^{14}C into kaurene and squalene in cell-free extracts of pea shoot tips. The system described would appear to be very useful in direct biochemical investigation of several problems of physiological interest, such as: effects of light quality, duration and timing of light and dark periods, and low temperature on gibberellin biosynthesis; and possible variations in gibberellin metabolism among dwarf and normal and early- and late-flowering varieties of *Pisum sativum*.

RESULTS AND DISCUSSION

Identification of Products

Incubation of enzyme preparations from pea shoot tips with MVA-2-^{14}C, ATP, $MnCl_2$ and $MgCl_2$ resulted in the enzymic production of kaurene, squalene and some unidentified products. In this system kaurene appears to migrate as a single radioactive product on TLC in hexane. This system is in contrast to that from castor bean preparations which produces five related diterpene hydrocarbons.[18,19] In the castor bean system, four of these radioactive products including kaurene migrate to the same position in hexane.[18]

Kaurene and squalene were identified by co-chromatography with authentic samples on TLC in three solvents (Figs. 1–3) and by recrystallization to constant specific radioactivity (Tables 1 and 2). The radioactive material originally chromatographing with kaurene (Fig. 1) was resolved into two peaks in all subsequent chromatograms (Fig. 2). The major zone matched the position of authentic kaurene. The minor peak appeared with a conversion product of the authentic kaurene, perhaps kauranol, a product of the hydration of kaurene. Non-enzymic conversion of kaurene was minimized by the addition of carrier non-radioactive kaurene before extracting the reaction mixtures and by doing the analyses as rapidly as possible. The absence of additional radioactive materials on these chromatograms indicates that under the conditions of these experiments the three diterpene hydrocarbons, beyerene, sandaracopimaradiene and trachylobane[18,19] do not accumulate in detectable quantities.

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Radioactive material from the kaurene region of TLC plates (equivalent to the kaurene region in Fig. 1) was mixed with pure authentic kaurene and recrystallized to constant specific radioactivity (Table 1). After an initial decrease, probably attributable to conversion of some kaurene prior to elution, the specific radioactivity of the sample remained constant through three recrystallizations.

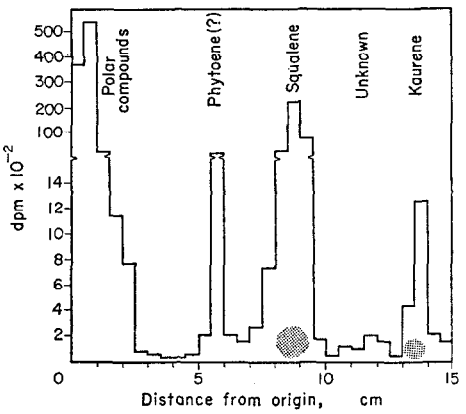

FIG. 1. RADIOACTIVE FRACTIONS PRODUCED FROM MVA-2-^{14}C BY A CELL-FREE ENZYME PREPARATION FROM PEA SHOOT TIPS.

Squalene also was identified as a product of MVA metabolism in these reactions by TLC (Figs. 1 and 3) and by making the hexahydrochloride according to the method of Heilbron *et al.*[21] and recrystallizing the hot-acetone soluble isomerides to constant specific radioactivity (Table 2).

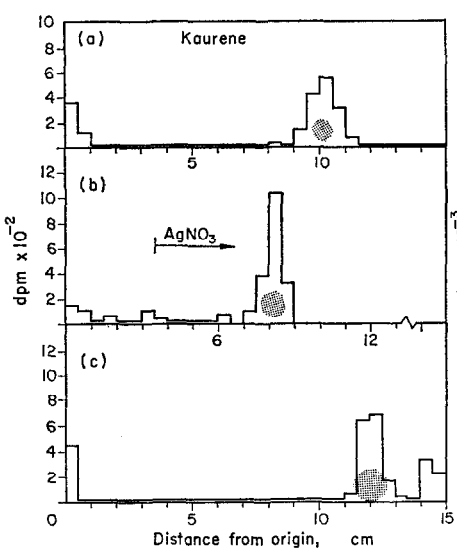

FIG. 2. TLC OF PRESUMPTIVE *ent*-KAURENE IN THREE SYSTEMS.

