AN ABSTRACT OF THE THESIS OF

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Title: THE BIOSYNTHESIS AND METABOLISM OF (-)-KAURENE IN CELL-FREE EXTRACTS OF IMMATURE PEA SEEDS

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Thomas C. Moore

Procedures were developed for assaying the biosynthesis of (-)-kaurene-^{14}C and other intermediates in gibberellin (GA) synthesis from mevalonic acid-^{14}C in cell-free enzyme extracts of immature pea (Pisum sativum L. cv. Alaska) seeds. This system was utilized to investigate three aspects of GA biosynthesis: (1) apparent capacities for (-)-kaurene biosynthesis in extracts of pea seeds at various stages of development; (2) localization of the enzymes which catalyze (-)-kaurene biosynthesis in immature pea seeds; and (3) further metabolism of (-)-kaurene in extracts of immature pea seeds.

It was readily demonstrated that mevalonic acid was incorporated into (-)-kaurene and also into squalene (identification tentative) in extracts of immature seeds. The synthesis of (-)-kaurene was shown to be approximately linear with time through 75 minutes at 30°C, to vary directly with enzyme concentration, and to be dependent on ATP. Both

Mg^{2+} and Mn^{2+} stimulated the reaction, but Mn^{2+} was a much better activator than Mg^{2+}. The optimum pH was 7.1.

The capacity to convert mevalonic acid to (-)-kaurene, as measured in cell-free extracts, was found to vary markedly with the stage of seed development. Using enzyme extracts prepared from seeds at different stages of development, it was observed that the activity increased from a very low initial level to a maximum at about 13 days after anthesis, or when the seeds had attained about half-maximum fresh weight.

Comparative assays of (-)-kaurene biosynthesis and accumulation in cell-free extracts of excised seed coats, cotyledons and embryonic shoot-root axes revealed that the enzymes responsible for (-)-kaurene biosynthesis apparently are localized exclusively in the cotyledons. The rates of (-)-kaurene biosynthesis in extracts of isolated cotyledons were higher than those observed in extracts of whole seeds. The enzymes responsible for the synthesis of squalene were present in both the seed coats and the cotyledons.

The enzymes catalyzing the synthesis of (-)-kaurene from mevalonic acid were present in the soluble fraction of these extracts, whereas one preliminary experiment indicated that the enzymes catalyzing (-)-kaurene oxidation were localized in a microsomal fraction. The rates of (-)-kaurene synthesis and accumulation were greater in the 100,000 x g supernatant fraction than in the 40,000 x g supernatant fraction of extracts of excised cotyledons, whereas the opposite relationship was observed with extracts of whole seeds.

Repeated efforts to demonstrate cell-free metabolism of exogenous (-)-kaurene resulted in uniformly negative results. However, when (-)-kaurene was formed in situ from mevalonic acid in cell-free mixtures containing 10, 000 x g supernatant from cotyledon extracts, its oxidation was observed. The metabolism of (-)-kaurene formed in situ was enzymic, or at least heat labile, and was markedly sensitive to inhibition by carbon monoxide. The difference in (-)-kaurene accumulation between carbon monoxide-inhibited preparations and non-inhibited preparations was greater in extracts which were prepared by homogenizing the tissues in the presence of insoluble polyvinylpyrrolidone (PVP) than in extracts prepared without PVP. Although these results are not considered to provide direct evidence, they are interpreted to mean that (-)-kaurene metabolism in these extracts is protected from inhibition by insoluble PVP.

The inability of the enzyme extracts to metabolize exogenous (-)-kaurene remains incompletely understood. Two possible reasons for this failure are: (a) binding of the (-)-kaurene to non-catalytic protein; and (b) a possible requirement in this system for some intimate functional association between the enzyme systems catalyzing (-)-kaurene biosynthesis and the enzyme systems catalyzing (-)-kaurene oxidation. Particular significance is ascribed to the latter possibility on the basis of limited indirect evidence.

The products formed from mevalonic acid in the experiments designed to investigate the metabolism of (-)-kaurene formed in situ

were isolated and tentatively identified as geranylgeraniol, (-)-kaurenol, (-)-kaurenal, and (-)-kaurenoic acid. The identifity of (-)-kaurenol was confirmed by co-crystallization of the radioactive product with authentic kaurenol to constant specific radioactivity. While (-)-kaurenol, (-)-kaurenal, and (-)-kaurenoic acid were synthesized from mevalonic acid-^{14}C as the exogenous substrate, it is assumed, of course, that (-)-kaurene was a direct precursor of all three products.

The Biosynthesis and Metabolism of (-)-Kaurene in Cell-free Extracts of Immature Pea Seeds

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Ronald Charles Coolbaugh

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APPROVED:

Redacted for Privacy

Associate Professor of Botany
in charge of major

Redacted for Privacy

Head of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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Typed by Muriel Davis for Ronald Charles Coolbaugh

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THE BIOSYNTHESIS AND METABOLISM OF (-)-KAURENE IN CELL-FREE EXTRACTS OF IMMATURE PEA SEEDS

GENERAL INTRODUCTION

Gibberellin Biosynthesis

The gibberellins (GA's) are a group of plant hormones which possess extremely powerful growth regulatory activity. Chemically they are tetracyclic diterpenoid compounds. Gibberellins were first isolated from culture filtrates of the ascomycete fungus Gibberella fujikuroi (Saw.) Wr. (Fusarium moniliforme Sheld., the asexual or imperfect stage), and have now been isolated from many diverse kinds of plants, including bacteria, fungi, algae, ferns, gymnosperms and angiosperms. A total of 28 GA's have been identified and characterized. Among the most important recent reviews of various aspects of the chemistry, biochemistry and physiology of the GA's are those by Cross (1968), West et al. (1968), and Lang (1970).

Control of the biosynthesis and metabolism of GA's in vivo in seed plants, by manipulation of physical environmental factors or treatment with growth regulating chemicals, potentially is a valuable practice in efforts to control the growth and development of economically and esthetically valuable species of seed plants. Of course, detailed knowledge about the pathway of GA biosynthesis and the

natural controls operating on this pathway is prerequisite to enlightened efforts to control growth and development.

The first studies on the biosynthesis of GA's, which is the subject of this dissertation, were done with the fungus Gibberella fujikuroi. Cross et al. initially proposed the basic isoprenoid structure of GA's in 1956, and shortly thereafter Birch, Rickards and Smith (1958) confirmed this hypothesis by showing that both mevalonic acid-2-^{14}C (MVA)[1/] and acetate-1-^{14}C could be incorporated into GA's in this organism. The pathway for gibberellin biosynthesis from MVA, as presently proposed on the basis of all available evidence, is illustrated in Figure 1. The intermediary role of kaurene[2/] was first demonstrated by Cross et al. (1964) when they demonstrated that kaurene-17-^{14}C was incorporated directly into gibberellic acid (GA_3) by the fungus. Similar results were obtained subsequently for kaurenol (Graebe et al., 1965), kaurenal (Dennis and West, 1967), kaurenoic acid and 7-β-OH-kaurenoic acid (West et al., 1968). Geranylgeranyl pyrophosphate and copalyl pyrophosphate have been shown

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[1/] Mevalonic acid-2-^{14}C is abbreviated as MVA throughout this dissertation.

[2/] The trivial names kaurene, kaurenol, kaurenal, kaurenoic acid, and hydroxy-kaurenoic acid are used in place of the actual chemical designations (-)-kaur-16-ene, (-)-kaur-16-en-19-ol, (-)-kaur-16-en-19-aldehyde, (-)-kaur-16-en-19-oic acid, and (-)-7β-OH-kaur-16-en-19-oic acid. With the two exceptions of natural products of the fungus or of higher plants and the authentic reference samples, all references to kaurene and its derivatives refer to the ^{14}C-labelled compounds.

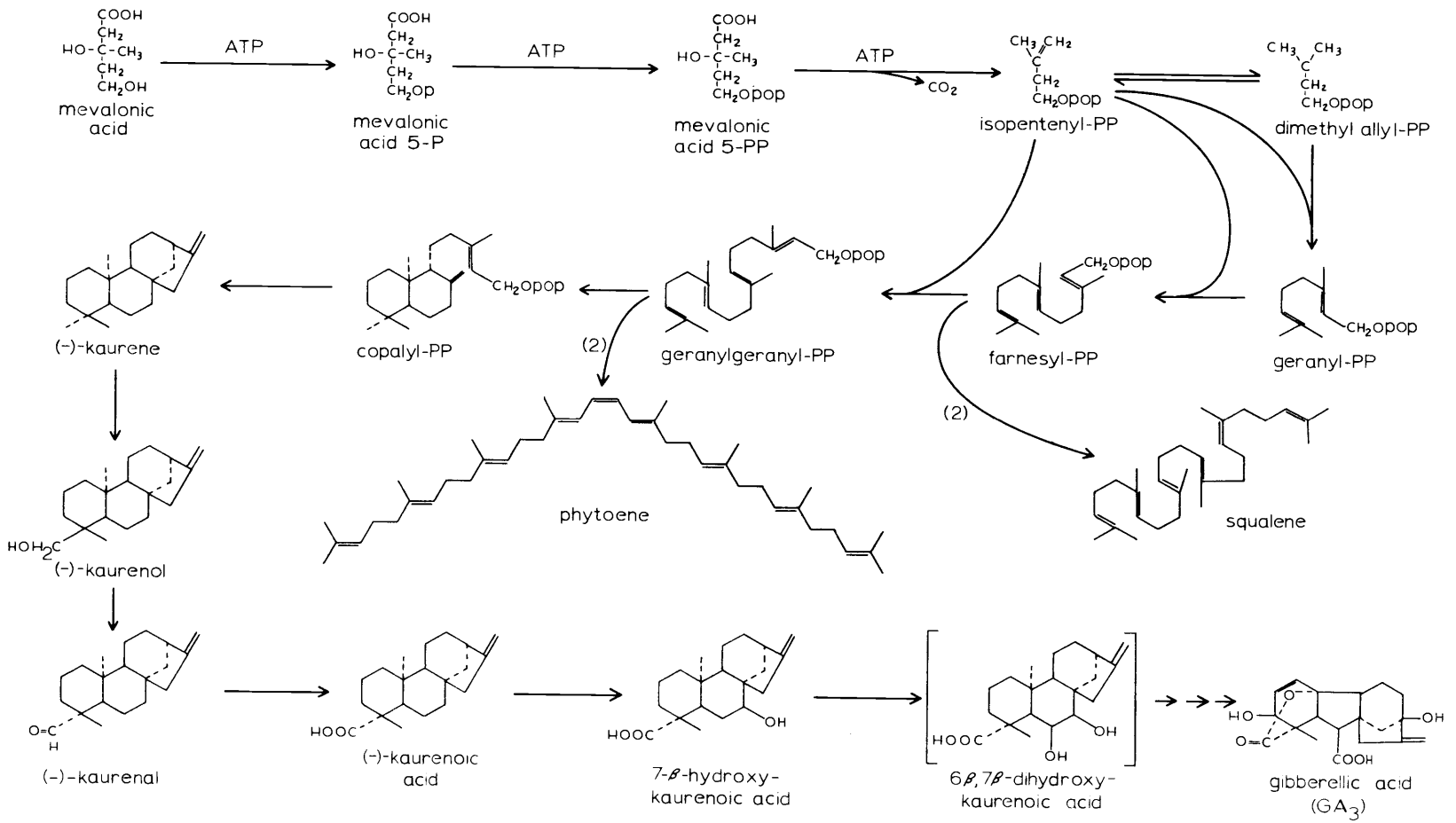

Figure 1. Pathway of gibberellin biosynthesis.

to be intermediates in this pathway, since both compounds can be readily converted to kaurene by extracts of Gibberella fujikuroi, Echinocystis macrocarpa and Ricinus communis (Upper and West, 1967; Shechter and West, 1969; Robinson and West, 1970b).

Kaurene, kaurenol, kaurenoic acid, hydroxy-kaurenoic acid, and dihydroxy-kaurenoic acid have all been shown to be natural products of the fungus (Cross et al., 1963; Cavell and MacMillan, 1967; Hanson and White, 1969; Cross, Stewart and Stoddart, 1970). Furthermore, all of these compounds have been shown to possess biological activity in higher plant tissues (Katsumi et al., 1964; Jones, 1967; West et al., 1968; Cross, Stewart and Stoddart, 1970). Cross, Stewart and Stoddart (1970) have recently shown 6β, 7β-dihydroxy-kaurenoic acid to be a natural product of the fungus Gibberella fujikuroi, but they were unable to observe its incorporation into gibberellic acid in cultures of the fungus. They have postulated that a derivative of the dihydroxy-acid, possibly a 6-pyrophosphate, is a precursor of gibberellins in this fungus.

Although all these compounds are thought to be intermediates in GA biosynthesis in higher plants, as well as in the fungus, no higher plant tissue or extract has ever been reported to convert any of these intermediates to a compound which could be positively identified as a GA. However, the intermediary role of these compounds in this pathway in higher plants is supported by four lines of evidence:

(1) The isolation of kaurene, kaurenol, and kaurenoic acid from higher plant tissues (Appleton, McCrindle and Overton, 1968; Sitton, Richmond and Vaadia, 1967; Hendrick and Jefferies, 1964;

Krishnaswami et al., 1969; Mihashi et al., 1969).

(2) The GA-like activity of these compounds in some higher plant tissues (Katsumi et al., 1964; Murakami, 1968; Petridis, Verbeek and Massart, 1966; Jones, 1968).

(3) The development of a cell-free enzyme system from the endosperm-nucellus of immature seeds of wild cucumber (Echinocystis macrocarpa Greene) (a tissue which is known to contain large quantities of known GA's) which will incorporate MVA into kaurene, kaurenol, kaurenal, kaurenoic acid, and hydroxy-kaurenoic acid in sequence and irreversibly (see West et al., 1968).

(4) The development of a cell-free enzyme system from Brassica oleracea leaves which incorporates kaurenoic acid-^{14}C into a compound that behaves chromatographically like a GA in two solvent systems (Stoddart, 1969).

Substantial progress toward the development of cell-free systems from higher plant tissues capable of GA biosynthesis was first made when Graebe et al. (1965) reported on the incorporation of MVA into kaurene, kaurenol, and geranylgeraniol in cell-free extracts of endosperm-nucellus of immature seeds of Echinocystis macrocarpa Greene. Dennis and West (1967) subsequently showed that this system also produces kaurenal, kaurenoic acid, and some other acids. One of the other acids later was identified as hydroxy-kaurenoic acid (West et al., 1968). In this system, these products

can be formed from MVA or any of the earlier intermediates. Anderson and Moore (1967) described the incorporation of MVA into kaurene in cell-free extracts of immature pea seeds, and more recently, Graebe (1968) demonstrated the synthesis of kaurene, in addition to squalene and phytoene, in cell-free preparations from immature pea seeds and pea fruits. Graebe (1969) has also reported on the synthesis of kaurene from MVA by cell-free enzyme extracts of immature seeds of Cucurbita pepo.

Some progress has also been made recently toward the development of cell-free systems from tissues other than immature seeds which are capable of GA biosynthesis. Robinson and West (1967, 1970a, 1970b) have described a cell-free enzyme system from germinating castor bean (Ricinus communis L.) seeds which incorporates MVA into kaurene as well as four other diterpene hydrocarbons. And, as noted previously, Stoddart (1969) reported on a cell-free enzyme extract from Brassica leaf chloroplasts which converts kaurenoic acid into a compound similar to a GA.

Localization of Enzyme Systems Catalyzing Gibberellin Biosynthesis

Evidence for the localization of enzyme systems catalyzing gibberellin biosynthesis is limited largely to data on the content of extractable GA-like substances of various tissues and data obtained

from cell-free enzyme systems capable of some of the reactions involved in GA biosynthesis. Both of these types of evidence are of limited significance and subject to variable interpretation. Of course, GA's are translocatable and are not metabolically inert, hence the presence of a certain quantity of GA-like material in an extract of a particular tissue may be falsely indicative of the amount of GA synthesis, if any, in that tissue. Neither does incorporation of MVA into kaurene or kaurenoic acid in a cell-free enzyme extract necessarily mean that these intermediates are converted to GA's in the tissue. Only by a combination of several approahces can the biosynthesis and metabolism of GA's in vivo be fully understood.

The indirect evidence for GA biosynthesis in immature seeds is becoming overwhelming. Baldev, Lang and Agatep (1965) showed that pea seeds developing in excised pods in culture increased in content of GA-like materials. This increase in content of extractable GA-like materials was shown to be quite sensitive to a very specific inhibitor of GA biosynthesis, AMO-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride). AMO-1618 inhibits specifically the cyclization of geranylgeranyl pyrophosphate to copalyl pyrosphosphate (Shechter and West, 1969). The development of cell-free enzyme systems from immature pea seeds and fruits and from Echinocystis macrocarpa seeds capable of incorporating MVA into kaurene, and in the latter case into some

oxidized products of kaurene, supports the hypothesis that GA biosynthesis occurs in these immature seeds and fruits. Developing seeds and fruits of several species contain approximately 100 times as much extractable GA-like material as other parts of the same plants on a per fresh weight or per plant basis (Radley, 1958; and cf. Anderson, 1968 and Baldev, Lang and Agatep, 1965; Corcoran and Phinney, 1962).

Localization of GA biosynthesis in a specific tissue or organ is difficult. The data of Graebe (1968) indicate that gibberellin biosynthesis potentially occurs in the pea pod. However, very little GA-like material was observed in extracts of the pods or fruit walls of Lupinus succulentus and Echinocystis macrocarpa, respectively (Corcoran and Phinney, 1962). And it has been observed that the fruit usually attains its maximum size some time before the maximum content of extractable GA-like material occurs. It is possible that, in the case of legumes, GA biosynthesis occurs independently in the pod and ovules. Graebe et al. (1965) localized the enzymes catalyzing kaurene synthesis in the endosperm-nucellus tissue of immature Echinocystis macrocarpa seeds.

The localization of enzymes responsible for kaurene biosynthesis in immature pea seeds has not been determined. Radley (1958) reported that the seed coat and cotyledons of immature runner bean seeds contain approximately equal concentrations of extractable

GA-like materials, while the embryo axis contains somewhat less. However, Corcoran and Phinney have reported extracting nearly 100 times more GA-like material from immature cotyledons than from seed coats at the same stage of development in Lupinus succulentus (1962).

The most direct evidence for the biosynthesis of GA's in vegetative plant parts was presented by Jones and Phillips (1966) when they demonstrated that more GA-like material diffused out of sunflower root and shoot apices than could be extracted. Furthermore, extraction of the plant material after the diffusion period yielded amounts of GA-like substances which were nearly equal to amounts obtained from similar plants before diffusion. The presence of GA-like materials in root exudates also was reported by Skene (1967), Carr, Reid, and Skene (1964), Phillips and Jones (1964) and Sitton, Richmond and Vaadia (1967). Sitton, Richmond and Vaadia (1967) demonstrated that the concentrations of GA-like materials in root exudates increased on the third day after excision, and also were able to show that MVA-^{14}C was incorporated into a compound tentatively identified as kaurenol in excised apical sections of sunflower roots in vitro.

The development of a cell-free enzyme system from isolated Brassica leaf chloroplasts which incorporates kaurenoic acid into a compound which behaves chromatographically like a GA (Stoddart,

1969) also supports the supposition that GA biosynthesis occurs in stem apices.

