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Presence of Endogenous *ent*-Kaurene in a Microsomal Preparation from *Cucurbita maxima* L. Endosperm and Implications for Kinetic Studies of *ent*-Kaurene Oxidase

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Abstract. Microsomal and soluble cell-free extracts prepared from liquid endosperm of *Cucurbita maxima* L. were found to contain high concentrations of endogenous *ent*-kaurene and *ent*-kaurenol by gas chromatography-mass spectrometry-chemical ionization with deuterated internal standards. Increases in the levels of *ent*-kaurenol, *ent*-kaurenoic acid, and *ent*-7α-hydroxykaurenoic acid are correlated with a decline in the amount of endogenous *ent*-kaurene following a 10 min incubation of microsomes with NADPH and FAD. The rate of oxidation of radiolabeled *ent*-kaurene by the microsomal fraction was determined, and the need to account for endogenous substrate is shown. Endogenous *ent*-kaurene present in soluble extracts had the effect of diluting the [^{14}C]*ent*-kaurene synthesized from [^{14}C]mevalonic acid, resulting in reduced specific radioactivity of the product. The dilution of [^{14}C]*ent*-kaurene was more pronounced in extracts with higher endogenous *ent*-kaurene levels or when the reactions were run in the presence of O_2 and NADPH. Evidence is presented that suggests differential metabolism of endogenous *ent*-kaurene and radiolabeled *ent*-kaurene in both microsomal and soluble extracts.

Kaurene oxidase catalyzes the oxidation of kaurene to kaurenol, an early step in the gibberellin biosynthetic pathway (Fig. 1). Studies on kaurene oxidase usually involve assays using radiolabeled kaurene as a substrate (Ashman et al. 1990, Coolbaugh et al. 1978, Murphy and West 1969). Conversion of ^{3}H- or ^{14}C-labeled kaurene to radioactive products is used to estimate kinetic parameters for the enzyme. This technique assumes that the exogenous labeled kaurene represents all the substrate and is readily available to the enzyme. However, kaurene is a natural product in liquid cultures of *Gibberella fujikuroi* (Cross et al. 1963), liquid endosperm of *Cucurbita maxima* (Hedden and Graebe 1981), and elongating stem internodes from *Zea mays* L. (Suzuki et al. 1992), three systems used to study kaurene metabolism. Kaurene also accumulates in shoots of *Oryza sativa* L. (Moore et al. 1988) and germinating caryopses of *Hordeum vulgare* L. (Großelindemann et al. 1991). In cell-free extracts from pea seeds, there is evidence for the differential ability of in situ-synthesized [^{14}C]kaurene, as compared to exogenously added [^{14}C]kaurene, for the enzyme (Moore et al. 1972), possibly due to the involvement of a kaurene carrier protein (Hanson et al. 1980, Moore et al. 1972). Others have suggested (Graebe and Ropers 1978, Hedden 1983) that kaurene was not complexed to a protein but rather associated with microsomal fragments not sedimented by ultracentrifugation. Even without invoking a carrier mechanism, it is still possible that newly synthesized kaurene produced in the cytoplasm and/or plastids (Coolbaugh 1983) is preferentially metabolized.

In order to accurately estimate the kinetics of kaurene oxidase activity, the contribution of endogenous kaurene to the total substrate concentration must be recognized. It is also relevant that [^{14}C]-kaurene used in most studies is synthesized from

