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PARTICIPATION OF NONCATALYTIC 'CARRIER' PROTEIN IN THE METABOLISM OF KAURENE IN CELL-FREE EXTRACTS OF PEA SEEDS*

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Key Word Index—*Pisum sativum*; Leguminosae; biosynthesis; diterpenes; (−)-kaurene; gibberellins; mixed function oxidases.

Abstract—Kaurene-^{14}C, when synthesized from mevalonate-2-^{14}C in reaction mixtures containing ATP, Mg^{2+}, Mn^{2+} and 100 000 *g* supernatant of extracts of cotyledons of immature pea (*Pisum sativum* L. cv. Alaska) seeds, became bound in a large proteinaceous aggregate (estimated $> 15 \times 10^6$ daltons). The kaurene-protein complex was readily produced also by incubating exogenous kaurene directly with 100 000 *g* supernatant. The complex served as a substrate for carbon monoxide-sensitive oxidation by heat-labile preparations of pea seed microsomes under conditions where free exogenous kaurene-^{14}C did not undergo enzymic oxidation. These results suggest that a noncatalytic 'carrier' protein fraction, possibly solubilized from endoplasmic reticulum, combine with kaurene and render the lipophilic diterpene accessible to oxidation by mixed function oxidases in the microsomal fraction. The kaurene-protein complex fractions isolated on columns of agarose also contained squalene, which underwent enzymic oxidation under the same conditions as did kaurnene.

INTRODUCTION‡

IT WAS reported in a previous paper[1] that the tetracyclic diterpene kaurene and three oxidized derivatives which also serve as intermediates in gibberellin biosynthesis[2]—kaurenol, kaurenal and kaurenoic acid—were synthesized from MVA-2-^{14}C in cell-free reaction mixtures containing 10 000 *g* supernatant of extracts of cotyledons of immature pea (*Pisum sativum* L. cv. Alaska) seeds. At that time it was already known that the enzymes catalysing the biosynthesis of kaurene occur in particle-free supernatant fractions of homogenates of the liquid endosperm of immature *Echinocystis macrocarpa* seeds,[3] seedlings of *Ricinus communis*,[4] immature seeds of *Cucurbita pepo*,[5] immature seeds and fruits of *Pisum sativum*[6,7] and the fungus *Gibberella fujikuroi*.[8] Further transformations of kaurene to kaurenol,

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‡ *Abbreviations used*: MVA = mevalonic acid; kaurene, kaurenol, kaurenal and kaurenoic acid refer specifically to (−)-kaur-16-ene, (−)-kaur-16-en-19-ol, (−)-kaur-16-en-19-al and (−)-kaur-16-en-19-oic acid, respectively.

[1] R. C. COOLBAUGH and T. C. MOORE, *Phytochem.* **10**, 2401 (1971).
[2] A. LANG, *Ann. Rev. Plant Physiol.* **21**, 537 (1970).
[3] C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **242**, 3285 (1967).
[4] D. R. ROBINSON and C. A. WEST, *Biochemistry* **9**, 70 (1969).
[5] J. E. GRAEBE, *Planta* **85**, 171 (1969).
[6] J. E. GRAEBE, *Phytochem.* **7**, 2003 (1968).
[7] R. C. COOLBAUGH and T. C. MOORE, *Phytochem.* **10**, 2395 (1971).
[8] I. SHECHTER and C. A. WEST, *J. Biol. Chem.* **244**, 3200 (1969).

kaurenal and kaurenoic acid in the *E. macrocarpa* system had been shown to require catalysis by mixed function oxidases in the particulate microsomal fraction.[9,10]

Enzymic oxidation of exogenous kaurene-^{14}C and kaurenal-^{14}C to expected products was readily demonstrated in preparations of microsomes from *E. macrocarpa*.[9,10] However, free exogenous kaurene did not undergo enzymic oxidation in cell-free preparations from pea seeds under any conditions which were tested.[1] Indirect evidence for enzymic oxidation was obtained only when kaurene was synthesized *in situ* from MVA in reaction mixtures containing 10 000 *g* supernatant. In seeking to explain these results, the hypothesis was advanced[1] that kaurene oxidation by microsomal enzymes in the system from pea cotyledons depends upon the substrate being bound to a (heat labile) moiety, i.e. a 'carrier protein', which is somehow functionally associated with the soluble enzymes which catalyse kaurene biosynthesis.

Such a hypothesis is not without precedent in terpene biochemistry. Scallen *et al.*[11–13] and Ritter and Dempsey[14–16] have characterized rather extensively a noncatalytic carrier protein, 'Sterol Carrier Protein' (SCP), which they have reported to be involved in the conversion of squalene to cholesterol by microsomal enzymes of rat liver. This paper presents evidence for analogous participation of a noncatalytic, soluble protein carrier in the conversion of kaurene to other diterpenoid intermediates in gibberellin biosynthesis by microsomal enzymes in cell-free extracts of immature pea seeds.