The intracellular localization of GA biosynthesis also is a subject of interest and importance. West and his associates have shown conclusively that kaurene biosynthesis from MVA-^{14}C in extracts of E. macrocarpa occurs in the soluble fraction of that tissue (Upper and West, 1967; Oster and West, 1968), whereas the oxidations which convert kaurene to hydroxy-kaurenoic acid occur in the microsomal fraction of this endosperm tissue (Dennis and West, 1967). Anderson and Moore (1967) reported on the incorporation of MVA into kaurene in the 40, 000 x g supernatant of immature pea seed homogenates, and Graebe (1968) measured significant kaurene synthesis activity in the 100, 000 x g supernatant of immature pea seed and pea fruit homogenates. The data of Stoddart (1969) would indicate that at least a part of the leaf gibberellins are synthesized in the chloroplasts of mesophyll chlorenchyma.

Purpose of the Study

Although there are several systems now available from higher plant tissues which are capable of incorporating MVA into kaurene, the major work in this area has been done with the liquid endosperm tissue of Echinocystis macrocarpa Greene. In fact, the only system reported to be capable of metabolizing kaurene is the system from

E. macrocarpa. It should be noted that the E. macrocarpa material routinely utilized by West and his associates is endosperm-nucellus from immature seeds, and that this material is predominantly liquid, free-nuclear endosperm. At the present time, the Echinocystis system is incapable of converting kaurene to a GA (West et al., 1968). Developing seeds of a number of plants have been shown to be a rich source of GA's, and there has been much interest in the relation between GA content and the growth of seeds and fruits (Baldev, Lang and Agatep, 1965; Corcoran and Phinney, 1962; Murakami, 1961; Ogawa, 1963) as well as the possible role of GA's stored in seeds in the subsequent growth of seedlings developing from them (Barendse et al., 1968; Moore, 1967).

This study was undertaken to develop a cell-free enzyme system from immature pea seeds which will incorporate MVA into kaurene, and perhaps other intermediates in GA biosynthesis, and to use this system for further investigating three rather distinct but related aspects of gibberellin biosynthesis:

(1) The apparent capacities for kaurene biosynthesis in extracts of immature pea seeds at various stages of development.

(2) The localization of the enzyme systems which catalyze kaurene biosynthesis in immature pea seeds.

(3) The further metabolism of kaurene in extracts of immature pea seeds.

These three topics are discussed in the following three sections, with the characterization of the cell-free enzyme system for kaurene biosynthesis included in the first section.

KAURENE BIOSYNTHESIS IN CELL-FREE EXTRACTS OF PEA SEEDS AT DIFFERENT STAGES OF DEVELOPMENT

Introduction

The first substantial progress toward the development of cell-free enzyme systems from higher plant tissues capable of GA biosynthesis was made when Graebe et al. (1965), working in the laboratory of Dr. Charles A. West at the University of California at Los Angeles, reported on the incorporation of MVA-^{14}C into geranylgeraniol, kaurene, and kaurenol in extracts of the endosperm-nucellus of immature seeds of wild cucumber (Echinocystis macrocarpa Greene). Subsequently, West and his associates have studied rather thoroughly the biosynthesis and metabolism of kaurene in this system. Oster and West (1968) reported on the isolation and purification of geranylgeranyl pyrophosphate and its conversion to kaurene. Upper and West (1967) characterized the reactions from geranylgeranyl pyrophosphate to kaurene, and Dennis and West (1967) showed further that kaurenol, kaurenal and kaurenoic acid were formed irreversibly and in sequence from kaurene in the Echinocystis system. West et al. (1968) reported the identification of hydroxy-kaurenoic acid as a product of kaurenoic acid metabolism. Shechter and West (1969) demonstrated that copalyl pyrophosphate can be converted to kaurene

in extracts of E. macrocarpa, as well as in extracts of germinating Ricinus communis seeds and in extracts of the fungus Gibberella fujikuroi. And, Murphy and West (1969) characterized more thoroughly some of the oxidative reactions involved in the metabolism of kaurene in cell-free extracts of Echinocystis endosperm-nucellus.

Several other cell-free systems from higher plant tissues which are capable of kaurene biosynthesis have been reported. Anderson and Moore (1967) reported on cell-free biosynthesis of kaurene in extracts of immature pea (Pisum sativum L.) seeds. Graebe (1969) described a cell-free system from immature seeds of Cucurbita pepo which will incoporate MVA into kaurene in a yield of about 40% of the active isomer. This yield is comparable to that obtained by extracts of E. macrocarpa. Kaurene has been reported to be one of five very similar diterpenoid products of MVA metabolism in extracts of germinating seeds of Ricinus communis (Robinson and West, 1967, 1970a, 1970b).

In view of the fact that the cell-free enzyme system from immature pea seeds is the only system yet described from a cellular tissue[3/] which produces kaurene as the only diterpenoid hydrocarbon

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[3/] According to Hayward (1948, p. 368-369), the pea (Pisum sativum L.) embryo occupies a small part of the embryo sac during early embryogenesis, the remainder being filled with liquid (free-nuclear) endosperm. However, by the time the seed is 11-12 days old, the endosperm has been entirely digested, and the seed consists exclusively of the embryo and the seed coat. In these investigations pea seeds of 10-18 days of age were utilized.

metabolite of MVA, this system was chosen for the present study. Developing pea seeds are known to contain relatively very large concentrations of gibberellin-like materials (Baldev, Lang and Agatep, 1965), and highly uniform plants can be grown in the greenhouse. The Alaska variety of Pisum sativum was chosen for these studies because of the short time (approximately 30 days) required for flowering and because a large amount of physiological research has been done on the gibberellin relations in this variety (e.g. Moore, 1967; Ecklund and Moore, 1968).

This section describes the optimum assay conditions for a cell-free enzyme system prepared from immature Alaska pea seeds which incorporates MVA-^{14}C into kaurene-^{14}C, and the apparent changes in the rate of kaurene biosynthesis during the development of these seeds. A brief report on these investigations has been published previously (Coolbaugh and Moore, 1969).

Methods and Materials

Source and Purity of Reagents

Mevalonic acid-2-^{14}C lactone (sp. act. 5.02 mc/mmole) in benzene solution was purchased from CalBiochem. The benzene was removed under a stream of nitrogen and the lactone was hydrolyzed by treating overnight with 100% excess NaOH equivalents, after which

the MVA was diluted with glass re-distilled water to a concentration of approximately 0.01 μc/μl. Adenosine triphosphate (ATP) was purchased from Sigma Chemical Company. AMO-1618 was purchased from Enomoto Company, Redwood City, California. Samples of (-)-kaurene were generously supplied by Dr. L.H. Briggs, University of Aucklund, New Zealand, and Dr. Charles A. West, University of California, Los Angeles, California. Squalene was purchased from Nutritional Biochemical Corporation, and geraniol and farnesol from International Chemical and Nuclear Corporation. Polyvinylpyrrolidone (PVP) (insoluble Polyclar-AT) was purchased from General Aniline Film Corporation, Graselli, New Jersey. Constituents of the liquid scintillation counting solutions, 2,5-diphenyloxazole (PPO), p-bis-2-(5'phenyloxazolyl)-benzene (POPOP) and triphenyl-p-terphenyl were purchased from Packard Corporation. Toluene-^{14}C was purchased from Packard Corporation. Silica Gel G was purchased from Brinkmann Instruments Company. All other chemicals were reagent grade, and all organic solvents were redistilled before use.

Culture of Plants

Alaska peas (Pisum sativum L.; W. Atlee Burpee Company, Riverside, California) were grown in a greenhouse where the light and temperature regime consisted of a 16-hour photoperiod, at

approximately 20-27°C and a light intensity of 800-1000 ft-c, and an 8-hour nyctoperiod at approximately 16-18°C. The natural light intensity was supplemented with and the photoperiod extended by Gro-Lux fluorescent lamps. Seeds were planted in water-saturated vermiculite in plastic pots. The plants were irrigated alternately with water and complete nutrient solution throughout the periods of culture. After 8-10 days, the seedlings in each pot were thinned to leave 10 quite uniform plants. The fruits were harvested and the seeds taken from the pods between the 10th and the 18th days after flowering.

Preparation of Enzyme Extract

Freshly harvested pea seeds were homogenized in cold 0.1 M phosphate buffer (1 g fresh wt/ml buffer), pH 7.1, containing 50 μg/ml each of penicillin G and streptomycin sulfate, using a pre-chilled mortar and pestle. Homogenates were centrifuged at 40,000 x g for 15 minutes and the resulting supernatant was used as the enzyme source. Fresh enzyme extracts were used, except in the indicated experiments in which an enzyme extract was frozen in liquid nitrogen and stored at -70°C for up to two months prior to use.

Reaction Conditions and Product Isolation

A complete reaction mixture routinely contained 0.05 μmole of

MVA-2-^{14}C, 0.75 ml enzyme extract (representing the enzyme extracted from about 3.5 seeds, or approximately 2.75 mg of a micro-Kjeldahl determined nitrogen), 0.5 μmoles of ATP, 3 μmoles of $MnCl_2$, and 0.6 ml of 0.1 M potassium phosphate buffer at pH 7.1 in a total of 1.6 ml. Reaction mixtures were incubated for 75 minutes at 30°C. The reactions were stopped by adding 3 ml of acetone, and the reaction mixtures were extracted twice with 1-ml portions of benzene. The combined organic extracts were then evaporated under reduced pressure using a rotary-film evaporator. Each residue was extracted three times with 0.2 ml of acetone, and the entire 0.6 ml of extract was applied to a 5 x 20 cm glass plate coated with a 250μ-layer of silica gel G. Thin-layer chromatograms were developed routinely in hexane. After the solvent front had advanced 15 cm from the origin, the plates were removed from the solvent and scanned to locate the radioactive products.

Product Identification

Identification of (-)-kaurene. The ^{14}C-product which was suspected to be kaurene from preliminary thin-layer chromatography was co-chromatographed on thin-layer plates with authentic kaurene in four solvent systems. The four solvent systems used were: (1) pure hexane; (2) hexane/methanol (99/1); (3) petroleum ether (b.p. 30-60°C)/benzene (96/4); and (4) hexane/benzene (7/3). The first

and third solvent systems were used with silica gel G plates which had been activated in the oven at 100°C for one hour. The second and fourth solvent systems were used on silica gel G plates impregnated with silver nitrate. The silver nitrate-impregnated plates were prepared by slurrying 30 g of dry gel with 60 ml of 3% or 10% (w/w) $AgNO_3$ solution. The radioactive substances were located by scanning, and the samples of authentic compounds by charring. Indirect evidence for the presence of kaurene as a product of these reactions was obtained by incubating typical reaction mixtures with different quantities of the inhibitor AMO-1618. In these experiments, the AMO-1618 was dissolved in 0.1 M potassium phosphate buffer (pH 7.1), and 0.1 ml of the inhibitor solution was added to each reaction mixture in place of 0.1 ml of buffer.

A third method for the identification of kaurene as a product of these reactions was the formation of the hydration product of kaurene, kauranol. Authentic kaurene and presumptive kaurene from reaction mixtures were subjected separately to hydration by the method of Dennis and West (1967). Kaurene was reacted for two days in 3 N hydrochloric acid in a water-methanol solution (4/1) at room temperature. After the reactions were stopped, the products were extracted from both reaction mixtures with acetone and benzene and co-chromatographed in hexane on two thin-layer chromatograms, with the solvent front advancing 15 cm. The plates were then

rechromatographed in either hexane/propanol (98/2) or benzene/ethyl acetate (9/1) with the solvent front advancing 10 cm. The radioactive substances were located by scanning, and the authentic products were located by spraying the plate with 5% sulfuric acid in ethanol and then heating for 10 minutes at 100°C.

Tentative identification of squalene. Presumptive squalene-^{14}C was co-chromatographed in four solvent systems with authentic squalene. The solvent systems used in these experiments were: (1) hexane; (2) petroleum ether (b. p. 30-60°C)/benzene, (96/4); (3) dichloromethane/acetone, (99/1); and (4) benzene/ethyl acetate, (9/1). The radioactive products were located by scanning, and the authentic squalene was located by charring, as described above.

Radioassay Procedures

Thin-layer chromatograms were scanned to detect radioactive compounds with a Packard Radiochromatogram Scanner, Model 7201. The appropriate bands of silica gel were removed from the chromatograms and placed in liquid scintillation vials each of which contained 10 ml of counting solution. The liquid scintillation fluid routinely used contained 0.3 mg of p-bis-2'-(5'-phenyloxazolyl)-benzene and 30 mg of triphenyl-p-terphenyl per 10 ml of toluene. The radioactivity measurements were made with a Packard Tricarb Liquid Scintillation Spectrometer, Model 3375. The liquid scintillation

data are expressed in disintegrations per minute (dpm); the counting efficiency was approximately 85%.

Nitrogen Determinations

The total nitrogen content of two 0.5-ml aliquots of each enzyme extract was measured by a micro-Kjeldahl procedure (Horwitz, 1965).

Results

Identification of Products

After isolation of the products and chromatography in pure hexane, the radiochromatograms showed three distinct peaks. The radioactive product at R_f 0.85 was suspected to be kaurene, the product at R_f 0.35 was suspected to be squalene, and the radioactivity at the origin represented several polar compounds. The silica gel containing each of these zones of radioactive products was scraped from the glass plates, and the products were eluted with acetone and saved for identification.

Although kaurene is the only diterpene hydrocarbon which has been shown to be synthesized from MVA in cell-free systems from three different varieties of pea seeds, and the appearance of radiochromatogram scans of products obtained from these reactions appeared identical to those reported by Anderson and Moore (1967),

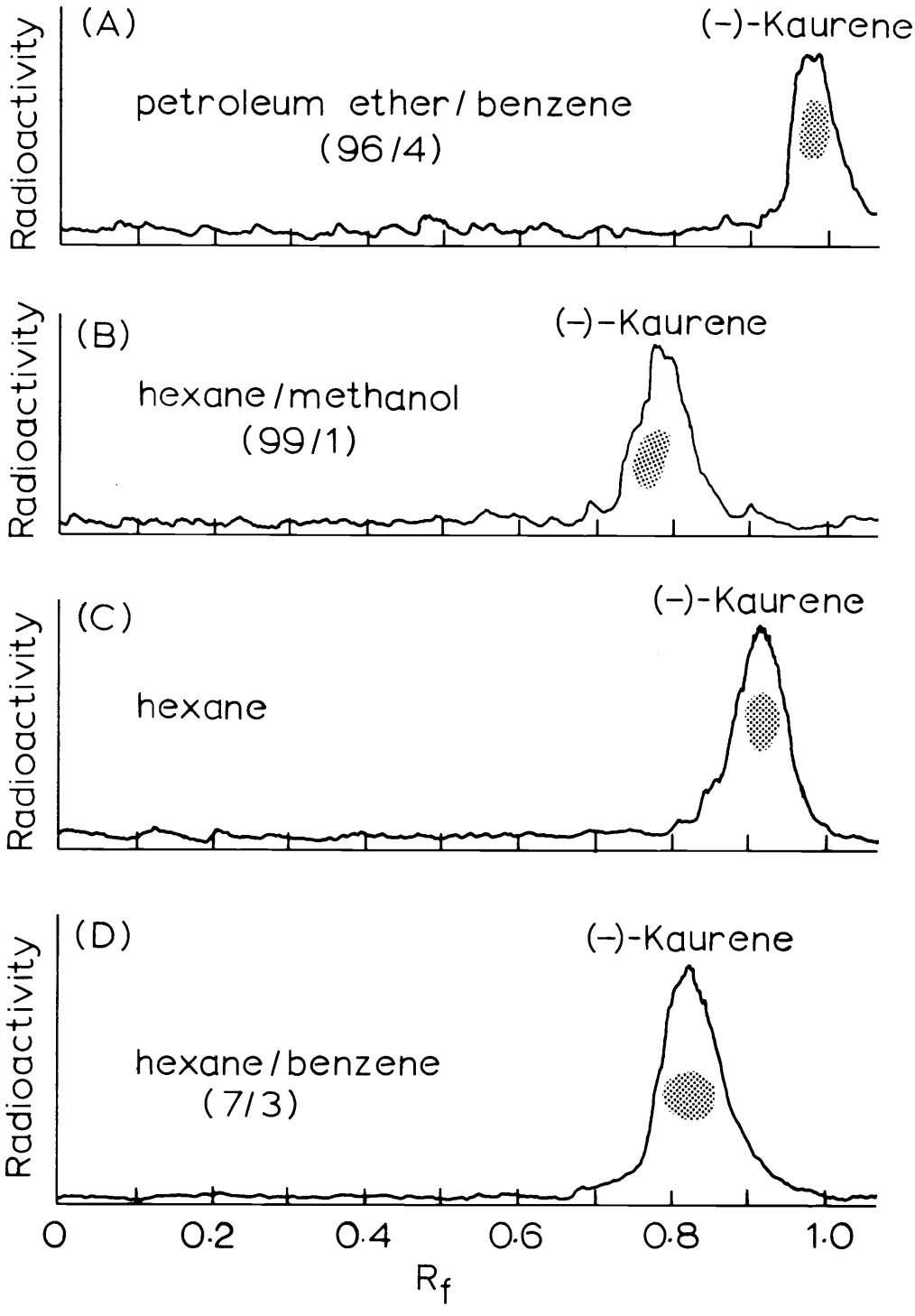
the identity of the presumptive kaurene from this system was confirmed by three methods. Presumptive kaurene-^{14}C was co-chromatographed with authentic kaurene in four solvent systems. In each case, the radioactive material migrated to the same R_f as the authentic kaurene (Figure 2).

The effects of a wide range of concentrations of AMO-1618 on the yield of presumptive kaurene-^{14}C in cell-free extracts were investigated. As expected, one μg/ml AMO-1618 nearly completely inhibited the formation of kaurene (Figure 3).

When both authentic kaurene and the presumptive kaurene were hydrated and then co-chromatographed on silica gel G plates in two solvent systems, the radioactive product again exhibited identical mobility to one of the products from the authentic sample (Figure 4). There was an additional product in the extract from authentic kaurene, but this was presumed to be a contaminant of that sample. These three lines of evidence in combination with the evidence from the other two cell-free systems from immature pea seeds which have been described--gas chromatography and the formation of another derivative (Anderson and Moore, 1967) and co-crystallization (Graebe, 1968)--are considered conclusive evidence for the identification of kaurene as a product of MVA metabolism in this enzyme system.

The product from these reactions which migrated to R_f 0.35 in hexane is thought to be squalene-^{14}C. Squalene has been identified

Figure 2. Strip chart tracings of radiochromatograms of presumptive kaurene-^{14}C which was co-chromatographed with authentic, non-radioactive kaurene on silica gel G in four solvent systems. The thin-layer plates utilized in B and D were developed with 10% and 3% solutions of $AgNO_3$, respectively, and dried prior to the application of samples.