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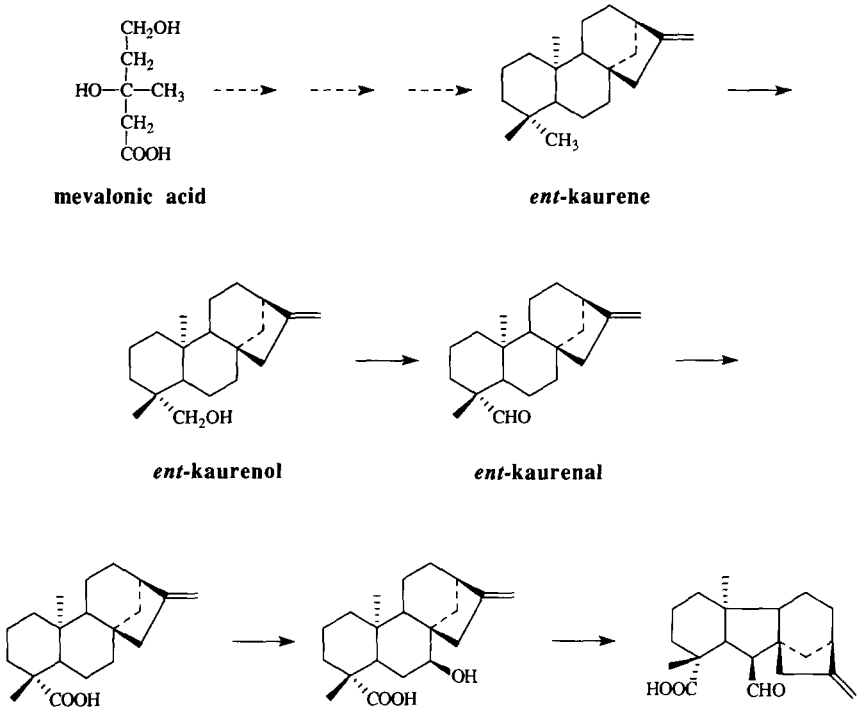
Abbreviations: Kaurene, *ent*-kaur-16-ene; MVA, mevalonic acid; kaurenol, *ent*-kaur-16-en-19-ol; kaurenoic acid, *ent*-kaur-16-en-19-oic acid; EtOAc, ethyl acetate; MeOH, methanol; GC-MS-CI, gas chromatography-mass spectrometry-chemical ionization; 13-OH KA, *ent*-13-hydroxykaur-16-en-19-oic acid; 7α-OH kaurenoic acid, *ent*-7α-hydroxykaur-16-en-19-oic acid; kaurenal, *ent*-kaur-16-en-19-al; Me(x), methyl ester of x; TMS(x), trimethylsilyl ether or ester of x; GA(x), gibberellin A(x)



mevalonic acid ***ent*-kaurene**

***ent*-kaurenol** ***ent*-kaurenal**

***ent*-kaurenoic acid** ***ent*-7α-hydroxykaurenoic acid** **GA_{12} aldehyde**

Fig. 1. GA biosynthetic pathway from mevalonic acid to GA12.

[^{14}C]mevalonic acid using soluble enzyme preparations from *G. fujikuroi* or *C. maxima* that contain endogenous kaurene. Indeed, we found that biosynthesized [^{14}C]kaurene can be heavily diluted. We therefore set out to determine the endogenous kaurene levels in our extracts.

Materials and Methods

Preparation of Cell-Free Extracts

Pumpkin (*Cucurbita. maxima* cv. Big Max) plants were field-grown in Fargo, North Dakota (1987, 1990) and in Lafayette, Indiana (1990). The developmental state of the seeds in growing fruit was followed using the procedures of Birnberg et al. (1986). Fruits were harvested when the mean cotyledon length was 20–50% of the length of the seed lumen, the stage in development strongly correlated with high enzyme activity (Graebe 1972). Crude extracts from liquid endosperm (S_{10}) were prepared according to the procedures of Graebe (1972). The S_{10} extract was centrifuged at 150,000 *g* for 1.5 h to yield a "soluble" high speed supernatant (S_{150}).

Microsomes were prepared from liquid endosperm of plants grown in Lafayette, Indiana (1990). S_{10} was prepared as before, except dialysis was omitted. The S_{10} was centrifuged at 150,000 *g* for 1.5 h to yield a microsomal pellet (P_{150}). The microsomes were suspended in 100 mM Tris-HCl buffer (pH 7.5) containing 20% glycerol (vol/vol) and protease inhibitors (Bowen et al., 1972). The volume of the P_{150} fraction was made equal to that of the S_{10}, and insoluble precipitates were removed by centrifugation at 10,000 *g* for 10 min. Enzyme extracts were prepared at 4°C. Extracts were stored in liquid N_2 or at −70°C without significant loss of enzyme activity. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Oxidation of Kaurene by Microsomes