RESULTS

Binding of Kaurene-^{14}C to Soluble Protein and to Microsomal Fraction in vitro

Several experiments were performed to ascertain with what component, if any, of cell-free extracts kaurene-^{14}C would bind (Table 1). When kaurene-^{14}C was formed from

TABLE 1. ASSOCIATION OF KAURENE-^{14}C WITH SOLUBLE PROTEIN AND WITH MICROSOMES *in vitro*

Reaction conditions prior to ultracentrifugation	Ultracentrifugation at 100 000 *g* (min)	Kaurene-^{14}C in system (dpm)	Distribution after centrifugation (dpm) Supernatant	Pellet
Kaurene-^{14}C formed from MVA-2-^{14}C in S_{10} for 60 min	60	2017	52	1401
Exogenous kaurene-^{14}C incubated in S_{10} for 15 min	60	no det.	122	1638
Kaurene-^{14}C formed from MVA-2-^{14}C in S_{100} for 60 min	60	8250	6200	2000
	120	8250	5250	1910
^{14}C-KP incubated with resuspended microsomes	60	835	51	680
Exogenous kaurene-^{14}C incubated with resuspended microsomes	60	1500	130	1330

[9] D. T. DENNIS and C. A. WEST, *J. Biol. Chem.* **242,** 3293 (1967).
[10] P. J. MURPHY and C. A. WEST, *Arch. Biochem. Biophys.* **133,** 395 (1969).
[11] T. J. SCALLEN, M. W. SCHUSTER and A. K. DHAR, *J. Am. Oil Chem. Soc.* **47,** 85A (1970).
[12] T. J. SCALLEN, M. W. SCHUSTER and A. K. DHAR, *Federation Proc.* **29,** 673 Abs (1970).
[13] T. J. SCALLEN, M. W. SCHUSTER and A. K. DHAR, *J. Biol. Chem.* **246,** 224 (1971).
[14] M. C. RITTER and M. E. DEMPSEY, *Federation Proc.* **29,** 673 Abs (1970).
[15] M. C. RITTER and M. E. DEMPSEY, *Biochem. Biophys. Res. Commun.* **38,** 921 (1970).
[16] M. C. RITTER and M. E. DEMPSEY, *J. Biol. Chem.* **246,** 1536 (1971).

MVA-2-^{14}C in reaction mixtures containing 10 000 *g* supernatant (S_{10}) for 60 min and the reaction mixture subsequently centrifuged at 100 000 *g* for 60 min, practically all the acetone-benzene extractable kaurene-^{14}C was recovered from the microsomal pellet. The same result was obtained when exogenous kaurene-^{14}C was incubated with S_{10} for 15 min prior to ultracentrifugation. If kaurene-^{14}C was produced from MVA-2-^{14}C in a particle-free 100 000 *g* supernatant (S_{100}) mixture and the reaction mixture then was centrifuged at 100 000 *g* for 2 hr, most of the extractable kaurene-^{14}C remained in the supernatant. However, experiments to be described later revealed that under those circumstances the kaurene was bound to a soluble protein fraction, designated kaurene-protein complex (KP). When ^{14}C-KP, which was isolated by gel filtration chromatography, was incubated with resuspended microsomes in phosphate buffer and the mixture then centrifuged at 100 000 *g* for 60 min, nearly all the extractable kaurene-^{14}C again was recovered from the pellet fraction.

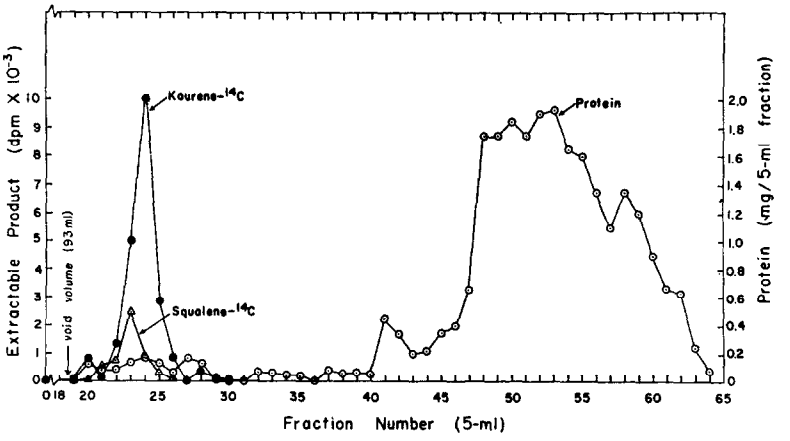

FIG. 1. ELUTION PROFILE OF 100 000 *g* SUPERNATANT PROTEIN AND ENDOGENOUSLY SYNTHESIZED KAURENE-^{14}C AND SQUALENE-^{14}C ON BIO-GEL A-5m AGAROSE.