(A)
(-)-Kaurene
petroleum ether/benzene
(96/4)
Radioactivity
(B)
(-)-Kaurene
hexane/methanol
(99/1)
Radioactivity
(C)
(-)-Kaurene
hexane
Radioactivity
(D)
(-)-Kaurene
hexane/benzene
(7/3)
Radioactivity
0
0.2
0.4
0.6
0.8
1.0
R_f

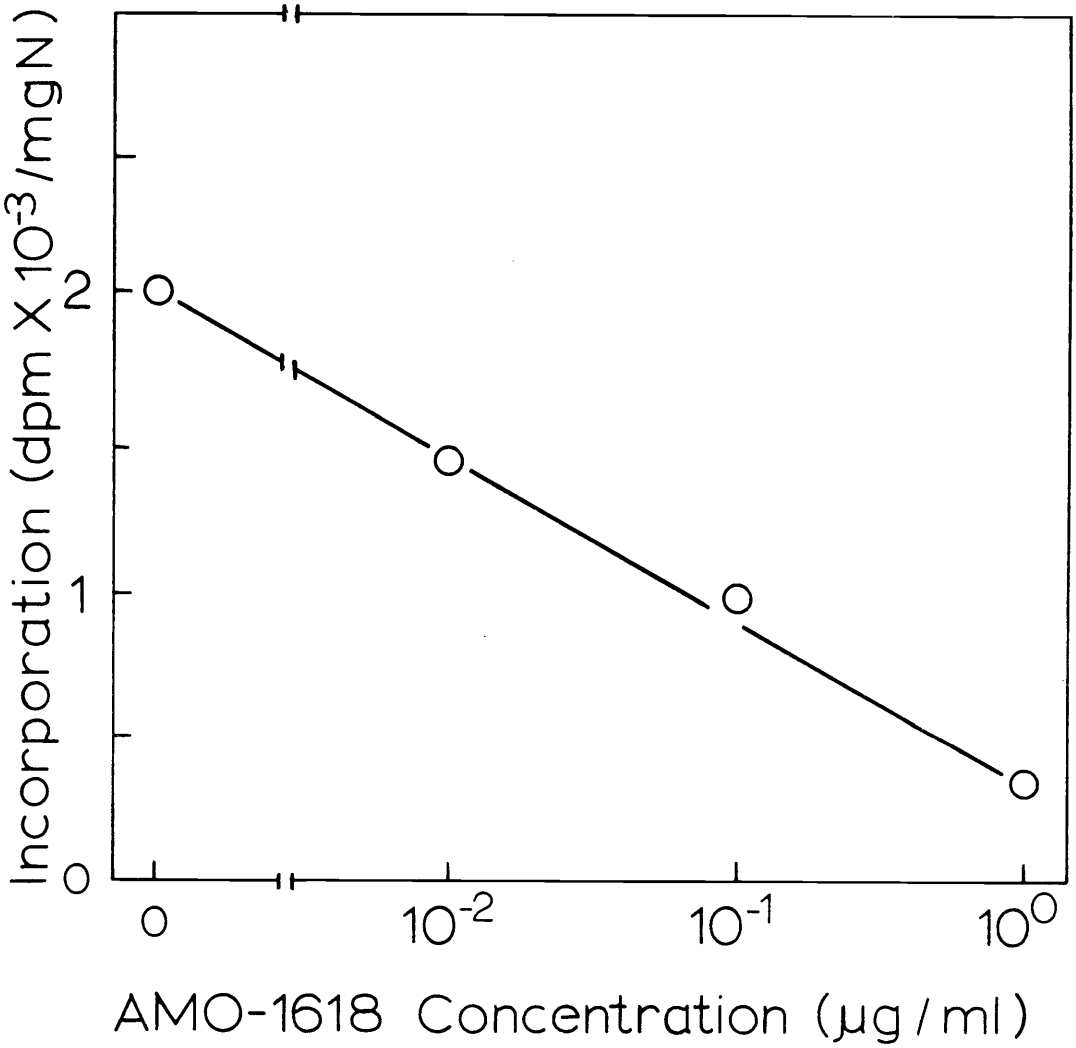


Figure 3. The effect of various concentrations of AMO-1618 on the incorporation of mevalonic acid-2-^{14}C into kaurene in cell-free extracts of immature pea seeds.

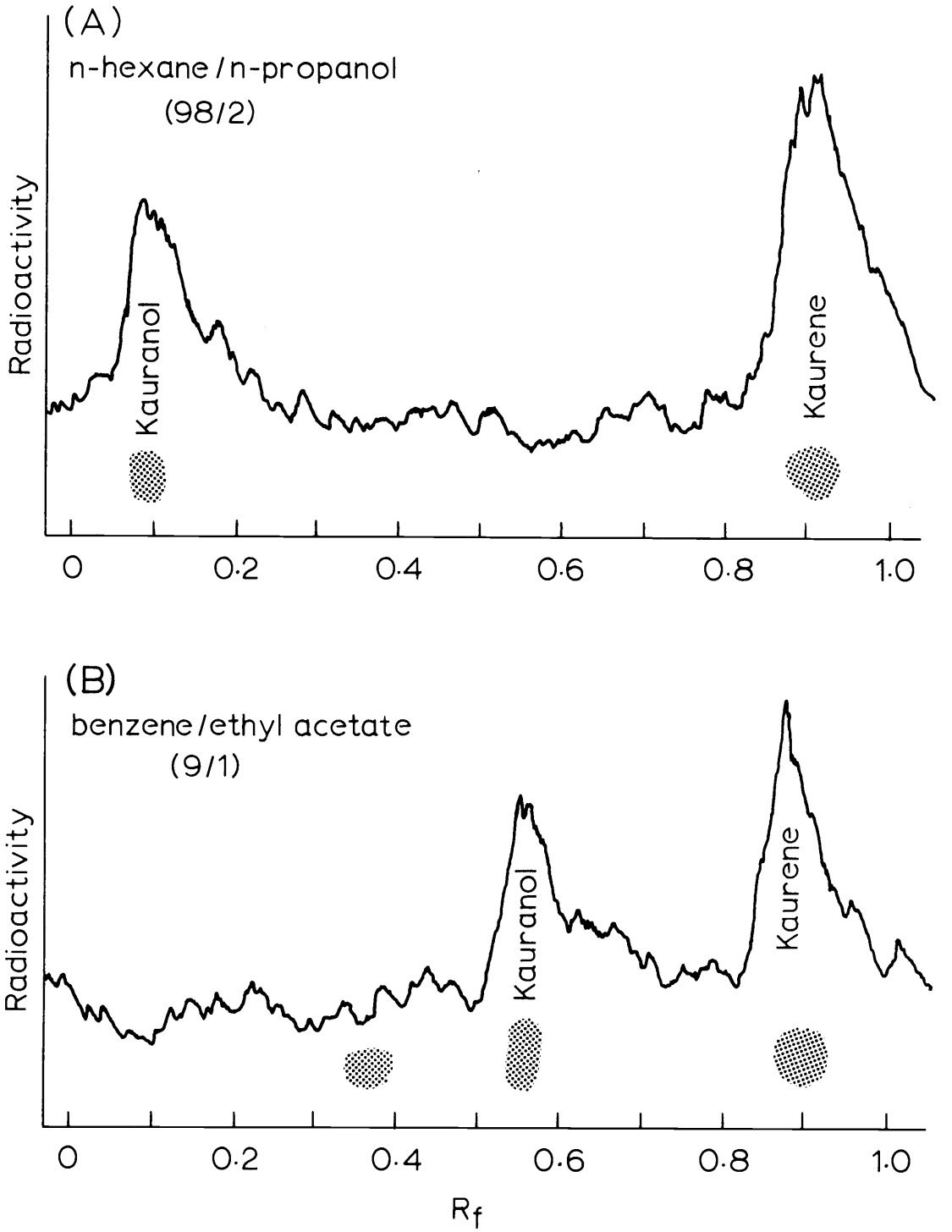


Figure 4. Strip chart tracings of radiochromatograms of the products of acid methanol treatment of presumptive kaurene-^{14}C and authentic, non-radioactive kaurene which were developed on silica gel G in two solvent systems.

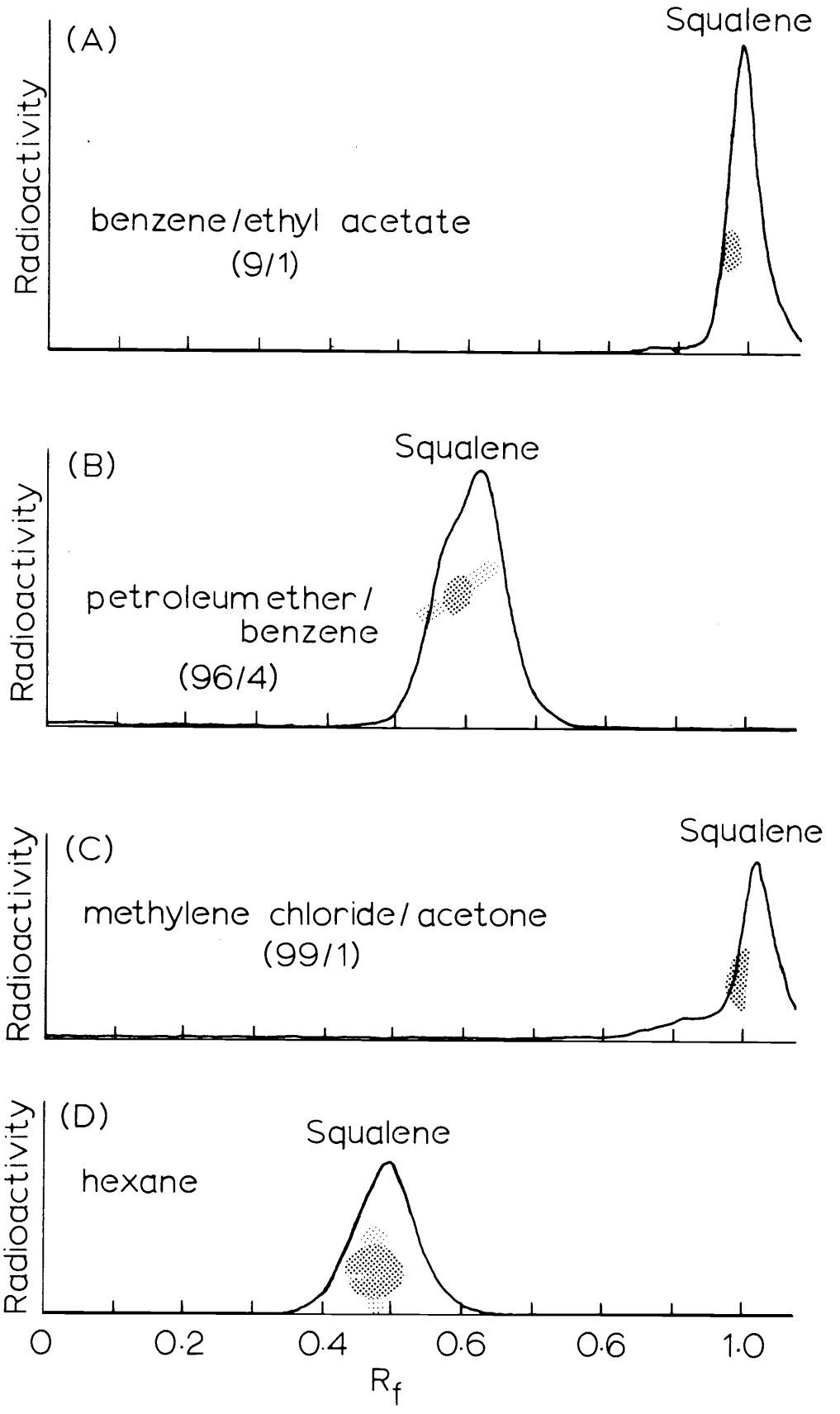
as a product of MVA-^{14}C metabolism in immature pea fruits and very young pea seeds (Graebe, 1968). This product was co-chromatographed with authentic squalene in four solvent systems. As indicated in Figure 5, the radioactive product was observed at an identical R_f as the authentic squalene in all of these systems. This ^{14}C-product is therefore tentatively identified as squalene.

On several occasions the radioactive material which remained at the origin of thin-layer plates after chromatography in hexane was eluted and chromatographed in other solvent systems in attempts to identify other products of these reactions. However, none of the other expected products, including geraniol, farnesol, or kaurenol was ever observed. It should be noted that on one occasion, when the pea seeds were homogenized in a Sorvall Omnimixer and the enzyme extract prepared as usual, four peaks were observed on the radiochromatogram scan after chromatography in hexane. The additional peak, which was located between the origin and squalene, may have represented phytoene which could have been synthesized by enzymes which were solublized by the more vigorous homogenization. However, no attempt was made to identify this compound.

Optimum Conditions for the Assay

Using routine reaction mixtures as described in the Methods section, several experiments were done to characterize the system

Figure 5. Strip chart tracings of radiochromatograms of a product of mevalonic acid-2-^{14}C metabolism, presumptive squalene-^{14}C, which were developed on silica gel G in four solvent systems. The position of authentic, non-radioactive squalene, which was co-chromatographed with radioactive product in each case, is outlined on each figure.


(A)
Squalene
Radioactivity
benzene/ethyl acetate
(9/1)
(B)
Squalene
Radioactivity
petroleum ether/
benzene
(96/4)
(C)
Squalene
Radioactivity
methylene chloride/acetone
(99/1)
(D)
Squalene
Radioactivity
hexane
0
0·2
0·4
0·6
0·6
1·0
R_f

more completely and to ascertain the optimum conditions for assaying cell-free kaurene biosynthesis. The rate of incorporation of MVA into kaurene was directly proportional to enzyme concentration through approximately 0.75 ml of enzyme extract (Figure 6), which is the amount used in all subsequent experiments, and the reaction rate was constant through 90 minutes at 30°C (Figure 7). Based on the data shown in Figure 8, a nearly saturating concentration of 0.03 mM MVA was selected for use in all other experiments. Incorporation of MVA into kaurene is dependent upon ATP, and the concentration of ATP very critically affects the rate of incorporation (Figure 9). Mn^{2+} stimulated the incorporation of MVA into kaurene much more than Mg^{2+} (Figure 10). These were the only cations tested. In experiments not reported in detail, it was found that by using successively higher concentrations of each of these cations, the Mg^{2+} and Mn^{2+} curves converged at a concentration of about 4 mM because of the decreasing effectiveness of Mn^{2+} above about 2 mM.

The optimum pH for kaurene formation in the crude cell-free extracts is about 7.1, and the activity declines sharply above pH 7.3 (Figure 11). The pH curves were obtained using six separate enzyme extracts, all of which were prepared simultaneously from one randomized sample of seeds. Six phosphate buffers were prepared by mixing appropriate volumes of 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 until the desired hydrogen-ion concentrations were obtained. The

Figure 6. Incorporation of mevalonic acid-2-^{14}C into kaurene-^{14}C at various enzyme concentrations. One ml of extract contained 3.28 mg nitrogen, as determined by a micro-Kjeldahl method.

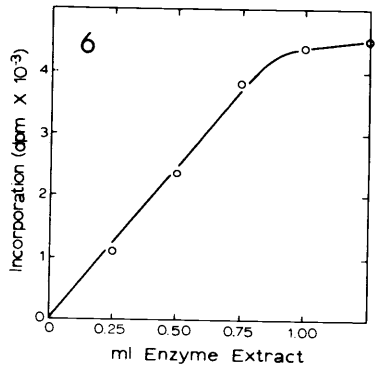
Figure 7. Time-course of incorporation of mevalonic acid-2-^{14}C into kaurene-^{14}C.

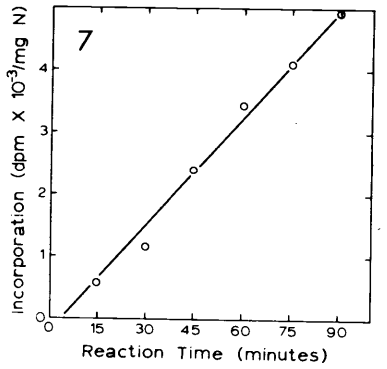
Figure 8. Effect of substrate concentration on the conversion of mevalonic acid-2-^{14}C to kaurene-^{14}C.

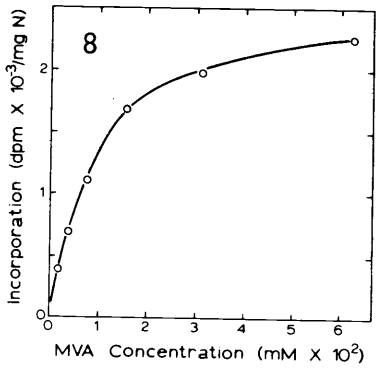
Figure 9. Effect of ATP concentration on the incorporation of mevalonic acid-2-^{14}C into kaurene-^{14}C.

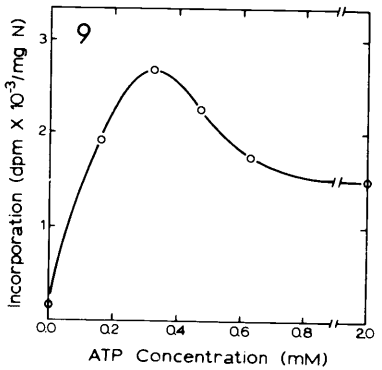
Figure 10. Effects of Mn^{2+} and Mg^{2+} on the conversion of mevalonic acid-2-^{14}C to kaurene-^{14}C. Each cation was added as the chloride salt.

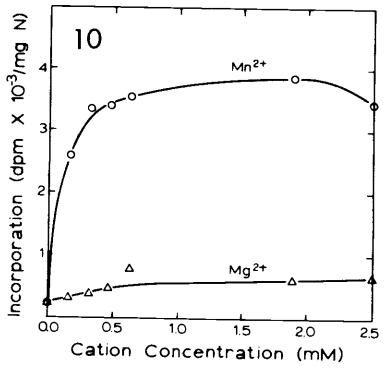
Figure 11. Effect of hydrogen-ion concentration on the incorporation of mevalonic acid-2-^{14}C into kaurene-^{14}C.

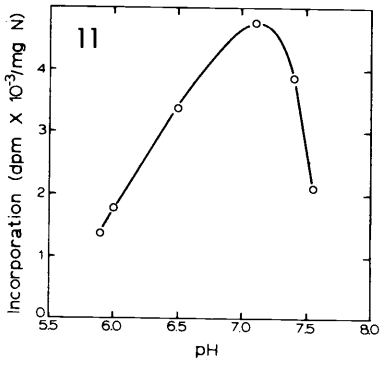

6
Incorporation (dpm X 10-3)
0
1
2
3
4
0
0.25
0.50
0.75
1.00
ml Enzyme Extract


7
Incorporation (dpm X 10-3/mg N)
0
1
2
3
4
0
15
30
45
60
75
90
Reaction Time (minutes)


8
Incorporation (dpm X 10-3/mg N)
0
1
2
0
1
2
3
4
5
6
MVA Concentration (mM X 10²)


9
Incorporation (dpm X 10-3/mg N)
1
2
3
0.0
0.2
0.4
0.6
0.8
2.0
ATP Concentration (mM)


10
Incorporation (dpm X 10-3/mg N)
Mn2+
Mg2+
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
Cation Concentration (mM)


11
Incorporation (dpm X 10-3/mg N)
1
2
3
4
5.5
6.0
6.5
7.0
7.5
8.0
pH

pH of each reaction mixture was measured both before and after incubation and did not vary more than 0.1 of a pH unit during this time. Thus the graph represents the activity obtained at the measured pH. It was interesting to note, incidentally, that the saturating concentrations of both Mn^{2+} and ATP changed from about 0.4 mM to about 0.3 mM when the pH of the reaction mixtures was changed from 6.5 to 7.1.