The rate of [^{14}C]kaurene oxidation was determined in microsomal preparations (P_{150}) from liquid endosperm. Reaction mixtures consisted of 20 μL P_{150} (0.6 μg protein μl^{-1}), 28 pmol [^{14}C]kaurene (10.0–12.0 MBq μmol^{-1}) dissolved in 50 μl acetone containing 0.1% Tween-20, 0.5 mM NADPH, 0.05 mM FAD, and 100 mM Tris buffer (pH 7.5) in a total volume of 0.5 ml. Duplicate reactions were incubated in open vials on a shaking (150 rpm) water bath at 30°C for 0–30 min. Reactions were stopped by the addition of 0.5 ml acetone. Mixtures were extracted once with 0.5 ml benzene:acetone (3:1, vol/vol) and twice with 0.5 ml EtOAc. The organic fractions were combined, dried under vacuum, and dissolved in 1.3 ml MeOH:H_2O (9:1, vol/vol). Samples were injected onto a Beckman ODS 4.6 × 250 mm C_{18} reversed-phase HPLC column using a BAS LC 241 autoinjector. Samples were chromatographed at 1 ml min^{-1} using an isocratic program (Hazebroek and Metzger 1990). The pH of the H_2O/MeOH mixture was adjusted to 2.5 with H_3PO_4. Radiolabeled reaction products were estimated by a Beckman model 171 radioisotope detector. Identification of radioactive peaks was accomplished by comparison of the retention times with those of authentic standards.

Biosynthesis of Kaurene in Soluble Cell-Free Extracts

[^{14}C]Kaurene was biosynthesized from R-[2-^{14}C]mevalonic acid lactone (2.0 MBq μmol^{-1}, Amersham) using soluble enzymes in S_{10} and S_{150} extracts. Five milliliter reaction mixtures contained 17.9 μM[^{14}C]MVA (prepared from hydrolysis of the lactone with 7.4 N NH_4OH for 2 h at 50°C), 100 mM phosphate buffer (pH 7.1), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 3 mM ATP, and 1 ml S_{10} or S_{150}. The reactions were incubated under N_2 at 30°C for 16 h. Duplicate reactions were run for the S_{10} or S_{150} from seeds of two different pumpkins.

To determine the effect of O_2 and NADPH on [^{14}C]kaurene synthesis, 2 ml reactions were incubated as previously described except with 1.7 μM[^{14}C]MVA and 1 ml S_{10}. In this case, five replicate reactions were incubated without NADPH under N_2, and five reactions were incubated under air with the addition of 1.0 mM NADPH.

In all experiments, the reaction mixtures were extracted three times with one volume of EtOAc. The organic fractions from all the replicates were combined and dried under N_2. The residue was dissolved in 200 μl MeOH and subjected to reversed-phase HPLC on a Whatman 9 × 250 mm Magum C_{18} column using a gradient elution described elsewhere (Metzger and Hazebroek 1989). The final yield of [^{14}C]kaurene was determined by liquid scintillation. The specific activity of [^{14}C]kaurene was determined by GC-MS (Bowen et al. 1972).

Quantitative Analysis of Endogenous Kaurenoids

The levels of endogenous kaurenoids in the P_{150} fraction were determined by GC-MS-CI. In addition, endogenous kaurenoid levels were measured in 10 pooled 0.5-ml replicate reaction mixtures, each containing 20 μl P_{150} after they had been incubated 10 min with 333 Bq [^{14}C]kaurene (10.0 MBq μmol^{-1}), 0.5 mM NADPH, 0.05 mM FAD, and 100 mM Tris-HCl buffer (pH 7.5).