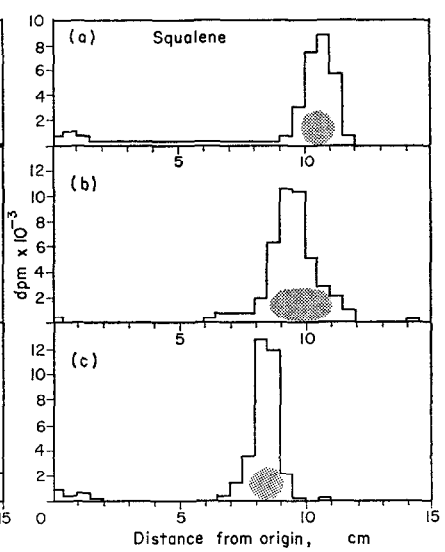

FIG. 3. TLC OF PRESUMPTIVE SQUALENE IN THREE SYSTEMS.

The other products resulting from these reactions were not identified in the present study. However, the product of R_f 0·23 in the hexane system is thought to be phytoene on the basis of its chromatographic behavior and its absence in reaction mixtures not containing Mn^{2+}.

TABLE 1. CO-CRYSTALLIZATION OF PRESUMPTIVE AND AUTHENTIC *ent*-KAURENE

Sample	Mass (mg)	Net dpm/mg	Sample	Mass (mg)	Net dpm/mg
Original mixture	65·3	132	Second crystals	16·8	77
First crystals	32·0	77	Third crystals	5·6	71

Kaurene-^{14}C was extracted from 32 combined reaction mixtures each of which was prepared as described in the Experimental. The C_6H_6-acetone extract was chromatographed on thin layers of silica gel G in hexane. A total of 8600 dpm of kaurene-^{14}C was isolated, combined wth 65·3 mg of nonradioactive kaurene and recrystallized from MeOH. Numbers represent the mean dpm of duplicated samples of crytsals.

Requirements of the System

Optimum conditions for the production of kaurene were found to be using 0·75 ml enzyme extract (*ca.* 1·2 mg protein), 1 mM Mg^{2+}, 1 mM Mn^{2+}, 3 mM ATP, 2 mM dithiothreitol, 100 μM chloramphenicol, 70 μM MVA-2-^{14}C and 75 mM KH_2PO_4–K_2HPO_4 in a total volume of 1·0 ml. Table 3 shows some of the characteristics of the system. ATP and a divalent cation were required for both kaurene and squalene synthesis. Under the conditions used, Mn^{2+} was a more effective activator than Mg^{2+}, a result consistent with previous results with cell-free preparations from two varieties of pea seeds[9,11] but in contrast to the cation requirement in other cell-free systems.[2,10,15] The results with NADPH and AMO-1618 are similar to those of Graebe.[10] NADPH had no effect on kaurene synthesis and stimulated squalene formation. And, as expected, AMO-1618 almost completely inhibited kaurene biosynthesis without having any significant effect on squalene accumulation. More kaurene and squalene accumulated in reaction mixtures incubated in an atmosphere of CO than in those incubated in air. The omission of CO never completely precluded the accumulation of these products, but a partial decrease was consistently observed. Atmospheres of argon and N_2 were equally effective as CO. Graebe[10] also reported increased yields when soluble enzyme extracts of pea fruits were incubated in N_2. The omission of dithiothreitol from reaction mixtures reduced the yield of kaurene 51 % and that of squalene by 62 %. In all other experiments dithiothreitol was included in the grinding buffer. PVP and chloramphenicol also were routinely used in the grinding process; the effect of their deletion has not yet been determined. The percentages incorporation of radioactivity into kaurene and squalene in complete systems run anaerobically routinely were < 1 % and 1–2 % respectively.

Estimates based on the data from cell-free systems indicate that the apparent level of net kaurene-synthesizing capacity per shoot tip (Table 3, complete reaction mixture) is approx. 150-fold less than the apparent level per pair of cotyledons of an immature pea seed.[12] These estimates agree at least roughly with the general observations that the amounts of gibberellins obtainable from immature pea seeds by extraction[22] greatly exceed (at least 100-fold) the amounts obtainable from pea seedling shoots by extraction or diffusion techniques.[23,24]

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EXPERIMENTAL

Source and purity of reagents. Mevalonic acid-2-^{14}C lactone (sp. act. 7·1 mCi/mmol) in benzene solution was purchased from Amersham/Searle Corporation, Des Plaines, Illinois. The benzene was removed under N_2 and the lactone was hydrolyzed by treating eight hr with 100% excess NaOH. Polyvinylpyrrolidone (PVP) (insoluble Polyclar-AT) was prepared for use as previously described.[13] Kaurene was a generous gift from Dr. R. K. Clark, Jr., of Abbott Laboratories, North Chicago, Illinois. All other chemicals were reagent grade and all solvents were redistilled before use.