Apparent Changes in the Rate of Kaurene Biosynthesis During Seed Development

The capacity to convert MVA to kaurene, as measured in cell-free extracts, varied markedly with the stage of seed development (Figure 12). Using enzyme extracts obtained from ovules of first-formed fruits at different stages of seed development, it was seen that the activity increased from a very low initial level to a maximum at about 13 days after anthesis, or when the seeds had attained about half-maximal fresh weight, and then declined as the seeds approached maximum diameter and fresh weight. In other experiments, some variation (about one day) was observed in the time sequence of the development of the seed as well as in the day upon which maximum incorporation of MVA into kaurene occurred. This variation probably was caused by fluctuations in the greenhouse temperature during the summer months. In these experiments peas were planted at 1- or 2-day intervals, and all the groups of seeds were harvested and assayed simultaneously. Only fruits which had developed from first-formed flowers

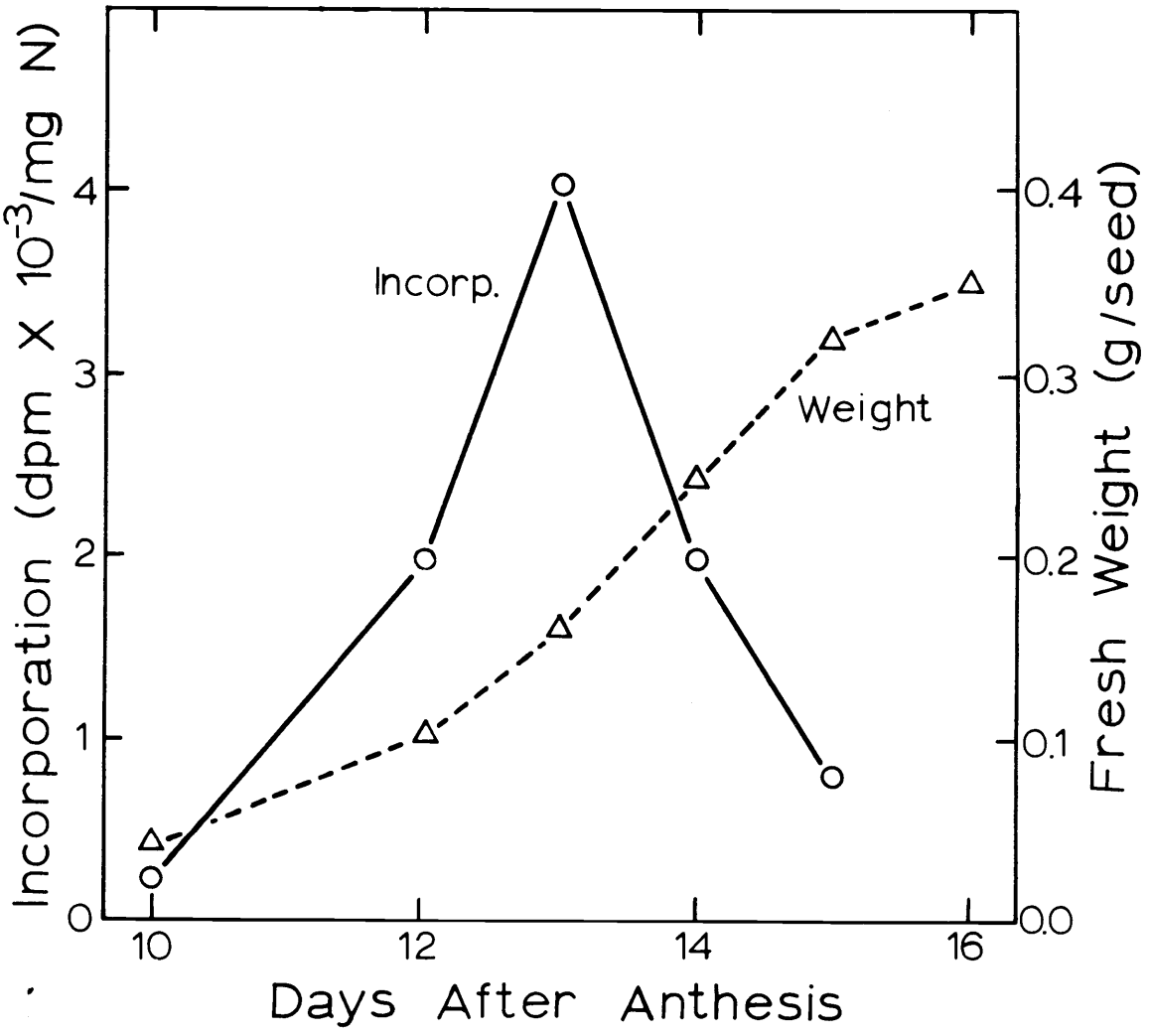


Figure 12. Apparent changes in the rate of kaurene biosynthesis during the development of pea seeds.

were used. It was noted that the pods achieved their final length during the first 10 days after anthesis. On about the 10th day, the fruits were flat and the developing ovules were extremely small (approximately 50 mg/ovule). The maximum incorporation of MVA into kaurene, as already noted, was obtained on approximately the 13th day after anthesis. The seeds attained maximum fresh weight on about the 16th day after anthesis. Hence, the apparent maximum capacity for kaurene biosynthesis in immature seeds was observed after the pods had reached final length and before the developing ovules reached maximum fresh weight. The incorporation of MVA into kaurene is expressed in Figure 12 as dpm incorporated per mg N; however, the same relationship is also seen when the data are calculated as dpm incorporated per seed. Thus, the mean dpm incorporated per seed were 223, 725, 2400, 1717, and 1020 at 10, 12, 13, 14, and 15 days after anthesis, respectively.

Discussion

Product Identification

The results on the identification of products of MVA metabolism in extracts of immature Alaska pea seeds agree well with the results of Anderson and Moore (1967) for Tall and Dwarf Telephone peas and those of Graebe (1968) for peas of the variety "Schnabel". These

investigators also reported that kaurene was the only diterpene hydrocarbon product observed to be formed and that its formation was strongly inhibited by AMO-1618. Kaurene is also the only diterpene hydrocarbon product of MVA metabolism in extracts of immature seeds of Echinocystis macrocarpa Greene (Graebe et al., 1965) and of Cucurbita pepo L. (Graebe, 1969). Robinson and West (1967, 1970a and 1970b) have reported that kaurene is one of five cyclic diterpene hydrocarbons formed from MVA in extracts of germinating Ricinus communis L. seedlings; and that the synthesis of four of these five compounds is inhibited by AMO-1618.

The inhibition of synthesis of the radioactive product by AMO-1618 in these experiments (Figure 2) supports the identification of this product as kaurene. However, since the synthesis of three other similar compounds, beyerene, sandaracopimaradiene, and trachylobane, is also known to be sensitive to this inhibitor, it was necessary to attempt to exclude these materials from the products which were isolated in the present investigations. Conventional thin-layer chromatography systems have been shown to be inadequate for the resolution of the diterpene compounds in question (Robinson and West, 1970b). Therefore, thin-layer chromatography was performed on silver nitrate-impregnated layers of silica gel according to the methods of Robinson and West (1970b). Co-chromatography of

the presumptive kaurene with samples of the authentic compound in four solvent systems revealed the presence of only one radioactive product which exhibited coincident mobility with authentic kaurene in all cases (Figure 3).

It is interesting to note that the same concentration of AMO-1618 which nearly completely inhibited kaurene biosynthesis in Telephone peas (Anderson and Moore, 1967), one μg/ml, also nearly completely inhibited the formation of this product in the present study with Alaska peas (Figure 2). A similar inhibition of kaurene biosynthesis in the Echinocystis system was obtained with 100 μg/ml (Dennis, Upper and West, 1965). Robinson and West (1970b) reported that 100 μg/ml completely inhibits the formation of kaurene from GGPP in extracts of R. communis. A similar concentration of AMO-1618 has also been shown to inhibit GA biosynthesis in the fungus G. fujikuroi (Kende, Ninnemann and Lang, 1963). Surprisingly, Graebe (1968) reported that 1 mg/ml of AMO-1618 was required to cause a similar magnitude of inhibition of kaurene biosynthesis in the system from "Schnabel" pea seeds.

The third method used to confirm the identify of presumptive kaurene as a product of these incubation mixtures was to form a derivative of the radioactive product, and to compare its properties chromatographically with the properties of the derivatives formed from authentic kaurene under the same conditions. After reaction of authentic and presumptive kaurene in acid-methanol solution and subsequent

co-chromatography of the lipid soluble extracts of the reaction mixtures in two solvent systems, two radioactive compounds were resolved (Figure 4). These two radioactive compounds corresponded identically with the published R_f values for unreacted kaurene and the product of hydration, kauranol (Dennis and West, 1967). Two compounds isolated from the reaction mixture with authentic kaurene and detected by charring also moved to identical positions as the radioactive materials on these chromatograms. It is notable that in the benzene/ethyl acetate (9/1) solvent system, a third compound was observed from the reaction of authentic kaurene which did not correspond to the position of a radioactive compound. This material is presumed to be due to a contaminant of the authentic kaurene. Thus, on the basis of three independent methods, it is concluded that the radioactive product isolated from these enzyme extracts which appears at R_f 0.85 on chromatograms developed in hexane is (-)-kaurene.

The identification of squalene as a radioactive product of MVA metabolism in these enzyme extracts was not proved, but the presence of a product which behaves chromatographically like squalene in four solvent systems was demonstrated. This compound was present in all experiments, and its accumulation was completely dependent upon ATP. Preliminary experiments showed that the substrate concentration was not saturating for the formation of this product, yet this compound was by far the most abundant product in all

experiments. It can be noted on the chromatogram scans presented in Figure 5, that there was enough of this material in the extracts to give a very distinct colored spot when the plates were sprayed with H_2SO_4 and heated. A compound which moved to R_f 0.6 on thin-layer plates developed in petroleum ether/benzene (96/4) (which is the R_f of the product separated here) was observed also by Graebe (1968) to be a product of MVA metabolism in extracts of another variety of peas and he identified the product as squalene. Graebe et al. (1965) also tentatively identified this compound as a product of cell-free metabolism of MVA in extracts of embryo tissue in E. macrocarpa. Baisted (1970) identified hydrocarbons and sterols as products of in vivo MVA metabolism in developing Alaska pea seeds. Since squalene is a precursor of sterols, it can be assumed that at least part of the hydrocarbon isolated in that study was squalene.

Rigorous identification of other isoprenoid compounds in these reaction mixtures was not attempted, but no product which behaved chromatographically like geraniol or farnesol was observed. Some evidence for the appearance of geranylgeraniol as a product in these incubations is presented in the following section on cell-free kaurene metabolism. Phytoene was not typically a product of these reactions, but in one experiment where the seeds were ground vigorously in a Sorvall Omnimixer a product was observed which could have been phytoene. The fact that phytoene is a precursor of carotenoids would

suggest that its formation may occur in the chloroplasts. Cell-free phytoene synthesis has been shown to occur in chloroplast extracts (e.g. Jungalwala and Porter, 1967; Charlton, Treharne and Goodwin, 1967; Shah et al., 1968). It would not be surprising, therefore, if the synthesis of this compound did not occur in homogenates which are prepared by a gentle grinding in a mortar and pestle but did occur in homogenates prepared by a method which would break more of the chloroplasts. The homogenates of pea seeds which were reported by Graebe (1968) to incorporate MVA into phytoene were prepared by grinding in a mortar and pestle with sand, a procedure which is much harsher than that used in the present studies.

Characterization of Kaurene Biosynthesis

Several experiments were designed to determine the optimum conditions for assaying cell-free biosynthesis of kaurene. It was shown that the reaction is approximately linear with enzyme concentration and time through 0.75 ml and through 90 minutes (Figures 6 and 7, respectively). A rough calculation from the data in Figure 6 indicates that the enzyme extract from one pea seed is capable of forming approximately one nmole of kaurene in 75 minutes under the conditions of this assay. This calculation is based upon the assumptions that there is not a significant pool of MVA or any intermediate between MVA and kaurene in the seeds and that four molecules

of MVA are incorporated into one molecule of kaurene. It was noted in all experiments that there was apparently a short lag period in the reaction before kaurene began to accumulate (Figure 7). This is in agreement with the data of Anderson and Moore (1967), as well as with those of Robinson and West (1970b), and is not very surprising since the formation of kaurene from MVA requires at least 9 reactions which would be expected to take some time.

A nearly saturating concentration of 0.03 mM MVA was selected for use in all experiments. Doubling the concentration of MVA did not lead to a significant increase in the amount of kaurene which accumulated (Figure 8). The incorporation of MVA into kaurene was shown to be dependent upon ATP, and the concentration of ATP very critically affected the rate of incorporation (Figure 9). This result for ATP is consistent with the data of Graebe (1968) for a similar system. In Graebe's system, an ATP generating system increased the activity about 30% in one experiment. The results are in agreement also with the results of Shechter and West (1969), who were able to show that high ATP levels in extracts of Gibberella fujikuroi caused the accumulation of copalyl pyrophosphate, the immediate precursor of kaurene. It therefore appears that the concentration of ATP regulates the biosynthesis of kaurene and presumably GA's at least at one site, the conversion of copalyl pyrophosphate to kaurene.

In agreement with the results of Anderson and Moore (1967), Mn^{2+} stimulated the incorporation of MVA into kaurene much more than Mg^{2+} (Figure 10). This finding agrees also with the report by Loomis and Battaile (1963) that Mn^{2+} is a better activator than Mg^{2+} of mevalonic kinase from pumpkin seedlings, and the finding by Nandi and Porter (1964) that Mn^{2+} is a better activator than Mg^{2+} for the carrot root enzyme which catalyzes the synthesis of geranyl-geranyl pyrophosphate from isopentenyl and farnesyl pyrophosphates. These systems all are inhibited by high concentrations of Mn^{2+}. Graebe (1968) reported that Mg^{2+} stimulated kaurene production, whereas Mn^{2+} in the presence of Mg^{2+} did not stimulate. Although the results are not presented here in detail, it was found in the present work that by using successively higher concentrations of each cation, the Mg^{2+} and Mn^{2+} curves converged at a concentration of about 4 mM because of the decreasing effectiveness of Mn^{2+} above about 2mM. Another interesting observation in these experiments was that usually when both Mn^{2+} and Mg^{2+} were used in low concentration in a reaction, the accumulation of kaurene was less than if Mn^{2+} were used alone. This result is in close agreement with the result of Anderson and Moore (1967). Upper and West (1967) have characterized the enzymes from E. macrocarpa which catalyze the reactions from geranylgeranyl pyrophosphate to kaurene. They have shown that this system requires a divalent cation, and that Mg^{2+} evokes the highest

activity, with Co^{2+} yielding about 2/3 of maximum activity and Mn^{2+} being a very poor activator.

These data show that there is an apparent difference in the cation requirements between the system reported here and the E. macrocarpa system. There is not sufficient evidence, however, to make a comparison between the present system and that developed by Graebe (1968) from pea seeds. It is known that phosphate ions inhibit endogenous phosphatase activity, and this may account for some of the differences observed here, since phosphate (0.1 M) buffer was used in these experiments, while Tris buffer was used by Graebe (1968). Apparent differences in cation requirements may be artifacts of these preparations, since all systems are admittedly crude, with impure enzymes.

The optimum pH for kaurene formation in the crude cell-free extracts is about 7.1 and the activity declines sharply above pH 7.3, as shown in Figure 11. The pH used in the system from Tall and Dwarf Telephone peas by Anderson and Moore (1967) was 7.4. The pH optimum for the conversion of geranylgeranyl pyrophosphate to kaurene in E. macrocarpa extracts as reported by Upper and West (1967) was pH 6.6, with less than a third of the maximum activity observed at pH 7.3. These data are not directly comparable with the data presented here, since the optimum pH reported for that system is only for the activity of two enzymes, whereas the pH optimum

observed in this study is the combined optimum for at least 9 reactions. It is interesting also that the pH optimum for conversion of geranylgeranyl pyrophosphate to kaurene in extracts of Ricinus communis as reported by Robinson and West (1970b) was approximately 7.1.

Apparent Changes in the Rate of Kaurene Biosynthesis During Development

The capacity to convert MVA to kaurene, as measured in cell-free extracts, varied markedly with the stage of seed development. As shown in Figure 12, the activity increased to a maximum at about 13 days after anthesis, or when the seeds had attained about half-maximum fresh weight, and then declined as the seeds approached maximum diameter and fresh weight. This variation occurred whether the data were plotted on the basis of dpm incorporated per mg N or on the basis of dpm incorporated per seed. The same relationship between development and enzyme activity was observed whether the parameter of development was fresh weight of seeds, days after anthesis, or seed diameter. It was found in later experiments that fresh weight was the most convenient and probably the most reliable parameter for this type of experiment.

Graebe et al. (1965) reported young seeds of E. macrocarpa to be most active in metabolism of MVA. The differences in seed types do not allow a direct comparison of the data, but they observed the

highest incorporation of MVA into the lipid fraction in seeds when the cotyledons were not yet visible and the seed cavity was mostly filled with endosperm, and a decline in activity as the seeds matured and the cotyledons formed. In contrast, pea seeds apparently have a relatively low capacity for kaurene biosynthesis during early morphological development, when liquid endosperm is present (see footnote on p. 14). Cell-free extracts of these seeds exhibit maximum capacity for kaurene biosynthesis at approximately the developmental stage when the endosperm is depleted. Furthermore, no kaurene-synthesizing activity has been demonstrable in the liquid endosperm fraction of very young pea seeds.

It is interesting to compare the results on kaurene formation in cell-free extracts of Alaska pea seeds with the available data on the extractable GA content of seeds at various stages of development. In immature seeds of Echinocystis macrocarpa Greene, Lupinus succulentus Dougl. (Corcoran and Phinney, 1962), Phaseolus vulgaris L. (Corcoran and Phinney, 1962; Skene and Carr, 1961), Lupinus luteus L. (Ogawa, 1963), and Pharbitis nil Chois. (Murakami, 1961) the maximum amounts of extractable GA's are found when the seeds have attained about half their maximum fresh weights. Preliminary extraction data from experiments not reported here in detail indicate the same result for pea seeds. Thus, the quantity of extractable GA and the apparent rate of kaurene biosynthesis from MVA both appear

to increase sharply during development of leguminous seeds and to reach maximum values when the seeds have attained about half-maximal fresh weight.

Graebe (1968) obtained comparatively low incorporation of MVA into kaurene using cell-free enzyme extracts prepared from very young pea fruits and even lower incorporation with immature seeds from almost fully grown pods. A direct comparison of the results obtained in the present study with Graebe's is difficult; however, the results obtained here (Figure 12), showing relatively low activity in very immature seeds, are consistent with Graebe's suggestion that the kaurene-synthesizing activity which he observed with very young fruits may reflect synthesis in the pod. Just as the growth curves for the legume pod and seed do not coincide in time (Corcoran and Phinney, 1962), maturation of the seed lagging behind that of the pod, it seems likely that the time-courses of kaurene and GA synthesis and GA accumulation in the pod and seed are similarly related.

LOCALIZATION OF KAURENE BIOSYNTHESIS IN PEA SEEDS

Introduction

There have been few studies on the localization of the enzyme systems for gibberellin biosynthesis in higher plants. Several studies have been made on the extractable GA content of various tissues and organs in some plants, but these data are indirect at best, since GA's are not metabolically inert and are translocatable. Probably the largest GA concentrations occur in the ripening seeds of many plants. Seeds of peas contain about 100 times as much GA-like material as stems, roots and leaves of older plants. Baldev, Lang and Agatep (1965) have shown that if the immature fruits of peas are grown in culture, the seeds increase in weight as well as in content of extractable GA-like materials, which means that GA biosynthesis occurs in the fruit of peas as they develop. Of course, cell-free biosynthesis of kaurene in extracts of pea seeds also lends support to this organ as a site of GA biosynthesis. As noted in the Discussion section (above), Graebe (1968) has reported higher levels of cell-free kaurene biosynthesis in extracts of young fruits than in very immature pea seeds. These data, when taken with the data of Figure 12 in the previous section, would suggest that GA biosynthesis may occur independently in the seed and the pod. Radley (1958) investigated the

gibberellin contents of various parts of immature runner bean seeds, and she showed that the seed coats and cotyledons of this plant contain similar amounts of GA. However, Corcoran and Phinney (1962) reported nearly 100 times more GA-like material in extracts of immature cotyledons of Lupinus succulentus than in seed coats of that plant. In this study, a similar accumulation of GA-like material was observed in the endosperm tissue of E. macrocarpa.