Before extraction of the kaurenoids, 300 ng [^{2}H]kaurene and 200 ng each of [^{2}H]kaurenol, [^{2}H]kaurenoic acid, and 13-OH kaurenoic acid (steviol) were added to 0.2 ml P_{150} as internal standards. Kaurenoids were extracted from the P_{150} fraction by partitioning against one ml of hexane:acetone (3:1, vol/vol) followed by two 1 ml volumes of EtOAc. The organic fractions were combined and dried under N_2. The residues were dissolved in 200 μl MeOH and subjected to reversed-phase HPLC using an 8 × 100 mm Waters Nova Pak C_8 reversed-phase column. The column was eluted with a H_2O-MeOH gradient. The initial solvent composition of 70% MeOH was held for 1 min, and then the percentage of MeOH was increased linearly to 100% over the next 10 min where it was maintained for 10 min. The flow rate was 2 ml min^{-1}. Fractions containing 7β-OH kaurenoic acid and 13-OH kaurenoic acid (3.5–5 min), and those containing kaurenol and kaurenoic acid (8–10 min) were collected, dried, methylated with ethereal diazomethane, and redissolved in 10 μl of *N*-methyl-*N*-trimethylsilylfluoracetamide. The fraction containing kaurene (11–13 min) was collected, dried and redissolved in 10 μl acetonitrile. Kaurenoids in the P_{150} fraction following the 10 min reaction were extracted in the same manner, except a 10% aliquot of the kaurenol–kaurenoic acid fraction before derivatization was loaded on a 0.25 mm silica gel G TLC plate (Analtech) and developed in benzene:EtOAc:7.4 N NH_4OH (80:20:1, vol/vol/vol). This solvent system separates kaurenol, kaurenal, and kaurenoic acid.

Five microliters of each final sample were analyzed by GC-MS-CI using a Finnegan ion trap mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph as described elsewhere (Metzger and Hazebroek 1989, Hazebroek et al. 1993). Absolute levels of endogenous kaurene, kaurenol, and kaurenoic acid were estimated using isotope dilution (Ashton et al. 1985).

Full-scan mass spectra were obtained and the ratio of the sum of the intensities of the labeled ions in the base peak clusters (m/z B + 3 through B + 6) from the ^{2}H-labeled internal standards and their unlabeled endogenous counterparts were used in determining kaurenoid levels. Adjustments in ion intensities were made to compensate for the natural isotope abundances. In the case of 7α-OH kaurenoic acid, ^{2}H-labeled internal standard was unavailable. Therefore, quantitation was done by calculating the ratio of m/z 317 in MeTMS 7α-OH kaurenoic acid to m/z 317 in MeTMS 13-OH kaurenoic acid and comparing this ratio against those from a standard curve of constant MeTMS 13-OH kaurenoic acid with varying amounts of MeTMS 7α-OH kaurenoic acid.

The amounts of endogenous kaurene in S_{10} and S_{150} fractions were determined in a similar manner as described above. Two hundred ng [^{2}H]kaurene and 243 Bq [^{14}C]kaurene (7.4 MBq μmol^{-1}) were added to 0.2 ml S_{10} and 0.5 ml S_{150}. Kaurene was extracted from the S_{10} and S_{150} samples with three equal volumes of EtOAc. Kaurene was purified from the EtOAc fraction using gradient-eluted HPLC as described before (Metzger et al. 1989). The kaurene-containing HPLC fraction was collected, dried, and redissolved in 10 μl acetonitrile.

Labeled Chemicals

[2,3,5,6,9,11,13,14-^{14}C]Kaurene (10.0–12.0 MBq μmol^{-1}) used in the microsomal enzyme assays was biosynthesized from RS-[4,5-^{14}C]mevalonic acid lactone (4.0 MBq μmol^{-1}, Amersham) by soluble enzymes from *Gibberella fujikuroi* (Hazebroek et al. 1991). [1,7,12,18-^{14}C]Kaurene (7.4 MBq μmol^{-1}) used in the quantitation of endogenous kaurenoids in S_{10} and S_{150} fractions was synthesized from R-[2-^{14}C]mevalonic acid lactone (2.0 MBq μmol^{-1}, Amersham) using the S_{150} from pumpkin liquid endosperm. ^{2}H-Labeled kaurene, kaurenol, and kaurenoic acid were synthesized from the respective C-16 norketones via the Wittig reaction (Gianfagna et al. 1983, Hazebroek and Metzger 1990). The resulting ^{2}H-labeled compounds were mixtures of analogs containing 1–4 ^{2}H atoms per molecule. 7α-OH Kaurenoic acid was extracted from leaves of *Solidago rigida* L. following the procedures used to extract kaurenoic acid from sunflower florets (Metzger and Hazebroek 1989). 13-OH Kaurenoic acid was obtained from leaves of *Stevia rebaudiana* (Gianfagna et al. 1983).