TABLE 2. RECRYSTALLIZATION OF SQUALENE HEXAHYDROCHLORIDE

Sample	Mass (mg)	Net dpm/mg	Sample	Mass (mg)	Net dpm/mg
Original mixture*	255·7	720	Second crystals	45·0	475
First crystals†	58·0	480	Third crystals	36·6	515

* Squalene. † Squalene hexahydrochloride.

Squalene-^{14}C was extracted from 39 combined reaction mixtures each of which was prepared as described in the Experimental, except that in this case only, the concentration of MVA-2-^{14}C was 19·4 μM and the specific activity was 12·9 mCi/mmol. The benzene-acetone extract was chromatographed on thin layers of silica gel G in hexane. A total of $3{\cdot}55 \times 10^5$ dpm of squalene-^{14}C was isolated; 52% of this was combined with 255·7 mg nonradioactive squalene, in acetone, and treated with HCl[21] to form the hexahydrochloride. The hot-acetone soluble isomerides were then recrystallized three times. Numbers represent the mean dpm of duplicate samples of crystals.

Plant material. Pea plants (*Pisum sativum* L. cv. Alaska; W. Atlee Burpee Company, Riverside, California) were cultured in water-saturated vermiculite in a greenhouse as previously described.[12] Enzyme extracts were prepared from the excised apical buds (including young leaves, leaf primordia and apical meristem above the sixth node) of pea seedlings harvested 12–14 days after planting. Plant material was either used immediately after harvest or stored in liquid N_2 (up to 4 weeks) until needed.

TABLE 3. REQUIREMENTS FOR *ent*-KAURENE AND SQUALENE SYNTHESIS FROM MEVALONATE-2-^{14}C IN CELL-FREE ENZYME SYSTEM

Conditions	Net yields of products (dpm/mg protein) Kaurene	Squalene	Conditions	Net yields of products (dpm/mg protein) Kaurene	Squalene
Complete	78	5543	Complete minus DTT	38	2123
Complete minus Mg^{2+}	83	4426	Complete minus CO	58	2428
Complete minus Mn^{2+}	29	1010	Complete (zero time control)	14	20
Complete minus Mg^{2+} and Mn^{2+}	24	6540	Complete plus NADPH	76	7900
Complete minus ATP	8	10	Complete plus AMO-1618	29	6210

Complete reaction mixtures, as described in the Experimental, were incubated 90 min at 30° then extracted with acetone–C_6H_6. The lipid extracts were co-chromatographed with authentic kaurene and squalene on silica gel G in hexane. After visualizing the authentic standards, the kaurene and squalene regions of the gel were removed and counted by liquid scintillation. Numbers represent the mean of duplicate reaction mixtures, and with the single exception of the result with DTT, all of these data have been confirmed in at least one other similar experiment.

Preparation of enzyme extracts. Excised shoot tips were frozen in liquid N_2 and ground into a fine powder in a mortar and pestle. When the powder had warmed slightly, PVP (0·5 g wet PVP per g fr. wt of tissue) and 0·1 M KH_2PO_4–K_2HPO_4 buffer (pH 6·8; 1·0 ml per g fr. wt) containing 100 μM chloramphenicol and 2 mM dithiothreitol were added and mixed to form a slush which was allowed to warm to 0–4°. The homogenate than was filtered 2× through four layers of cheese cloth and the filtrate was centrifuged 3×. The

first centrifugation was at a maximum force of 5000 *g* for a total of 100 sec to bring down the PVP and cellular debris. The first supernatant was then centrifuged for 15 min at 10 000 *g*. Finally, the 10 000 *g* supernatant was centrifuged at 78 000 *g* for 60 min, and the resulting supernatant was used as the enzyme source. All centrifugations were done at 0–4°.