In light of these fragmentary and predominantly indirect data on the localization of enzyme systems for GA biosynthesis in ripening fruits, an investigation was undertaken to determine the relative capacities of enzyme extracts prepared from various parts of developing pea seeds for the incorporation of MVA into kaurene. Of course, the evidence obtained in these experiments also is inconclusive and cannot be considered proof for GA biosynthesis in the respective parts, but, if kaurene biosynthesis occurs exclusively in one or more of these seed parts, it seems likely that the further conversion of kaurene to GA may occur in the same tissues because of the improbability of kaurene being translocatable.

It is also of interest to ascertain the relative solubility of the enzymes catalyzing kaurene biosynthesis and the cell fraction in which they occur. Upper and West (1967) have shown by differential centrifugation of enzyme extracts from E. macrocarpa seeds that the enzymes responsible for the conversion of geranylgeranyl pyrophosphate

to kaurene are in the "soluble" fraction, that is, the 100, 000 x g supernatant fraction. Robinson and West (1970b) reported that kaurene biosynthesis activity in Ricinus seedlings also was obtained in the "soluble" fraction. Graebe (1968) reported that the enzymes responsible for incorporation of MVA into kaurene, squalene, and phytoene were obtained from the 100, 000 x g supernatant of young pea fruits and seeds. And, finally, Shechter and West (1969) reported that in extracts of the fungus G. fujikuroi, kaurene accumulated in the 100, 000 x g supernatant.

The only other reports on the intracellular localization of enzymes involved in GA biosynthesis were by Stoddart (1968, 1969). Stoddart first reported detection of significant quantities of GA-like substances from the chloroplasts of leaves of Brassica oleracea and barley, and later reported on a cell-free enzyme preparation from sonicated Brassica leaf chloroplasts which incorporate kaurenoic acid-^{14}C into a compound which behaved chromatographically like a GA-like compound in those chloroplasts. This enzyme system obviously is particulate in nature, and Stoddart has discussed the possible significance of this chloroplast GA. While there is no direct evidence that the earlier steps in the GA pathway occur in the chloroplasts, the enzyme systems which catalyze the formation of geranyl-geranyl pyrophosphate are present in leaf chloroplasts, since Charlton, Treharne and Goodwin (1967) have reported on the synthesis

of phytoene in extracts of these organelles.

Methods and Materials

The methods and materials used in these studies were identical to those used in the previous section with the following notable exceptions.

The plant materials used in these experiments were developing Alaska pea seeds from 12-16 days old. Enzyme extracts were prepared from whole seeds and from separate parts--seed coats, cotyledons, and embryonic shoot-root axes.

The preparation of enzyme extracts in this series of experiments was the same as described in the preceding section with seed coats and cotyledons being homogenized in one ml of buffer per gram of fresh weight of the whole seed. Enzyme extracts from embryo axes were prepared with less buffer, as described in the Results section, in order to concentrate the enzymes. Each enzyme extract was prepared from freshly harvested seeds immediately prior to use. In the differential centrifugation experiments, the crude homogenates were prepared as usual and then centrifuged at the lowest force used, usually 10,000 x g. The supernatant from each successive centrifugation was divided into two parts, and one part was held in an ice bath while the other was centrifuged at a higher speed. All centrifugations were done at $0^{\circ}C$ in a Sorvall RC2B refrigerated centrifuge

with the exception of the 100, 000 x g centrifugations which were done in a Spinco Model L refrigerated preparative ultracentrifuge, with a number 40 angle head rotor.

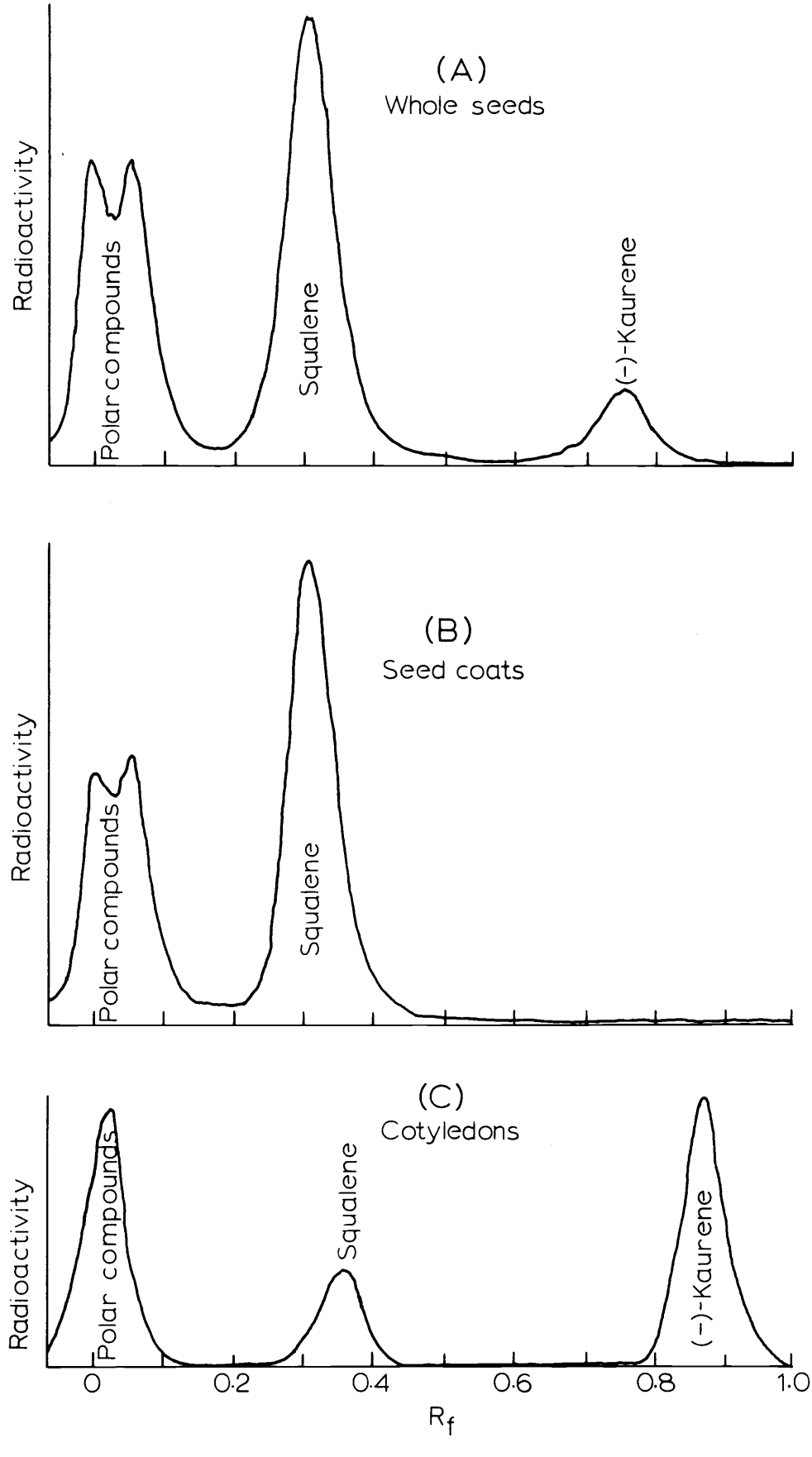
The protein concentration in each enzyme preparation was measured by the method of Lowry et al. (1951) using BSA as a standard.

Results

Localization of the Enzyme Systems Catalyzing Kaurene Biosynthesis in Immature Pea Seeds

Samples of whole seeds and excised seed parts were homogenized in phosphate buffer (1 ml buffer/gram fresh weight). After centrifugation of the enzyme extracts at 40, 000 x g for 15 minutes, routine assays of each type of enzyme preparation were conducted according to the procedures outlined in the previous section. The results of these experiments are shown in Figure 13 and in Table 1. It is clear that most, if not all, of the kaurene synthesis observed in extracts of these seeds occurred in the extracts of isolated cotyledons, with little or none occurring in the seed coat or in the embryo axis homogenates. It is also apparent that most of the synthesis of presumptive squalene in these seeds appears to have been localized in the seed coats, with a small amount occurring in the cotyledons. In later experiments, not reported here in detail, in which isolated cotyledons

Figure 13. Tracings of strip chart scans of radiochromatograms of products obtained from cell-free metabolism of mevalonic acid-2-^{14}C in extracts of: (A) whole seeds; (B) seed coats; and (C) isolated cotyledons, after thin-layer chromatography on silica gel G with hexane as the developing solvent.


(A)
Whole seeds
Radioactivity
Polar compounds
Squalene
(-)-Kaurene
(B)
Seed coats
Radioactivity
Polar compounds
Squalene
(C)
Cotyledons
Radioactivity
Polar compounds
Squalene
(-)-Kaurene
0
0·2
0·4
0·6
0·8
1·0
R_f

were used as an enzyme source, the ratio of squalene to kaurene was much higher than in these experiments.

Table 1. Localization of enzyme activities for kaurene and squalene biosynthesis in cell-free extracts of different parts of immature pea seeds.

Seed part	Kaurene		Squalene	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
	(dpm/mg protein)			
Whole seeds	2300	235	15100	8000
Cotyledons	11200	7500	4200	2000
Seed coats	11	8	16100	34400
Embryos	38	0	0	0
	(dpm/seed)			
Whole seeds	4160	420	27200	14300
Cotyledons	7180	2240	2680	620
Seed coats	18	13	26500	56800
Embryos	4	0	2	0

It must be emphasized that the protein content of the embryo axis extract actually was so low that it reasonably could not be expected to be active. Even when the axis tissues were prepared in one-third the amount of buffer of the other tissues, as was done in Experiment No. 2 in Table 1, the protein content of this preparation was only 0.25 mg/ml, whereas those of the cotyledons and the seed coats were 1.5 mg/ml and 8.5 mg/ml, respectively.

It is also interesting to note that more kaurene accumulated in the homogenates from isolated cotyledons than in those from whole seeds. A similar effect was observed with regard to presumptive squalene. Although more noticeable in the second experiment than in the first (Table 1), presumptive squalene accumulated more in isolated seed coat preparations than in extracts of whole seeds.

Determination of the "Soluble" Versus "Particulate" State of the Enzymes Catalyzing Karuene Biosynthesis

The results of two experiments using extracts of whole seeds and two experiments using preparations from isolated cotyledons are summarized in Table 2. The activity for kaurene synthesis in extracts from whole seeds increased as more of the particulate material was removed from the homogenates by centrifugation up to 40, 000 x g (Table 2). However, the 100, 000 x g supernatant consistently showed lower activity than the 40, 000 x g supernatant. The results of two separate experiments with isolated cotyledons indicated, on the other hand, that the accumulation of kaurene increased with increasing centrifugal force with which the enzymes were prepared, even up to 100, 000 x g. The data from Experiment No. 1 in Table 2 were taken from the time-course experiment shown in Figure 14, which shows that this result was consistent throughout 90-minute incubations with all three enzyme preparations.

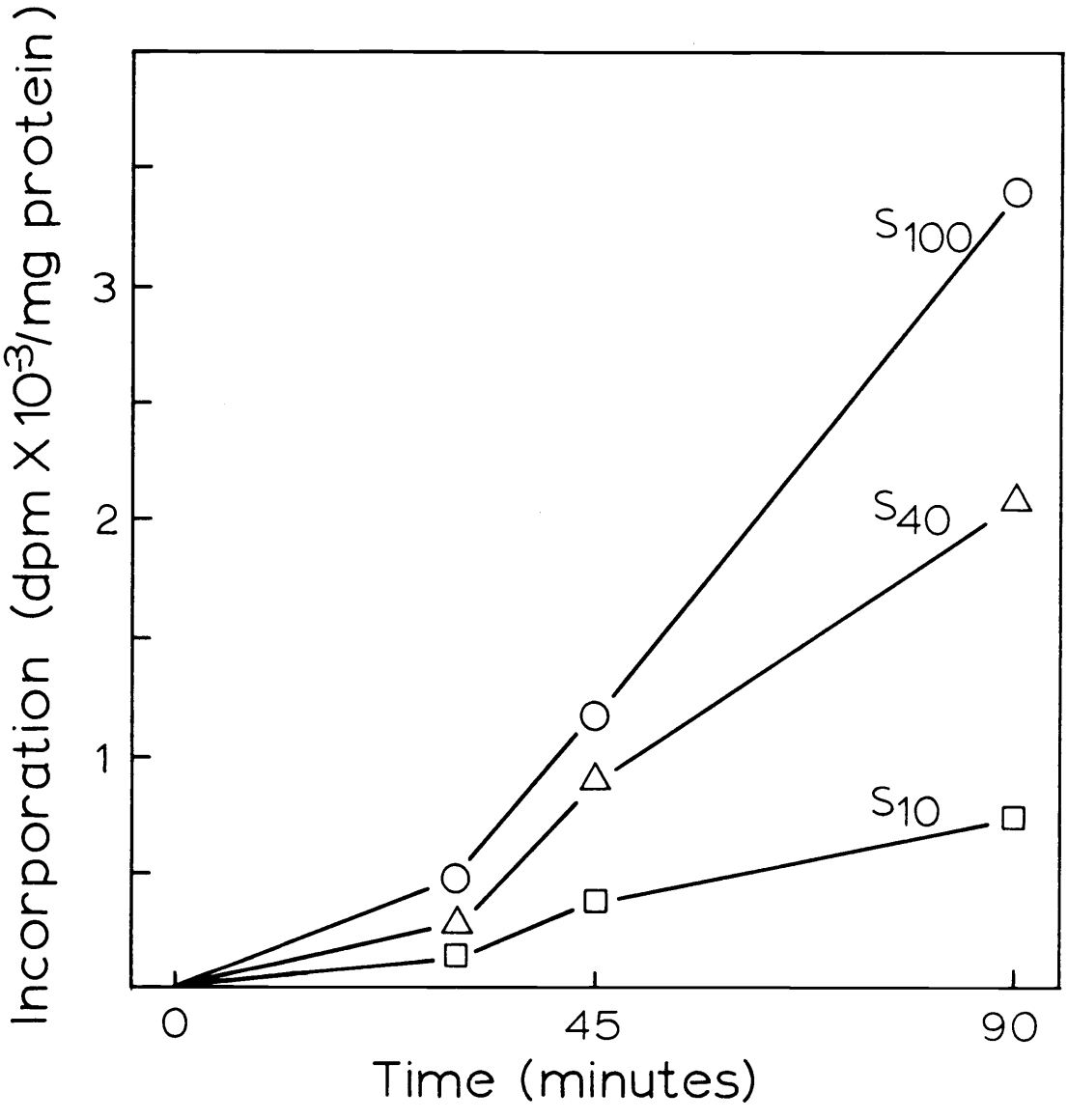


Figure 14. Time-courses of incorporation of mevalonic acid-2-^{14}C into kaurene-^{14}C by supernatant fractions of cotyledon extracts which were centrifuged at 10,000, 40,000 and 100,000 x g.

Table 2. Comparative biosynthesis and accumulation of kaurene in differentially centrifuged enzyme extracts from whole immature pea seeds and excised cotyledons.*

Fraction	Dpm incorporated/mg protein			
	Whole seed extract		Isolated cotyledon extract	
	Experiment 1	Experiment 2	Experiment 3	Experiment 4**
S_{10}	144	1130	720	270
S_{40}	655	4650	2060	835
S_{100}	648	2830	3200	1260

* It should be noted that the same relationships held true for all experiments when the data were expressed on a per-reaction-mixture basis.

** The data of Experiment 4 were for a 90-minute incubation; all others were for 75-minute incubations.

Discussion

Localization of the Enzyme Systems Catalyzing Kaurene Biosynthesis in Immature Pea Seeds

In view of the earlier report on the localization of kaurene and squalene biosynthesis in extracts of E. macrocarpa endosperm-nucellus and embryo, respectively (Graebe et al, 1965), and the conflicting data on the comparative extractable GA content of various parts of immature seeds (Radley, 1958; Corcoran and Phinney, 1962), the localization of enzymes responsible for kaurene and squalene synthesis in immature pea seeds was investigated. The results of these experiments indicated that the enzymes responsible for kaurene biosynthesis in these seeds are primarily localized in the cotyledons,

with little or no kaurene biosynthesis occurring in extracts of seed coats or preparations of isolated embryo axes. In contrast, the synthesis of presumptive squalene in these preparations was observed primarily in extracts of seed coats, with a small amount in the cotyledon extract and apparently none occurring in the embryo-axis extracts. The inability to observe MVA metabolism in extracts of isolated embryo axes may be due to the low concentration of protein in these extracts, or to an inherent incapability of these tissues to metabolize MVA at this stage of development.

It is interesting to note that more kaurene accumulated in the extracts of isolated cotyledons than in extracts of whole seeds. This result would indicate that something in the whole seed homogenate was either interfering with kaurene biosynthesis, or else contributing to the breakdown of kaurene once it was formed. A conceivable way by which the latter effect might be manifested is that the seed coats might contain enzymes for the further metabolism of kaurene. If these enzymes were very active, one would expect to see no accumulation of kaurene in the seed coat extracts. These enzymes might oxidize some of the kaurene formed in the whole seed extracts, and thus whole seed extracts would accumulate less kaurene than the extracts of isolated cotyledons. In order to test this hypothesis, an enzyme extract was prepared from isolated seed coats of immature pea seeds, and incubated in a routine reaction mixture for MVA

metabolism in the presence of a saturating atmosphere of carbon monoxide. Since carbon monoxide is known to inhibit cytochrome P-450, which is involved in the first oxidative step in the metabolism of kaurene in E. macrocarpa endosperm (Murphy and West, 1969), it was considered likely that kaurene would accumulate under those conditions if, indeed, kaurene were being synthesized in extracts of isolated seed coats. The results of this experiment are not presented in detail, but no kaurene was detected in these extracts. In other experiments, described in the following section, it was shown that exogenous kaurene added to extracts of whole seeds was not metabolized under the conditions of the present experiments. It therefore appears that there is some material present in extracts of whole seeds, which is not present in extracts of isolated cotyledons, that interferes with the synthesis of kaurene. This alternative was not tested further but may be either a direct effect of an inhibitor on one or more of the enzymes in this system, or simply a competition among enzymes for substrate or one or more intermediates common to two or more biosynthetic pathways. Of course, squalene biosynthesis is a good candidate for a pathway which would compete for substrate or intermediates in these reactions. The competition of squalene or sterol biosynthesis with kaurene biosynthesis for intermediates common to both pathways in these reactions was not investigated directly. However, it can be observed in Table 1 that the accumulation of squalene in the

seed coat extracts also exceeded that obtained in the whole seed extracts. This result would indicate that something in the whole seed extracts was interfering with squalene biosynthesis, as was observed with kaurene biosynthesis, although nothing is known about the further conversion of squalene to sterols in this system.

One other possible explanation for the data in Table 1, which indicate that kaurene and squalene accumulated more in extracts of isolated cotyledons and seed coats, respectively, than in extracts of the whole seeds, is that products formed in one tissue when combined with those from another tissue, upset a very delicate balance of controls. Dorsey and Porter (1968) have studied the in vitro inhibition of purified preparations of mevalonate kinase by terpenyl pyrophosphates, and they concluded that their results are "consistent with the suggestion that geranyl-PP and farnesyl-PP act as physiological controls over farnesyl-PP biosynthesis and ATP utilization." There probably are other possible explanations for the effect observed here, but the important conclusion from these experiments is that kaurene biosynthesis in extracts of these seeds was limited to extracts from isolated cotyledons, while presumptive squalene biosynthesis was observed in preparations from both cotyledons and seed coats.

The results on kaurene biosynthesis are in agreement with those of Corcoran and Phinney (1962), which showed that most of the extractable GA-like material present in immature Lupinus succulentus seeds

was present in the cotyledons of these seeds. The results of Radley (1958), are not consistent with those of Corcoran and Phinney (1962) or with the results obtained here. These differences may be due to inherent differences in the plant materials, or to differences in the stages of development chosen for study.