Results

Quantitation of Endogenous Kaurenoids in Microsomal Preparations

The levels of several kaurenoids present in a P_{150} enzyme preparation were determined by combined GC-MS using deuterated internal standards. The concentrations of kaurene, kaurenol, and kaurenoic acid were determined to be 96, 180, and 32 ng in 200

Table 1. Quantitation of endogenous kaurenoids in a P_{150} preparation.

	No reaction	After 10 min reaction
	(ng in 200 µl P_{150})	
Kaurene	96	ND
Kaurenol	180	186
Kaurenoic acid	32	45
7α-OH Kaurenoic acid	ND	2

Kaurenoid concentrations before and after a 10 min incubation with substrate and cofactors were determined using GC-MS-CI. Reactions were run in 0.5 ml volumes with 333 Bq (7.5 ng) [^{14}C]-kaurene and 20 µl P_{150}. Ten replicate reactions were pooled prior to quantitation to give 200 µl P_{150}.
ND, not detected.

µl P_{150}, respectively. No 7α-OH kaurenoic acid was detected (Table 1). As only 20 µl of enzyme were used per half milliliter reaction, the amounts of these substrates by the enzyme extracts in zero-time reactions were 9.6, 18, and 3.2 ng, respectively. Similar measurements were made on pooled reaction mixtures after 10 min incubations with substrates (including 7.6 ng [^{14}C]kaurene) and cofactors. Using these conditions, the amounts of unlabeled kaurenol, kaurenoic acid, and 7α-OH kaurenoic acid increased, while the level of unlabeled kaurene dropped below the limit of detection (Table 1).

Interestingly, radioisotope monitoring of the HPLC separation of products showed that only a fraction of the [^{14}C]kaurene added to the reaction mixtures was metabolized during the 10 min incubation. Only 14% of the [^{14}C]kaurene added to the reactions was oxidized, with [^{14}C]kaurenol as the major product and [^{14}C]kaurenal as a minor product (data not shown).

Effect of Endogenous Kaurene on the Apparent Kaurene Oxidase Activity

The presence of endogenous kaurene in microsomes significantly alters the appearance of data related to kaurene oxidase activity as measured by oxidation of labeled exogenous substrate. Figure 2 illustrates data from a routine time course experiment. This figure compares curves generated on the assumption that there is no endogenous kaurene in the microsomes (curve represented by open symbols); or the assumption that the endogenous kaurene in the microsomes and added labeled substrate are equally available to the oxidative enzymes (curve represented by closed symbols).

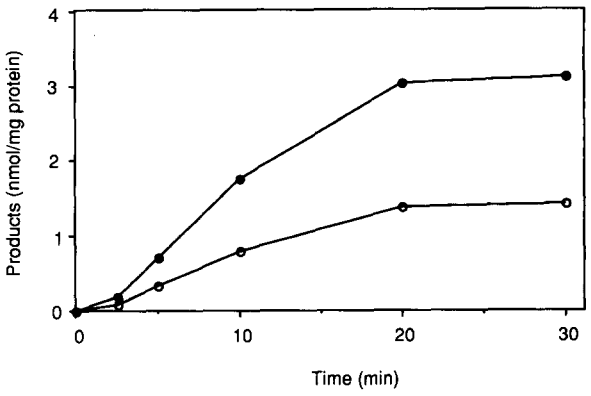


Fig. 2. Reaction time course for the oxidation of radiolabeled, endogenous, and total kaurene. Assays were run with 333 Bq [^{14}C]kaurene and 20 µl P_{150}. Dpm oxidized products recovered from reaction mixtures after indicated incubation times were converted to estimates of picomoles of oxidized products. Data represented by open circles were calculated assuming no endogenous kaurene in microsomes; closed circles represent same data calculating picomoles oxidized products assuming the known quantity of endogenous kaurene in 20 µl of microsomes is equally available to enzyme as added [^{14}C]kaurene.