Reaction conditions and product isolation. A typical reaction mixture for kaurene and squalene synthesis contained 0·75 ml enzyme extract, 1 mM Mg^{2+}, 1 mM Mn^{2+}, 3 mM ATP, 2 mM dithiothreitol, 0·1 mM chloramphenicol, 70 μM MVA-2-^{14}C and 75 mM KH_2PO_4–K_2HPO_4 in a total of 1·0 ml. In all cases where one or more of the components was omitted, its volume was replaced by water. Duplicate reaction mixtures were incubated 90 or 120 min at 30°. Reactions were stopped by adding 2 ml acetone (which contained 2·5 μg authentic kaurene in the indicated experiments) and extracted with three 1-ml portions of C_6H_6. The combined organic extracts were then evaporated to dryness under vacuum and transferred with three 0·2-ml portions of acetone to a TLC plate. 10 μg samples of authentic kaurene and squalene were applied to either side of the extract and the TLC plates were routinely developed in *n*-hexane. In most experiments kaurene and squalene were visualized with iodine vapors. However, plates made with silica gel G impregnated with $AgNO_3$ were sprayed with 5% H_2SO_4 in 95% EtOH and heated to 100° for 15 min to visualize the authentic samples. The bands of gel containing these materials were removed from the chromatograms and placed in liquid scintillation vials for counting as previously described.[12] In the experiments described in Figs. 1–3 the center portion of the chromatogram (containing the radioactive materials chromatographed from the extract) had been removed in 0·5 cm bands and stored in acetone before the co-chromatographed authentic samples were visualized.

Protein determinations. Protein determinations were made on portions of each enzyme preparation by the method of Lowry *et al.*[25] using bovine serum albumin as a standard.

Radioactive experiments. Incubation mixtures containing MVA-2-^{14}C (70 μM; sp. act. 7·1 mCi/mmol), $MnCl_2$ (1 mM), $MgCl_2$ (1 mM), ATP (3 mM), DTT (2 mM), chloramphenicol (100 μM), KH_2PO_4–K_2HPO_4 buffer (75 mM) at pH 6·8 and 0·75 ml enzyme extract (*ca.* 1·2 mg protein) in a total vol. of 1·0 ml were incubated for 120 min at 30° in an atmosphere of carbon monoxide. Reaction mixtures were stopped by adding 2 ml acetone containing 2·5 μg non-radioactive kaurene and extracted with three 1-ml portions of benzene. The combined lipid extract from 39 reaction mixtures was chromatographed in *n*-hexane with authentic kaurene and squalene. The central portion of each 0·5 cm segment of the silica gel G (the portion containing the chromatographed extract)was removed and extracted with acetone, while the remaining gel on the plate was treated with I_2 vapors to visualize the authentic kaurene and squalene. A portion of each extract was taken for counting to yield the histogram (Fig. 1) while the remainder was used for rechromatography as shown in Figs. 2 and 3. The stippled spots denote the positions of authentic kaurene and squalene. The dpm associated with the 0·5 cm regions of the chromatograms were determined by liquid scintillation spectrometry and represent the total dpm from the combined reactions.

Distribution of radioactive products derived from the kaurene region of the chromatogram (Fig. 1) after rechromatography on thin layers of: (A) silica gel G impregnated with 3% silver nitrate and developed in *n*-hexane–C_6H_6 (7 : 3) as described by Robinson and West;[18] (B) silica gel G impregnated with silver nitrate over 14·5 cm of the plate and developed in hexane–C_6H_6 (17 : 3) as described by Robinson and West;[19] and (C) silica gel G developed in *n*-hexane exactly as in Fig. 1. Stippled spots represent the positions of authentic kaurene which was co-chromatographed with the extract. Radioactivity measurements represent the calculated total from the initial reactions described above.

Distribution of radioactive products derived from squalene region of the chromatogram (Fig. 1) after rechromatography on thin layers of: (A) silica gel G developed in light petrol.–C_6H_6 (96 : 4); (B) silica gel G impregnated with 3% silver nitrate developed in C_6H_6–ethyl acetate (9 : 1); and (C) silica gel G developed in *n*-hexane is shown in Fig. 3. Stippled areas indicate the positions of authentic squalene which was co-chromatographed with the extract. The radioactivity represents the calculated total from the initial reactions described above.

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