The results on localization of kaurene and squalene biosynthesis in different parts of pea seeds differ rather strikingly from comparable data for E. macrocarpa seeds. In E. macrocarpa kaurene is formed in the endosperm tissue, and, in fact, oxidative metabolism of kaurene also occurs in this tissue. Squalene synthesis appears to be exclusively localized in the embryonic tissues. In pea seeds, kaurene biosynthesis appears to be exclusively localized in the cotyledons, whereas the synthesis of squalene occurs both in the seed coat and in the cotyledons. Nothing definite can be said from the data presented here about the metabolism of MVA in embryo-axis tissues, other than the fact that it was not observed. However, more than 100% of the radioactivity incorporated into kaurene and squalene in extracts from whole seed preparations was observed in the extracts from isolated cotyledons and seed coats, so it is highly unlikely that the enzymes present in the axis tissues contributed significantly to the metabolism of MVA in the whole seed extracts.

Just as very large differences in the rates of kaurene synthesis in extracts of whole seeds of various stages of development were

observed, it is possible that the sites or the relative rates of kaurene and squalene synthesis in the various seed parts may change during development; however, this was not investigated. The experiments reported in Table 1 were done with seeds which were approximately half-maximal fresh weight (0.17 and 0.20 g/seed in Experiments 1 and 2, respectively), which is the stage when the kaurene biosynthesis activity in the whole seed preparations was highest.

Determination of the "Soluble" Versus "Particulate" State of the Enzymes Catalyzing Kaurene Biosynthesis

Table 2 presents the results obtained in experiments on the differential centrifugation of extracts of immature pea seeds and pea cotyledons, respectively. These data, from four independent experiments, indicated that in extracts of whole seeds the activity for kaurene biosynthesis increased with increasing centrifugal force used to prepare the enzymes up to 40,000 x g and then decreased, whereas in extracts of isolated cotyledons this activity continued to increase even in the 100,000 x g supernatant. The latter result is consistent with the results of Upper and West (1967) for the kaurene synthase activity from E. macrocarpa.

Greater accumulation of kaurene with increasing centrifugal force used to prepare the enzyme extracts, as observed with cotyledon extracts, is consistent with the hypothesis that the enzymes

catalyzing kaurene biosynthesis are "soluble" in nature. The increasing activity in these preparations probably is due to the removal of particulate substances, possibly bound enzymes, which might compete for substrate or one of the intermediates with the kaurene-synthesizing enzymes, or perhaps contribute to the disappearance of kaurene once it has been formed. In initial experiments, the pathway of squalene biosynthesis was considered a good candidate for competing with kaurene synthesis for a substrate or intermediate. However, the data obtained in those experiments were quite variable, with regard to squalene biosynthesis, and no conclusions could be drawn from them. Thus it appears more likely that this effect is due to non-specific binding of the intermediates to some particulate substance. Of course, all of the intermediates from geranyl pyrophosphate to kaurene and squalene would be expected to be quite hydrophobic and therefore have a tendency to bind to non-catalytic proteins.

Another possible explanation for kaurene accumulation increasing with higher centrifugal forces used to prepare enzymes from isolated cotyledons is the further metabolism of kaurene by some particulate enzyme. In the case of E. macrocarpa it is known that the enzymes responsible for oxidation of kaurene are particulate in nature (Murphy and West, 1969). However, in these experiments, no products corresponding to kaurenol, kaurenal, or kaurenoic acid (the expected immediate products of kaurene metabolism) were ever observed.

And, although in one experiment (Table 4, following section), carbon monoxide, an inhibitor of enzymic kaurene oxidation, did increase the accumulation of kaurene more in a 10, 000 x g supernatant than in a 40, 000 x g supernatant, the difference was not enough to account for the differences observed here. Upper and West (1967) have reported that most of the phosphatase activity which attacks geranylgeranyl pyrophosphate in that system is found in the 100, 000 x g pellet. Thus differential centrifugation of phosphatase may account for some of the increased rate of kaurene biosynthesis with increasing centrifugal force used in preparation of the enzyme extracts (Figure 14 and Table 2).

The result for decreased activity of kaurene biosynthesis in the 100, 000 x g supernatant of extracts from whole seeds is unexpected and at present remains inexplicable. The only obvious hypothesis which is consistent with the data is that there is some substance in the soluble fraction of whole seeds which is not present in the soluble fraction of extracts of isolated cotyledons, which becomes active when the particulate material is removed, and which somehow inhibits the accumulation of kaurene. A mechanism for such a phenomenon is difficult to imagine, and was not investigated. It is not known whether this result is related to the data in Table 1, showing more kaurene accumulation in extracts of isolated cotyledons than in extracts of whole seeds. Whatever the reason for this unexpected

result, it has been shown that enzyme extracts prepared from isolated cotyledons are responsible for most if not all of the kaurene synthesis observed in extracts of immature pea seeds, and that the enzymes responsible for the incorporation of MVA into kaurene in these extracts are present in the 100, 000 x g supernatant.

A few very preliminary experiments were conducted in efforts to observe the biosynthesis of kaurene or other intermediates in GA biosynthesis in extracts of vegetative organs of germinating pea seedlings. As noted in the Introduction, kaurene biosynthesis in extracts of three-day-old seedlings of Ricinus communis was reported by Robinson and West (1967, 1970a, 1970b). It is not known where this synthesis is localized, since these studies were done with whole seedlings minus roots. The data of Sitton, Richmond and Vaadia (1967) certainly indicate the presence of enzymes responsible for kaurenol biosynthesis in young excised sunflower roots. There are other studies on the content of extractable and diffusible GA's of various plant parts which may be interpreted as indicating that GA biosynthesis occurs in root and shoot tips. One preliminary experiment, not reported here in detail, showed that homogenates from root tips of five-day-old pea seedlings, which were prepared in the presence of insoluble PVP, did not metabolize MVA to any significant extent, whereas extracts of young shoot tips prepared from the same plants in a similar manner did incorporate MVA into a compound

which moved to a similar R_f as kaurenoic acid on thin-layer plates developed in benzene/ethyl acetate (9/1). The development of a cell-free enzyme system from young shoot tips could be a very exciting step forward in investigations of GA biosynthesis, particularly if kaurenoic acid and its derivatives were the only products of MVA metabolism in the system. It might offer a new system for studying further steps in this biosynthetic pathway if, for some reason, the enzymes for the further conversion of hydroxy-kaurenoic acid cannot be isolated from immature seeds. It also would offer a system for the study of differences in GA biosynthesis during early stages of germination and growth. And, it would be a much easier system for detailed studies on control mechanisms and effects of environment on GA biosynthesis.

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

Introduction

The metabolism of kaurene has been reported in cell-free extracts of only one higher plant tissue. Using the liquid endosperm-nucellus of immature seeds of wild cucumber (Echinocystis macrocarpa Greene), West and his associates have very carefully characterized several reactions in kaurene metabolism. Their work began with the isolation of kaurene and kaurenol as products of MVA metabolism in these extracts (Graebe et al., 1965). A further examination of the products of the system yielded kaurenal, kaurenoic acid, and four unidentified acidic products (Dennis and West, 1967). One of these acids has since been identified as hydroxy-kaurenoic acid (West et al., 1968). After identification of each of these products, West's group then produced sufficient quantities of each of the intermediates to incubate them as substrates with the enzyme extract to demonstrate that each predicted reaction occurred irreversibly and in sequence (Figure 1). Murphy and West (1969) have since shown conclusively that these reactions are catalyzed by mixed function oxidase type enzymes (see Mason et al., 1965). The reactions only proceed in the presence of microsomal enzymes, and cytochrome P-450 is involved. Additional requirements for the reactions are molecular

oxygen and NADPH. West and his colleagues have further reported that each of these intermediates can be converted to GA by the fungus Gibberella fujikuroi (West et al., 1968). All of these compounds, with the exception of kaurenal, have been shown to be biologically active (e.g. Jones, 1968; Katsumi et al., 1964; Phinney et al., 1964; Cross, Stewart and Stoddart, 1970), possibly by virtue of being converted to gibberellins.

Very little other work has been done on kaurene metabolism or the reactions between kaurene and GA in higher plants. Sitton, Richmond and Vaadia (1967) reported on the incorporation of MVA into kaurenol in excised roots of sunflower seedlings, but did not report the isolation of kaurene from these tissues. As reported in the previous section on localization of kaurene biosynthesis, Stoddart has reported on the possible conversion of kaurenoic acid to a GA-like compound in cell-free extracts of sonicated Brassica leaf chloroplasts (Stoddart, 1969). Bennett, Lieber and Heftmann (1967) have shown that kaurene is converted into a related compound, steviol, when applied to leaves of Stevia rebaudiana. Steviol is also reported to possess some GA-like biological activity (Ruddat, Lang and Mosettig, 1963). Ruddat, Heftmann, and Lang (1965) had shown earlier that acetate-2-^{14}C could be incorporated into steviol when sprayed on the leaves of this plant. The significance of this compound remains to be determined though, since it has only been isolated from one species

of plant. Bennett, Ko and Heftmann (1966) have also provided evidence that exogenously applied kaurene is metabolized by Pharbitis nil seedlings. The metabolsim was significantly different in plants which were induced to flower and in non-induced plants. None of the products was identified in this study, but they were sufficiently characterized to exclude the presence of detectable quantities of any of the known GA's in the extracts.

In view of the very limited information on kaurene metabolism in plant material other than immature seeds of E. macrocarpa, these investigations were undertaken with the goal of developing a cell-free enzyme system from immature pea seeds which is capable of kaurene metabolism, in hopes of confirming and perhaps extending the results of West and his associates. This study seemed particularly desirable for several reasons:

(1) As noted above in the section on kaurene biosynthesis, the liquid endosperm tissue of E. macrocarpa is greatly different from the cellular cotyledonary tissue of pea seeds, and there may be significant differences in GA metabolism which are correlated with the striking anatomical differences in these two kinds of plant material.

(2) At last report (West et al. (1968), the cell-free enzyme system from E. macrocarpa had not been demonstrated to convert the unidentified acid metabolite of MVA or kaurene to any further

compounds. It is not known whether this failure reflects an inherent limitation of that tissue, or merely a technical problem in the preparation of the enzyme extracts. It is possible that the system from pea seeds could provide further information about the reactions beyond kaurenoic acid.

(3) The possibilities for studies on the control of GA biosynthesis using a system from developing pea seeds appear to be excellent, and information gained using the system would contribute substantially to the wealth of physiological data on gibberellin relationships in pea plants.

Methods and Materials

Plant Material

The plant materials used as enzyme sources in the experiments reported here were either whole seeds or excised cotyledons from immature pea seeds 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 the individual experiments in the Results section.

Source and Purity of Reagents

Mevalonic acid-2-^{14}C lactone (sp. act. 5.86 mc/mmole) in benzene solution was purchased from Amersham/Searle Corporation. The lactone was hydrolyzed as described in the first section. Authentic reference samples of (±)-kaurenol and (±)-kaurenoic acid were the generous gifts of Dr. Kenji Mori, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-Ku, 113, Japan. A very generous sample of 100 mg of (-)-kaurenoic acid was a gift 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 Dr. Otto Isler, Hoffmann-LaRoche and Company, Basel, Switzerland. All other reagents were as described in the first section.

Preparation of Enzyme Extracts

The enzyme extracts were prepared by grinding the seeds or excised cotyledons in ice cold buffer (1 ml buffer/gram fresh weight of whole seed) in a cold mortar and pestle. Phosphate buffer (0.1 M, pH 7.1) containing 50μg/ml each of streptomycin sulfate and penicillin G was used in all cases. In some cases, as described under Results, the seeds or cotyledons were ground in the presence of insoluble PVP or other additives such as glycerol or metabisulfite. PVP was

purified before use by boiling in 3 N HCl and then filtering and washing until neutral with glass distilled water. The PVP was then rinsed twice with phosphate buffer, pH 7.1, and finally centrifuged for 15 minutes at the same centrifugal force as the enzyme to be prepared. The wet PVP was then mixed with the plant tissue before grinding. A ratio of 0.5 g or 1.0 g of wet PVP per gram of plant material was used. Unless otherwise stated, these crude homogenates were centrifuged at 10,000 x g for 15 minutes, and the resultant supernatant was used as a source of enzymes.

In some cases the enzyme was prepared from frozen tissue and then the liquid enzyme preparation was again frozen in liquid nitrogen for use in other experiments. It worked out very well to make large batches of enzyme extract and slowly pipette the enzyme into a liter container of liquid nitrogen. When done in this way, the enzyme can be removed in small beads and stored in liquid nitrogen for at least several weeks without significant loss of activity. When stored in this form, a small portion of the enzyme can be removed and used without affecting the bulk of the material.

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 in the

first section. Up to 500 ml of enzyme extract were incubated with proportionate amounts of Mn^{2+}, ATP, MVA, and buffer. The incubations were for three hours instead of the typical 75 minutes, and the reaction mixtures were extracted as described in the first section with appropriate volumes of organic solvents. After the organic extracts were evaporated, they were transferred to several preparative thin-layer chromatograms. The chromatograms were developed in hexane, and the silica gel in the kaurene region of the plates was scraped from the plates and eluted with acetone. The kaurene so obtained was evaporated to dryness and stored in benzene solution in a refrigerator (4-6° C). The specific activities of two batches of kaurene-^{14}C were 2.8 and 3.6 μc/μmole based on the assumptions that four molecules of MVA are incorporated into one molecule of kaurene and that the pool sizes of MVA, kaurene, and intermediates between the two are negligible.

Incubations with Kaurene as Substrate

Two methods were used to present substrate quantities of kaurene to the enzyme extracts. By one method, exogenous kaurene was added directly to reaction mixtures; by the other method, kaurene was allowed to form *in situ* from MVA. 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 nitrogen.

Then 10 µl of either acetone, ethanol, 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 MVA in routine reaction mixtures (2 mM Mn^{2+}, 2 mM Mg^{2+}, 0.3 mM ATP, 0.03 mM MVA, and enzyme) and after 60 minutes, 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, it became impractical to measure for products, and the routine reactions were assayed only by measuring the disappearance of the substrate (kaurene) according to assay procedure number II or number III.

Assay procedure number I is the same as described by Dennis and West (1967), according to which the 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/ethyl acetate (9/1). The chromatography in hexane moves the kaurene to approximately R_f 0.85, while the other products are expected to remain at the origin. Then the second chromatography in benzene/ethyl acetate (9/1) separates the products on the basis of polarity. Kaurenal moves nearly to the front (10 cm), kaurenol moves to approximately R_f 0.5, kaurenoic acid moves to approximately R_f 0.15, and other more polar compounds remain at the origin. In these experiments, 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 this extraction, the aqueous phase was acidified with 0.4 N HCl to pH 2.5 and extracted twice with 1 ml of ethyl acetate. The combined organic extracts were evaporated to dryness and transferred to the center of the origin of a thin-layer (250 μ) plate of silica gel G. Typically, a small sample each of authentic kaurenol and/or kaurenoic acid was spotted to one side of the extract for comparison. After chromatography, the plates were scanned, sprayed lightly with a solution of sulfuric acid/ethanol (1/9), and heated in an oven for 10 minutes at 100°C. The colored spots corresponding to the authentic materials were noted on the radiochromatogram scan, and the plates were divided into 12 zones. The first 10 zones represent the R_f regions after chromatography in the second solvent, the 11th zone represents the first 0.5 cm of the kaurene region, and the 12th zone represents the rest of the kaurene

region. The gel from each zone was then trasnferred to a liquid scintillation vial and counted in a liquid scintillation spectrometer.

Assay procedure number II is a very simple but indirect measurement of kaurene disappearance. The procedure is simply to incubate the enzyme extract with a routine reaction mixture for kaurene biosynthesis which includes 0.05 μmoles of MVA-^{14}C, 0.75 ml of enzyme extract, 0.5 μmoles of ATP, 3 μmoles of Mn^{2+}, 3 μmoles of Mg^{2+}, and 0.6 ml of phosphate buffer at pH 7.1 in a total reaction volume of 1.6 ml. The addition of control reactions under a saturating atmosphere of carbon monoxide gas completes the assay procedure. The product isolation and measurement of radioactivity incorporated into kaurene are as outlined in the first section. The activity of the kaurene oxidizing enzymes is then estimated by the difference between the amount of kaurene which accumulates in carbon monoxide-inhibited preparations minus that accumulated in the normal preparations. This assay is admittedly crude and subject to several limitations. However, this assay has proved quite useful in determining the effects of a wide variety of additives and extraction procedures.

Assay number III depends not only upon the inhibition of the first reaction in kaurene metabolism by carbon monoxide, but also upon the inhibition of kaurene synthesis by the very specific inhibitor, AMO-1618. After kaurene biosynthesis has proceeded for 60 minutes in a routine reaction mixture, as described in the preceding

paragraph, AMO-1618 is added to make 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 minutes. Addition of a saturating atmosphere of carbon monoxide to some of the reaction mixtures then gives an additional control. At the end of the 120-minute incubation, all reactions are stopped by boiling, each mixture is extracted as described above for kaurene, and the extract is chromatographed on a thin-layer chromatogram in hexane, scanned, and counted. In this assay, the 60-minute incubation permits measurement of the accumulation of endogenous kaurene, while the other reactions give an estimate of the amounts of oxidation of kaurene which are sensitive and insensitive to carbon monoxide. This assay is subject to some assumptions which may or may not be true, but in spite of the limitations (which are noted in the Discussion) of this type of system, it has proven to be quite useful.

Thin-layer Chromatographic Analysis of the Products of MVA Metabolism

On two occasions the products of large-scale incubations (30 to 100 ml) with MVA as substrate were subjected to analysis by co-chromatography with authentic compounds in several thin-layer systems. All chromatography was done using thin layers (250-750 μ

thick) of silica gel G 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 a stream of nitrogen. The chromatograms were developed 15 cm and then scanned for radioactivity. The gel in the center of the plate, containing the radioactive compounds from the extract, was scraped from the plate into a centrifuge tube, while the remaining two strips of gel on the plate, containing the authentic materials, were sprayed with 10% sulfuric acid in ethanol and heated to 100°C for 10 minutes to detect the charred spots of the authentic compounds. The radioactive material corresponding to these spots was then eluted from the gel with acetone and spotted on another plate for further chromatographic analysis.

Co-crystallization of Presumptive and Authentic Kaurenol

The radioactive product of MVA metabolism in these experiments which behaved chromatographically like authentic kaurenol was purified in preliminary work by successive thin-layer chromatography in four solvent systems: hexane, benzene/ethyl acetate (9/1), hexane/ethyl acetate/propanol (82/15/3), and hexane/ethyl acetate/isopropyl ether (2/1/1), and finally rechromatographed in benzene/ethyl acetate (9/1). The radioactivity still remaining at an identical R_f with

authentic kaurenol on the last chromatogram was eluted and a small portion taken for radioactivity determination. The bulk of this radioactive material (approximately 40,000 cpm) was mixed with 60.2 mg of authentic kaurenol and recrystallized twice from methanol/water (6 ml/6 ml). During the first two crystallizations, the soluble material (approximately 12 ml) was decanted and the remaining crystals were dried under a stream of nitrogen before weighing. In these cases, a small amount of crystal was weighed and then transferred directly to a liquid scintillation vial. After the two crystallizations from methanol-water, the remaining crystals were dissolved in and crystallized from methanol. During these crystallizations, the crystals were filtered in the cold room. Each batch of crystals in the third through fifth crystallizations was weighed, and all crystals were transferred to a 10-ml volumetric flask and diluted to 10 ml with methanol. A small sample was then taken for counting.