Effect of Endogenous Kaurene on the Specific Activity of [^{14}C]Kaurene Synthesized from [^{14}C]MVA

Endogenous levels of kaurene in soluble (150,000 g supernatant) extracts of liquid endosperm from developing seeds from two different pumpkins were 46 and 164 ng/ml in S_{150} preparations (Table 2). The S_{10} fractions from the same two batches of seeds contained 405 and 1895 ng ml^{-1}, respectively. Endogenous kaurene present in these soluble enzyme preparations diluted the [^{14}C]kaurene synthesized from [^{14}C]MVA. This result is illustrated by comparison of the specific activities of the [^{14}C]kaurene made using these two different preparations. [^{14}C]Kaurene made using S_{150} had a specific activity 2.6–2.7 times higher than [^{14}C]kaurene synthesized using S_{10} (Table 2). The elevated specific activities of the S_{150} synthesized [^{14}C]kaurene were correlated with lower endogenous kaurene levels in the S_{150} fraction compared to the corresponding S_{10} fraction. An inverse correlation between specific activity and endogenous kaurene levels was found, even though the yield of [^{14}C]kaurene from [^{14}C]MVA was consistently lower in the S_{150} fractions.

The effect of O_2 and NADPH on the biosynthesis of [^{14}C]kaurene in S_{10} extracts was also determined. In the presence of both O_2 and NADPH, less [^{14}C]-kaurene with a lower specific activity accumulated compared to that in reactions run without O_2 and

Table 2. Effect of endogenous kaurene on the biosynthesis of [^{14}C]kaurene from [^{14}C]mevalonic acid.

	S_{10}		S_{150}	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Endogenous kaurene (ng ml)	405	1895	46	164
Yield of [^{14}C]kaurene (%)	13.7	9.0	5.4	5.3
Specific radioactivity of [^{14}C]kaurene (KBq μmol^{-1})	1745	1545	4776	3966

[^{14}C]Kaurene was synthesized from [^{14}C]mevalonic acid using dialyzed S_{10} preparations and S_{150} preparations with significantly different levels of endogenous kaurene.

NADPH (Fig. 3). [^{14}C]Kaurene, [^{14}C]kaurenoic acid, [^{14}C]GA_{12}, [^{14}C]GA_{12}-aldehyde, and [^{14}C]-squalene were all formed in the presence of O_2 and NADPH. However, only [^{14}C]squalene and two unidentified metabolites were made in their absence.

Discussion

The apparent rate of kaurene oxidase activity was affected by the presence of endogenous kaurene. In our reaction mixtures, endogenous kaurene originated from both the radiolabeled kaurene and the microsomal preparations themselves. Quantitation using GC-MS-CI revealed high levels of endogenous kaurene in the microsomes (Table 1), in confirmation of earlier observations (Hedden et al. 1983). In general, the most nonpolar kaurenoids were more abundant in the microsomal preparation. The relatively polar GA_{12} and 7β-hydroxykaurenolide were also found in the P_{150} fraction, but at very low levels (data not shown). The more polar 7α,12α-dihydroxykaurenolide had been detected previously in the high-speed supernatant (Hedden et al. 1983). It seems likely that the greater abundance of the more nonpolar compounds in the microsomal fraction is due to their greater affinity for the lipid membrane. This is also consistent with the observation that the kaurene remains with the protein during dialysis of extracts (unpublished results).

The relatively high levels of endogenous kaurene in the P_{150} fraction was reduced to below detectable levels by a 10 min reaction (Table 1), while only 14% of the added [^{14}C]kaurene was oxidized, indicating preferential oxidation of endogenous substrate. It is unlikely that the low percent oxidation of exogenous [^{14}C]kaurene was due to volatilization, since much greater oxidation can be obtained if the reactions are run for a longer duration (data not shown). The increase in endogenous kaurenoic acid and 7α-OH kaurenoic acid levels shows that reactions subsequent to kaurenol formation also occurred during the incubation. Furthermore, the fact that the total concentration of measured kaurenoids before the reaction was greater than that after the reaction suggests that steps after 7α-OH kaurenoic acid synthesis occurred in these microsomes as well. Changes in the endogenous levels of the kaurenoids during an enzyme assay can potentially influence both substrate competition and product inhibition, thus affecting rates of oxidation of exogenous radiolabeled kaurene.

The net result of the presence of kaurene in the microsomal preparation is a greater actual substrate concentration in an enzyme assay than that contributed by the exogenous substrate alone. In the simplest case, assuming that endogenous and exogenous kaurene are metabolized at equal rates in the lipophilic microsome membranes, total kaurene oxidation can be derived from the rate of exogenous kaurene oxidation and the absolute amount of endogenous and exogenous kaurene. When such calculations are made, estimates of kaurene oxidation reaction rates are adjusted significantly upward. Thus, calculations based on [^{14}C]kaurene oxidation alone would not only underestimate enzyme activity, but would seriously misrepresent the kinetic parameters. For example, calculating total oxidation does not alter the incubation time course of activity, but does result in about a doubling in reaction rates (Fig. 2). The situation is complicated further if differential oxidation of exogenous and endogenous kaurene occurs, which is almost certainly the case. These factors must be considered in enzyme studies based on the metabolism of radioactive substrate.

Another complication by the presence of endogenous kaurene in the cell-free extracts is the isotopic dilution of [^{14}C]kaurene biosynthesized from [^{14}C]MVA. The degree of isotopic dilution is controlled by the amount of endogenous kaurene in the enzyme preparation (Table 2). High endogenous kaurene in the S_{10} fractions significantly reduced the specific activity of [^{14}C]kaurene made with these extracts. Most of the endogenous kaurene was present in the pellet after ultracentrifugation, while the enzymes responsible for the conversion of MVA to kaurene remained in the supernatant. Thus, using the S_{150} fraction to synthesize [^{14}C]-kaurene resulted in products with higher specific activities despite lower yields compared to S_{10}.

The effect of endogenous kaurene on specific activity of [^{14}C]kaurene biosynthesized from [^{14}C]MVA was also observed in reactions run with and without O_2 and NADPH (Fig. 3). O_2 and NADPH are required for the oxidative steps be-