Carbon Monoxide Difference Spectra of Pea Seed Microsomes

Developing pea seeds of approximately half-maximum fresh weight were harvested and stored at -70° C until used. These seeds were then ground in phosphate buffer in a chilled mortar and pestle. The homogenate was centrifuged at 10,000 x g for 15 minutes in a Sorvall RC2B refrigerated centrifuge, and supernatant from this centrifugation was centrifuged again at 100,000 x g for 60 minutes in a

Spinco Model L preparative ultracentrifuge, using a number 40 angle head rotor. The pellet from this centrifugation, which contains the "microsomal fraction," was then resuspended in 5/7 of the original volume of the 10,000 x g supernatant. The enzyme extract so obtained was distributed between two cuvettes and a baseline was established. Then NADPH was added to each cuvette, and a new baseline was established. When the baseline was obtained, CO was bubbled through one cuvette, and the difference spectrum was observed between 390 nm and 470 nm. After the spectrum had been measured, a few crystals of dithionite were added to each cuvette and the spectrum was measured again. Finally, more CO was bubbled through the sample cuvette, and the spectrum was taken once more from 390 nm to 470 nm. All spectra were measured on a Cary Model 11 recording spectrophotometer.

Results

Cell-free Metabolism of Exogenous Kaurene

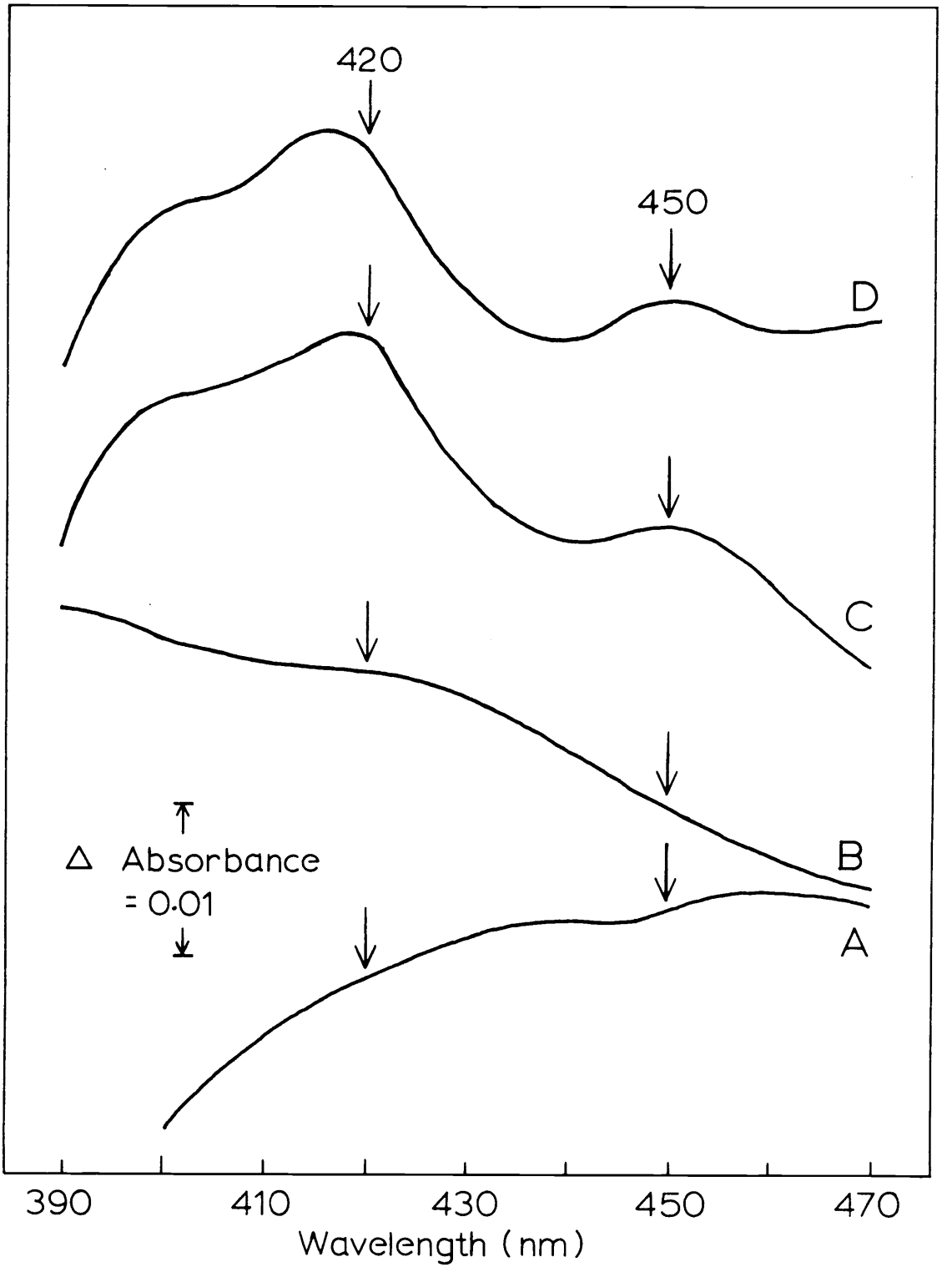
Repeated attempts were made to observe enzymic oxidation of exogenous kaurene under a wide variety of conditions. Experiments were performed using different concentrations of enzyme, substrate, NADPH, cations; different salt strengths; and using other additives such as NADP, NAD, NADH, ATP, dithionite, dithiothreitol,

metabisulfite, ascorbate, and glycerol. All these attempts resulted in failure. In all of 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 cpm 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 per minute, but certainly not enough for characterization experiments.

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 kaurene oxidizing enzymes. The involvement of cytochrome P-450 in the expected reactions, as reported by Murphy and West (1969), 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 Figure 15. It can be seen in these spectra that the addition of NADPH and CO to the enzyme extract did not cause a peak to appear at 450 nm. However, when dithionite was added to these enzyme preparations, peaks appeared promptly at 450 nm and

Figure 15. Carbon monoxide difference spectra of a microsomal fraction prepared from immature pea seeds. Microsomal enzyme preparation as described in text was incubated in the presence of 1 mM Mn^{2+} and 0.5 mM NADPH. (A) baseline, or difference spectrum between identical preparations. (B) difference spectrum after addition of CO to sample cuvette. (C) difference spectrum after addition of dithionite to both cuvettes. (D) difference spectrum after addition of more CO to sample cuvette.


420
450
D
C
B
A
Δ Absorbance
= 0·01
390
410
430
450
470
Wavelength (nm)

at 420 nm.

Cell-free Metabolism of Kaurene Formed in situ

Although the results in Figure 15 indicated that cytochrome P-450 was present in the enzyme extracts and that it was possible to reduce it with dithionite, attempts to observe cell-free oxidation of exogenous kaurene even in the presence of dithionite also yielded negative results.

However, examination of several different enzyme preparations for cell-free metabolism of kaurene formed in situ by assay procedures II and III soon led to some very interesting positive results. The data presented in Table 3 represent the results from one preliminary experiment on differential centrifugation of enzyme extracts. These data were obtained with an enzyme preparation from whole seeds, and show, at least tentatively, that CO did cause an increase (approximately 10%) in the amount of kaurene which accumulated in the 10,000 x g supernatant, whereas in the 40,000 x g supernatant, the difference between CO-inhibited and non-inhibited kaurene accumulation was very small.

The data presented in Table 4 are the results of two independent experiments on the effect of insoluble PVP on kaurene-oxidizing activity in enzyme extracts. The enzymes used in both of these experiments were obtained from isolated cotyledons. In both cases, CO

Table 3. Kaurene accumulation in the presence of carbon monoxide in 10, 000 x g supernatant and 40, 000 x g supernatant.*

Sample	Dpm incorporated/mg protein	
	Kaurene	Polar compounds
S_{10}+CO	2080	11050
S_{10}	1935	12280
Difference	+145	-1230
S_{40}+CO	3590	13400
S_{40}	3525	13600
Difference	+65	-200

* The homogenate used in this experiment was prepared in phosphate buffer (1 ml/g fresh weight) from whole seeds at approximately half-maximal fresh weight which had been frozen in liquid nitrogen and stored at -70°C for several weeks. It should also be noted that the same relationships held when the data were expressed on the basis of per reaction mixture.

Table 4. Kaurene accumulation in the presence of carbon monoxide in extracts prepared with and without PVP.

Fraction	Dpm incorporated/mg protein			
	Experiment 1*		Experiment 2**	
	Kaurene	Polar compounds	Kaurene	Polar compounds
S_{10}+PO_4+CO	7,380	11,600	4,850	11,000
S_{10}+PO_4	5,670	13,400	3,650	14,000
Difference	+1,710	-1,800	+1,200	-3,000
S_{10}+PVP+CO	11,000	18,400	4,600	13,700
S_{10}+PVP	7,400	25,200	3,100	21,000
Difference	+3,700	-6,800	+1,500	-7,300

* The buffer used to prepare the homogenate with PVP in Experiment 1 also contained 0.25 M ascorbate and 1 mM NaCN, all at pH 7.1. 1.5 g wet PVP were mixed with 9 ml buffer and 3 g of isolated cotyledons from freshly harvested seeds (50 seeds). The buffer used for homogenization of the material without PVP was 0.1 M phosphate at pH 7.1.

** The buffer used for both preparations in Experiment 2 contained only phosphate at pH 7.1. 2.5 g wet PVP was mixed with 11 ml of buffer and 4.9 g of isolated cotyledons from freshly harvested seeds (50 seeds).

caused a significant accumulation of kaurene in comparison with the controls, but the inclusion of PVP in the grinding buffer caused an even greater difference between the CO-inhibited preparation and the non-inhibited preparation. 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.

Using assay procedure III, as described under Methods and Materials, an experiment was conducted to determine the actual disappearance of kaurene, and the possible requirement for kaurene to be synthesized in situ before it can be oxidized in these preparations. The results of a representative experiment out of several which were performed are presented in Figure 16. Kaurene which was formed in situ in these reactions during the first 60 minutes, did partially disappear during the second 60-minute period. The disappearance of kaurene appeared to be enzymic, since a boiled control reaction mixture did not show any disappearance of kaurene. It is interesting that addition of 10,000 x g supernatant to the reaction after the first 60 minutes did not stimulate the metabolism of kaurene as compared to a reaction to which buffer was added. In one experiment, not reported here in detail, the addition of boiled enzyme after 60 minutes did not significantly affect the subsequent disappearance of kaurene, as compared to added buffer or added fresh enzyme. Two other interesting observations can be made concerning oxidation of kaurene

Figure 16. Disappearance of kaurene-^{14}C which was synthesized *in situ* in cell-free enzyme extracts. Routine reaction mixtures, each containing 0.03 mM MVA, 0.5 mM ATP, 3 mM Mn^{2+}, and 3 mM Mg^{2+} in a total reaction volume of 1.6 ml, were incubated for 60 minutes, 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 minutes, 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 minutes.

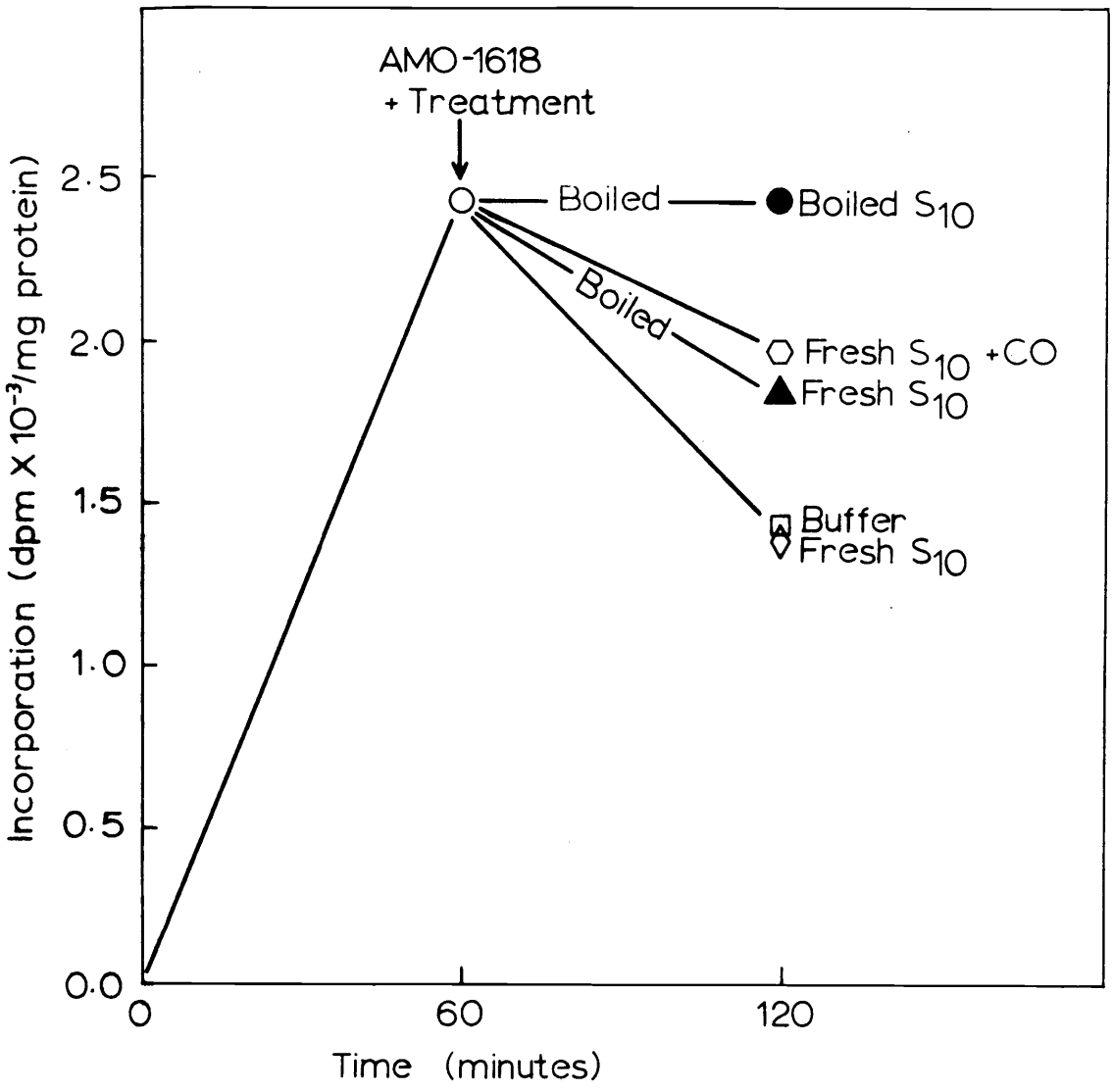
(⬡) 0.75 ml of fresh S_{10} enzyme added and reaction mixture held under CO atmosphere for additional 60 minute incubation.

(▲) reaction mixture boiled and 0.75 ml of fresh S_{10} enzyme added and incubated for an additional 60 minutes.

(◇) 0.75 ml of fresh S_{10} enzyme added and reaction mixture incubated for an additional 60 minutes.

(□) 0.75 ml of buffer added and incubated an additional 60 minutes.

In all cases, "S_{10} enzyme" is supernatant resulting from the centrifugation of extract from isolated cotyledons at 10,000 x g for 15 minutes at 0 to 4°C.


AMO-1618
+ Treatment
Boiled
Boiled S10
Boiled
Fresh S10 +CO
Fresh S10
Buffer
Fresh S10
Incorporation (dpm X 10-3/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
0
60
120
Time (minutes)

synthesized in situ: (a) carbon monoxide at least partially inhibited the metabolism of kaurene; and (b) when the enzyme was boiled after kaurene formation, and then more fresh enzyme was added, the metabolism of kaurene was much less than was observed with only the original enzyme present, or with the original enzyme plus additional enzyme added at 60 minutes.

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.

Identification of ^{14}C-Products

The polar products isolated from large incubations (30-100 ml) after chromatography in hexane and benzene/ethyl acetate (9/1) (Figures 17A and 17B) were divided into three fractions according to the numbering system of Graebe et al. (1965): Fraction 4 being the least polar compounds in benzene/ethyl acetate (9/1) and appearing from R_f 0.7 to 1.0; Fraction 3, the alcohols appearing from R_f 0.3 to 0.7; and Fraction 2, the acids and other very polar materials moving between R_f 0.0 and 0.3. Fraction 1 by this system is kaurene which 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.

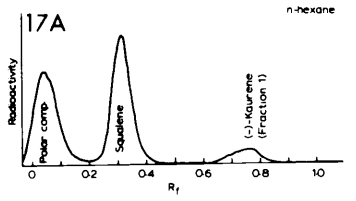
Figure 17. Chromatographic separation of products of MVA metabolism in cell-free enzyme extracts of immature pea seeds. A. Radiochromatogram scan of lipid-soluble products of MVA metabolism after chromatography in hexane. B. Radiochromatogram scan of polar compounds after elution from thin-layer chromatogram 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. (1965).

Figure 18. Thin layer chromatography of Fraction 2. A. Fraction 2 (see Figure 17B) 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 (Figure 18A) and rechromatographed in benzene/ethyl acetate (89/11). C. Material corresponding to the position of kaurenoic acid in Figure 18B was eluted from the gel and rechromatographed in benzene/acetic acid (95/5) with authentic kaurenoic acid.

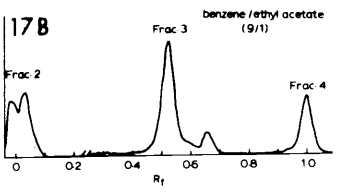
Figure 19. Thin-layer chromatography of Fraction 3. A. Fraction 3 (see Figure 17B) was rechromatographed in hexane/ethyl acetate/propanol (82/15/3). B. The radioactivity corresponding to the position of kaurenol in Figure 19A was rechromatographed in hexane/ethyl acetate/isopropyl ether (2/1/1) on silver nitrate-impregnated silica gel G. C. The radioactive material corresponding to kaurenol in Figure 19B was then rechromatographed in benzene/ethyl acetate (9/1).

Figure 20. Thin-layer chromatography of Fraction 4. A. Fraction 4 was eluted from the thin-layer chromatogram (Figure 17B) and rechromatographed in the same solvent system. B. The gel containing the radioactivity in Figure 20A was eluted and rechromatographed in hexane/propanol (98/2). Arrows point to the positions of published R_f values for kaurenal, which are similar to those obtained in earlier experiments with authentic kaurenal.