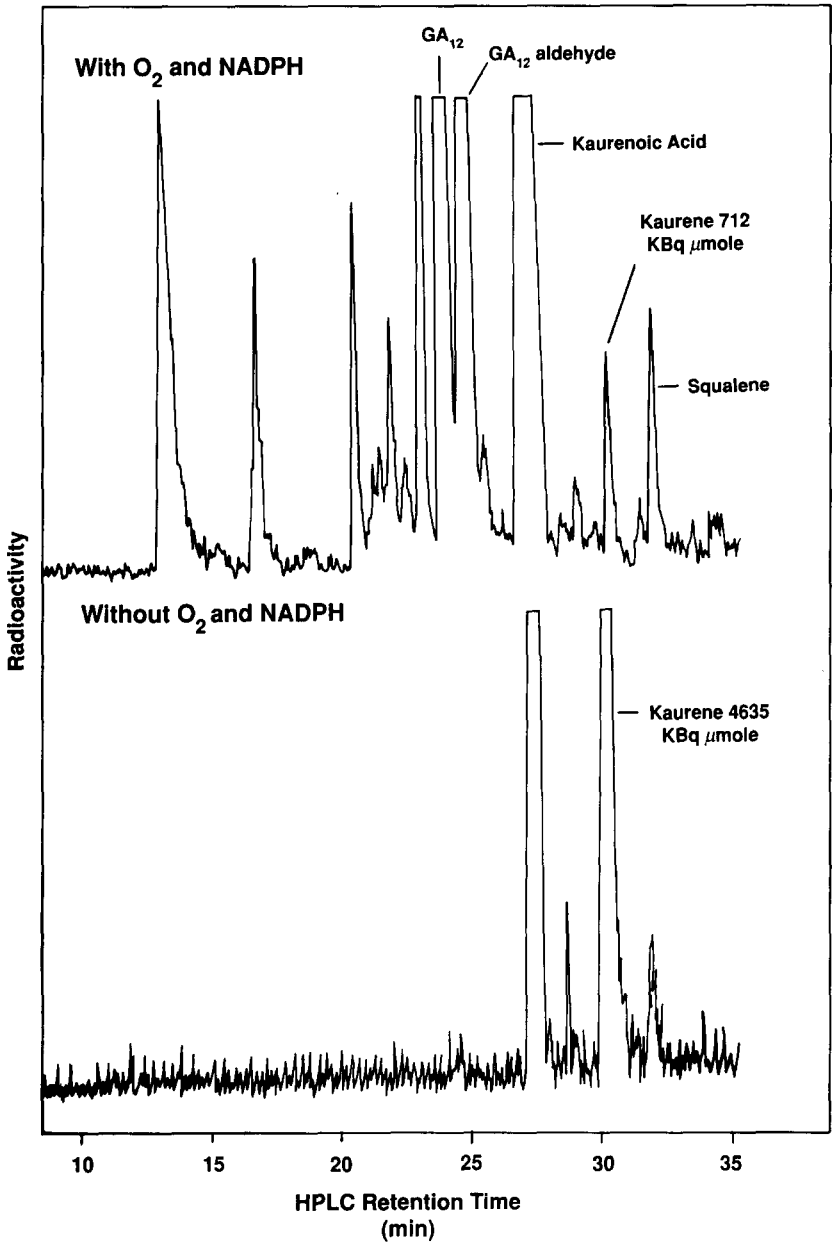


Fig. 3. Effect of O_2 and NADPH on the specific activity of [^{14}C]kaurene biosynthesized from [^{14}C]mevalonic acid. [^{14}C]Kaurene was synthesized from [^{14}C]mevalonic acid using a dialyzed S_{10} homogenate with and without O_2 and NADPH. Both chromatograms are shown in the same scale. The large peak at 27.35 min without O_2 and NADPH was shown by GC-MS not to be [^{14}C]kaurenoic acid.

yond kaurene (Hedden et al. 1983); and in reactions with both present, label was incorporated into several intermediates in the gibberellin biosynthetic pathway. The reduced amount of [^{14}C]kaurene accumulating under these conditions was diluted to a larger degree by endogenous kaurene, resulting in a lower specific activity. In the absence of O_2 and NADPH, more [^{14}C]kaurene accumulated and dilution was less. If all the kaurene in the S_{10} preparation was equally available for metabolism, the ratio of unlabeled to labeled kaurene (i.e. specific activity) would not be affected when oxidation was prevented by the lack of O_2 and NADPH. Since this was not the case, there was presumably more than one pool of endogenous kaurene with different potentials for metabolism. Furthermore, in reactions using exogenous [^{14}C]kaurene, endogenous kaurene is probably more readily oxidized than [^{14}C]kaurene added to a reaction mixture.

Without dilution by endogenous kaurene, the specific activity of synthesized [^{14}C]kaurene would be four times greater than that of the [^{14}C]MVA used in its synthesis, as four MVA molecules are required for each kaurene molecule (Fig. 1). Radiolabeling of gibberellin precursors has been reported to be close to the theoretical maximum by this and other laboratories (Bowen et al. 1972, Hazebroek and Metzger 1990; Hazebroek and Metzger 1991;

Großelindemann et al. 1991, Zeevaart et al. 1993). In the present study, however, a much lower amount of radiolabel was incorporated into kaurene. The cause(s) for the reduction in specific activity is not known. Although in a recent study the high-speed *C. maxima* supernatant was filtered prior to incubation (Zeevaart et al. 1993), this procedure was not necessary in the past to achieve near maximum labeling (Hazebroek and Metzger 1990; Hazebroek and Coolbaugh 1991). It is possible that the enzyme preparations used in the current study contained greater levels of endogenous kaurene than those used in previous studies, resulting in a lower specific activity. Significant variation in the amount of endogenous kaurene was found among our extracts (Table 2). It is also likely that the proportionally large amounts of soluble enzyme preparation added to our synthetic reactions using [^{14}C]MVA resulted in a lower level of incorporation of label into kaurene compared to that in other studies.

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