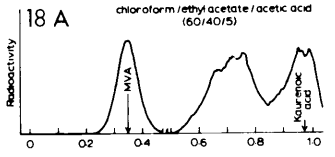
Initial chromatography of extract

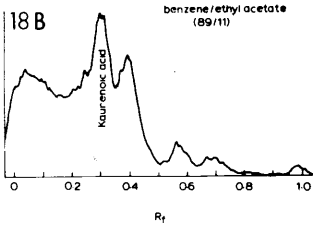


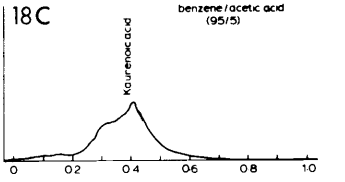
Rechromatography of polar compounds from A



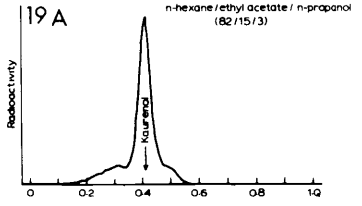
Rechromatography of Fraction 2

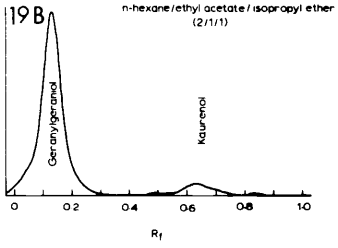


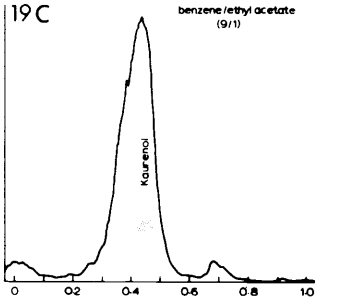




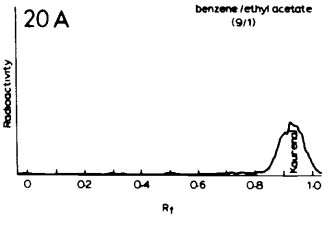
Rechromatography of Fraction 3

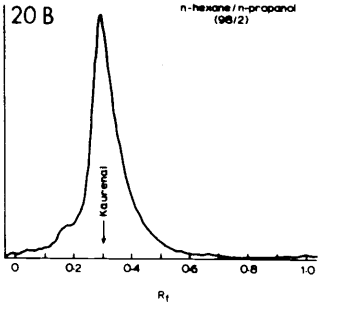






Rechromatography of Fraction 4





Fraction 2, which contains acids and other polar compounds was first chromatographed in chloroform/ethyl acetate/acetic acid (6/4/.5) to separate kaurenoic acid from MVA. As can be observed in Figure 18A, three peaks were resolved. The peak on this radiochromatogram at R_f 0.3 corresponds to the R_f of MVA, and the other two peaks represent at least two other less polar compounds. When the radioactive material at R_f 0.95, which corresponds to the position of kaurenoic acid, was scraped from the plate and rechromatographed in benzene/ethyl acetate (89/11), a peak did appear at R_f 0.3, which corresponds to the position of the authentic kaurenoic acid (Figure 18B). Furthermore, when the radioactive material corresponding to the position of kaurenoic acid on this chromatogram (Figure 18B) was scraped from this plate and eluted and finally rechromatographed in benzene/acetic acid (95/5), the radioactivity again coincided with the position of authentic kaurenoic acid (Figure 18C). No further analysis of this material was attempted.

Fraction 3, which should contain the alcohols, was purified by thin-layer chromatography in three additional solvent systems, as indicated in Figure 20. In each case, authentic kaurenol was co-chromatographed alongside the extract, and the gel containing the radioactive material, which moved with the authentic material, was scraped from the plate, eluted with acetone, and finally rechromatographed in the next solvent system. It can be seen (Figure 19A) that

the bulk of the radioactivity remained with kaurenol through chromatography in hexane/ethyl acetate/propanol (82/15/3). However, after chromatography in hexane/ethyl acetate/isopropyl ether (2/1/1) on silver nitrate-impregnated plates, two peaks were resolved, one corresponding to the position of authentic kaurenol and the major peak corresponding to the position of authentic geranylgeraniol (Figure 19B). The presumptive geranylgeraniol was not characterized further, but the presumptive kaurenol was rechromatographed in benzene/ethyl acetate (9/1) to determine the purity of this compound. It can be seen in Figure 20C that some of the presumptive kaurenol had been degraded during these successive analyses.

Fraction 4 was expected to contain kaurenal, as reported by Dennis and West (1967). Although no authentic kaurenal was available for co-chromatography, the radioactive material from this fraction did move to the appropriate R_f region in two solvent systems (Figure 20A and 20B). These R_f values were determined with a sample of kaurenal which was prepared chemically from kaurenol by the method of Murphy and West (1969), and which was purified by preparative thin-layer chromatography. The R_f values reported agree well with the values previously reported by Dennis and West (1967). It should be noted that kaurenal is a particularly labile compound; the sample prepared by chemical oxidation of kaurenol was degraded more than 50% after standing only 24 hours on a thin-layer plate. This material was

completely degraded after several weeks of storage at -20°C in acetone solution.

Co-crystallization of Presumptive and Authentic Kaurenol

The radioactive material which correspond to the R_f of authentic kaurenol in the final thin-layer chromatogram (Figure 20C) was mixed with approximately 60 mg of authentic kaurenol and recrystallized twice from methanol/water, and three times from methanol. The results from these crystallizations indicated that this mixture was recrystallized to constant specific radioactivity (Table 5). At least 60% of the radioactivity in the original mixture was retained with the crystals of authentic kaurenol.

Table 5. Co-crystallization of presumptive and authentic kaurenol.

Sample	Solvent	Net cpm/mg
Original mixture	methanol/water	619±10
First crystals	methanol/water	492±15
Second crystals	methanol/water	527±18
Third crystals	methanol	438± 1
Fourth crystals	methanol	425±12
Fifth crystals	methanol	412± 7

Discussion

Cell-free Metabolism of Exogenous Kaurene

Repeated attempts were made to observe enzymic oxidation of exogenous kaurene under a wide variety of conditions, all without success. In all these experiments, small amounts of kaurene disappearance were observed, but rarely was the activity of these compounds higher than in control preparations, indicating that the slight losses of kaurene were due almost entirely to non-enzymic reactions. In two large-scale experiments, where 10^5 cpm of kaurene was added as substrate, less then 1% of the kaurene was incorporated into compounds which behaved chromatographically like kaurenol and kaurenal. The inability of these extracts to metabolize exogenous kaurene to any significant extent remains incompletely understood. Two possible reasons for this failure are: (a) binding of the kaurene to non-catalytic protein; and (b) a requirement in this sytem for some type of intimate functional association between the enzymes catalyzing kaurene synthesis and the kaurene oxidizing enzymes; or to a combination of these effects. In view of evidence which is discussed later in detail, the failure of exogenous kaurene to be metabolized is thought tentatively to be due mainly to the latter of these possible causes.

The involvement of cytochrome P-450 in kaurene oxidation in E. macrocarpa endosperm extracts (Murphy and West, 1969) prompted an inspection of the carbon monoxide difference spectrum of pea seed microsomes. The presence of P-450 in a microsomal fraction was tentatively confirmed, and it was readily reduced by dithionite. The observed failure of exogenous NADPH to reduce P-450 is interpreted as meaning that either NADPH is not the natural donor in this system, or that some enzyme in the microsomal electron transport chain was not active in the preparation. The latter alternative seems most likely, since NADPH is the natural donor, or at least the most active donor, in most of the mixed function oxidase reactions which have been reported. The latter alternative also seems to be most likely in light of the vast evidence for the lability of P-450 and complexes containing P-450 in both animal and plant systems (e.g. Ichikawa, Uemara and Yamano, 1968; and Frear, Swanson and Tanaka, 1969). One attempt was made to bypass NADPH-reduction of P-450 by the addition of dithionite to a reaction mixture, but this effort also resulted in failure. Whereas data on the microsomal fraction reveal that P-450 was not reducible in that preparation by NADPH, the observed enzymic oxidation of kaurene synthesized in situ in 10,000 x g supernatants indicated that the cytochrome was apparently participating in redox reactions in those enzyme extracts.

Cell-free Metabolism of Kaurene Formed *in situ*

During further attempts to observe metabolism of kaurene in cell-free extracts of immature pea seeds, two new assays were developed for the measurement of kaurene disappearance. Both assays make use of kaurene formed from MVA *in situ* combined with the action of one or more inhibitors.

Assay procedure II makes use of carbon monoxide gas as an inhibitor. This assay admittedly is limited by the assumption that CO inhibits the first reaction in the oxidative metabolism of kaurene, and does not inhibit the production of kaurene from MVA in these preparations. The first step in the oxidative metabolism of kaurene in extracts of *E. macrocarpa* endosperm is the formation of kaurenol, and is strongly inhibited by CO (Murphy and West, 1969). And, as shown in Tables 4 and 5, this gas does not inhibit the accumulation of kaurene in extracts of pea seeds. Therefore, the difference in kaurene accumulation between inhibited and non-inhibited reaction mixtures containing the same enzyme extract is considered to be a true indication of the amount of kaurene which is metabolized by CO-sensitive enzymes. The preliminary results obtained with this assay (Table 4) indicated that the difference in kaurene accumulation between CO-inhibited and non-inhibited reactions was greater when 10,000 x g supernatant was used than when 40,000 x g supernatant was utilized.

This was true in spite of the fact that, as expected, far more kaurene accumulated in the presence of 40,000 x g supernatant than in the presence of the 10,000 x g supernatant. If, indeed, the difference between inhibited and non-inhibited kaurene accumulation is due to the enzymic degradation of kaurene, this result is indicative that particulate enzymes are involved in kaurene oxidation. This would be the expected result in view of the evidence for the occurrence of kaurene-oxidizing enzymes in the microsomal fraction in E. marocarpa (Murphy and West, 1969).

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. This result with PVP is not surprising when it is noted that Frear, Swanson and Tanaka (1969) 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 also thought to involve cytochrome P-450.

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 kaurene biosynthesis (e.g. Upper and West, 1967; Shechter and West, 1969; Anderson and Moore, 1967; Graebe, 1968) and carbon monoxide, an

inhibitor of kaurene oxidation (Murphy and West, 1969). Kaurene is allowed to be synthesized endogenously in 10,000 x g supernatant for 60 minutes, after which its further synthesis is blocked by AMO-1618. The use of carbon monoxide in a subsequent 60-minute incubation period permits measurement of enzymic oxidation of endogenous kaurene. By this procedure it was demonstrated that:

(1) Kaurene which is formed in situ in the first 60 minutes does disappear during the subsequent 60 minutes of the incubation in the presence of the original enzyme.

(2) The disappearance of kaurene during the second 60 minutes is dependent upon some heat labile substance(s).

(3) Adding fresh enzyme extract after 60 minutes does not stimulate the disappearance of kaurene.

(4) The disappearance of kaurene during the second 60 minutes is at least partially sensitive to carbon monoxide.

(5) Heat denaturation of the original enzyme after the first 60 minutes causes a reduced level of kaurene disappearance during the second 60 minutes, even when additional 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 four experiments, the difference in kaurene disappearance between the 60-minute incubation and the 120-minute incubation with added enzyme ranged from

25-66% of the kaurene present at 60 minutes. The differences in kaurene disappearance in these experiments was not correlated with the amount of kaurene which had formed in 60 minutes, 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. As expected, there was no difference between the 60-minute preparations and the extracts which were boiled and to which more boiled enzyme was added. The 60-minute preparations were actually held in the water bath with the other reaction tubes throughout the 120-minute period; then buffer was added before extraction, so that all extractions were from equal volumes of reaction mixtures. It is not known whether some breakdown of kaurene was occurring in all preparations simultaneously; only the amount of extractable kaurene was determined. The result with additional enzyme added after 60 minutes, as compared to buffer added at this time, indicated that the additional enzyme did not contribute to additional disappearance of kaurene. This result would imply that the enzymes originally present were present in saturating concentration. This is particularly interesting since, in one case, the enzyme concentration was nearly doubled, while in the other case the enzyme was diluted. It is interesting to spectulate that perhaps the kaurene which was formed at 60 minutes was already attached to the complex of oxidizing enzymes before the addition of more enzyme or buffer.

The inhibition of the kaurene disappearance by carbon monoxide varied from 70-120% in three experiments. This variability was probably due to variable effectiveness of the CO treatments, but may also be due to metabolism of kaurene which is insensitive to carbon monoxide. Murphy and West (1969) have shown that an atmosphere of 100% CO completely inhibits the conversion of kaurene to kaurenol in extracts of E. macrocarpa, but 90% CO only inhibits about 75%. The reactions described here were gassed for about 30 seconds before serum caps were placed on the tubes; then after stirring, the atmosphere above the reaction mixture was again replaced with CO for an additional 1 minute by means of two syringe needles. By this technique, it cannot be said with certainty that the atmosphere in these reaction mixtures was 100% carbon monoxide, but it was sufficient to cause a consistent inhibition of kaurene disappearance. It should also be noted that a variable amount of time (up to 20 minutes) elapsed between the addition of AMO-1618 to all reaction mixtures, and the administration of carbon monoxide gas.

The result obtained by heat denaturing the original enzyme and then adding fresh enzyme is very interesting. In two experiments, the amounts of disappearance of kaurene in these preparations were 28% and 37%, respectively. The disappearance of kaurene in these cases did not appear to be dependent upon the amount of kaurene present at 60 minutes. In these two experiments, it was clear that these

preparations catalyzed much less kaurene disappearance than the preparations in which the original enzymes were left intact (44% and 66%, respectively). A possible explanation for this effect 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 concept is compatible with the suggestion made previously 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 is added exogenously. It is interesting to note that Scallen, Schuster and Dhar (1970) have reported evidence for a " carrier" substance in the 105,000 x g supernatant of rat liver which is required for the conversion of squalene to sterol. This substance is heat labile and required in several microsomal oxidations in cholesterol biosynthesis.

Identification of Products of Cell-free MVA Metabolism

The products of MVA metabolism in large scale reaction

mixtures containing 10,000 x g supernatant as the enzyme extract were extracted from neutral buffer with acetone/benzene (3/1) and from acidified buffer (pH 2.5) with ethyl acetate and subjected to thin-layer chromatographic analysis. Preliminary thin-layer chromatography of these extracts has led to the separation of several radioactive products, among which were tentatively identified geranylgeraniol, kaurenol, kaurenal and kaurenoic acid. An unidentified product also was observed which was more polar than kaurenoic acid but less polar than MVA. The identification of kaurenol-^{14}C was confirmed by co-crystallization of the radioactive compound with authentic kaurenol to constant specific radioactivity. Further proof of the identity of the other products in these reaction mixtures was not obtained. It is notable that none of these products was observed in large scale reactions for kaurene biosynthesis using 40,000 x g supernatant of immature pea seed homogenates.

The identification of kaurenol and the tentative identification of other oxidized kaurene derivatives is not surprising, since each of these products has been identified as a product of MVA and kaurene metabolism in cell-free extracts of E. macrocarpa endosperm (Dennis and West, 1967). However, these products were obtained in the present case with extracts from immature pea seeds only with MVA as substrate, and not with exogenous kaurene. The intriguing 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 functionally associated with the "soluble" kaurene-synthesizing enzymes is the subject of continuing investigations.

SUMMARY AND CONCLUSIONS

The purposes of these investigations were to devise procedures and determine optimum conditions for assaying the biosynthesis of kaurene-^{14}C and other intermediates in GA biosynthesis from mevalonic acid-^{14}C in cell-free enzyme extracts of immature Alaska pea seeds, and to use this system to investigate three rather distinct but related aspects of gibberellin biosynthesis: (1) apparent capacities for kaurene biosynthesis in extracts of immature pea seeds at various stages of development; (2) localization of the enzymes which catalyze kaurene biosynthesis in immature pea seeds; and (3) further metabolism of kaurene in extracts of immature pea seeds.

It was readily demonstrated that MVA was incorporated into kaurene, and also into squalene (identification tentative), in extracts of immature Alaska pea seeds. The synthesis of kaurene was shown to be approximately linear with time through 75 minutes at 30°C, to vary directly with enzyme concentration, and to be dependent on ATP. Both Mg^{2+} and Mn^{2+} stimulated the reaction, but Mn^{2+} was a much better activator than was Mg^{2+}. The optimum pH was found to be 7.1.

The capacity to convert MVA to kaurene, as measured in cell-free extracts as described above, was found to vary markedly with the stage of seed development. Using enzyme extracts prepared from seeds at different stages of development, it was observed that the activity increased from a very low initial level to a maximum at about

13 days after anthesis, or when the seeds had attained about half-maximum fresh weight, and then declined as the seeds approached maximum diameter and fresh weight. When these data are compared with available data on extractable GA contents of various seeds, it is seen that the quantity of extractable GA and the apparent rate of kaurene synthesis from MVA both appear to increase sharply during the development of leguminous seeds and to reach maximum values when the seeds have attained about half-maximal fresh weight. Just as the growth curves for legume pod and seed do not coincide in time, maturation of the seed lagging behind that of the pod, it seems likely that the time courses of kaurene and GA synthesis and GA accumulation in the pod and seed are similarly related.

Utilizing enzyme extracts prepared from excised seed coats, cotyledons and embryonic shoot-root axes, it was determined that the enzymes responsible for kaurene biosynthesis apparently are localized exclusively in the cotyledons of pea seeds. It was observed that the rates of kaurene biosynthesis in extracts of isolated cotyledons were higher than those observed in extracts of whole seeds. This effect was not investigated, but it is assumed that there is some material in extracts of whole seeds which is not present in extracts of cotyledons, that interferes with kaurene synthesis. The enzymes responsible for the synthesis of squalene (identification tentative) in this system were distributed between the seed coats and the cotyledons.

These results are in contrast to those of Graebe et al. (1965) which showed that kaurene biosynthesis occurred only in extracts of the free-nuclear endosperm-nucellus tissue of immature Echinocystis macrocarpa seeds, and that squalene biosynthesis occurred only in extracts of embryo tissues.

Several experiments employing differential centrifugation of enzyme extracts were conducted. In extracts of whole seeds kaurene synthesis activity was higher in the 40, 000 x g supernatant than in the 100, 000 x g supernatant. However, in extracts of excised cotyledons, the activity was higher in the 100, 000 x g supernatant than in the 40, 000 x g supernatant. The unexpected but very reproducible results with differential centrifugation of extracts of whole seeds remain unexplained.

Repeated efforts to demonstrate cell-free metabolism of exogenous kaurene resulted in uniformly negative results. However, when kaurene was formed in situ from MVA, its oxidation was observed. One preliminary experiment indicated that this metabolism is localized in the microsomal fraction, but this result was not confirmed. It was shown in two independent experiments that the difference in kaurene accumulation between carbon monoxide-inhibited preparations and non-inhibited preparations was greater in extracts which were prepared by grinding with insoluble polyvinylpyrrolidone (PVP) than in extracts prepared without PVP. Although these

experiments are not considered to provide direct evidence, they are interpreted tentatively to mean that kaurene metabolism in these extracts is protected from inhibition by the inclusion of insoluble PVP in the grinding medium. Other data indicated that the metabolism of kaurene formed in situ was enzymic, or at least heat-labile, and at least partially inhibited by carbon monoxide.

The products of MVA metabolism in these cell-free enzyme extracts were isolated and tentatively identified as geranylgeraniol, kaurenol, kaurenal, and kaurenoic acid. The identity of kaurenol was confirmed by co-crystallization of the radioactive product with authentic kaurenol to constant specific radioactivity. While kaurenol, kaurenal and kaurenoic acid were synthesized from mevalonic acid-^{14}C as the exogenous substrate, it is assumed, of course, that kaurene was a direct precursor of all three compounds. No evidence was found for the accumulation of geraniol or farnesol as products of MVA metabolism in these enzyme extracts. These products of MVA metabolism in extracts of pea seeds thus appear to be identical to those found in extracts of E. macrocarpa (Dennis and West, 1967).

The inability of the enzyme extracts to metabolize exogenous kaurene remains incompletely understood. Two possible reasons for this failure are: (a) binding of the kaurene to noncatalytic protein; and (b) a possible requirement in this system for some type of intimate functional association between the enzymes catalyzing kaurene

synthesis and the enzymes catalyzing kaurene oxidation.

Regardless of the inability of these enzyme extracts to metabolize exogenous kaurene, they are capable of forming kaurene, kaurenol, and probably kaurenal, kaurenoic acid and other compounds which have been shown to be intermediates in GA biosynthesis in the fungus Gibberella fujikuroi and which probably are intermediates also in this pathway in higher plants. These studies are being continued to confirm the identify of kaurenal and kaurenoic acid, and to attempt to identify further intermediates in this pathway. Of course the long term goals of this research are to achieve cell-free biosynthesis of GA's in order to study the natural controls operative on the synthesis and metabolism of these hormones.

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