Kaurene-^{14}C and squalene-^{14}C were produced from mevalonate-2-^{14}C in reaction mixtures containing S_{100} enzyme extract for 60 min. Then 6·2 ml of reaction mixture containing 25·6 mg of protein, 31 723 dpm of kaurene-^{14}C and an undetermined amount of squalene-^{14}C was applied to a 3·9 × 29 cm column of agarose (exclusion limit $\simeq 5 \times 10^6$) and eluted with potassium phosphate buffer (0·1 M, pH 7·1) in 5-ml fractions at a flow rate of 1·5 ml/min. Eluate fractions were analysed for kaurene-^{14}C and squalene-^{14}C by acetone–benzene extraction, TLC and liquid scintillation counting. Protein determinations were by the method of Lowry *et al.*[20] In the experiment illustrated 67% of the kaurene-^{14}C present in the original sample was recovered in fractions 22–26 (110–130 ml elution volume); the amount of protein in those fractions was 0·6 mg, which represents 2·2% of the total protein applied to the column. Fractions 22–26 also contained a total of 4760 dpm of squalene-^{14}C.

These results (Table 1) are in agreement with the hypothesis that, under normal conditions of its biosynthesis from MVA *in vitro*, kaurene binds to a protein component of S_{100} and that the carrier protein renders the kaurene accessible to microsomal oxidases. Noteworthy also is the fact that exogenous kaurene-^{14}C became bound to microsomes resuspended in phosphate buffer (0·1 M, pH 7·1) independently of the soluble fraction of enzyme extracts (Table 1). However, the free hydrocarbon does not undergo enzymic oxidation under the latter conditions.[1]

Isolation of Kaurene-Protein Complex by Gel Filtration Chromatography

Kaurene-^{14}C was synthesized from MVA-2-^{14}C in reaction mixtures containing S_{100} enzyme extract, and the reaction mixtures subsequently were fractionated on columns of agarose. Eluate fractions were analyzed for protein, acetone–benzene extractable kaurene-^{14}C and, in some cases, extractable squalene-^{14}C. On agarose with exclusion limits of approximately $1{\cdot}5 \times 10^6$, 5×10^6 (Fig. 1) and 15×10^6, all the kaurene-^{14}C recovered was associated with protein which was eluted immediately after the void volume. In the experiment represented in Fig. 1, 67% of the total kaurene-^{14}C applied to the column was recovered with 2·2% of the total protein in five 5 ml fractions. In all cases where analyses were made also for squalene, this triterpene was found to be also associated with protein in eluate fractions that either closely coincided with or overlapped those containing kaurene-^{14}C.

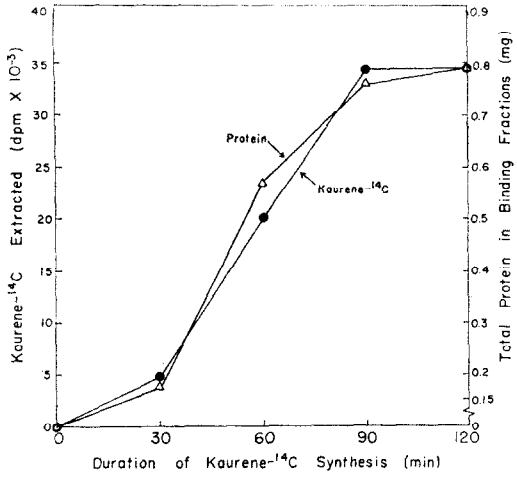

FIG. 2. TIME COURSE OF FORMATION OF KAURENE-^{14}C-PROTEIN COMPLEX IN S_{100} ENZYME EXTRACTS OF IMMATURE PEA COTYLEDONS.

Kaurene-^{14}C was synthesized from MVA-2-^{14}C in standard reaction mixtures (see Experimental) for the indicated times. At the end of each time interval 6·25 ml of reaction mixture containing approximately 25 mg of protein and kaurene-^{14}C was applied to a column of Bio-Gel A-5m and eluate fractions were analysed as described in Fig. 1. The amounts of kaurene-^{14}C and protein shown at each time are the totals recovered in eluate fractions 22–26 (110–130 elution volume). Approximately 70% of the kaurene-^{14}C applied to each column was recovered from kaurene-^{14}C-protein complex. The time course of accumulation of squalene-^{14}C in the protein aggregate of fractions 22–26 was not precisely determined; however, the total extractable squalene-^{14}C isolated from fractions 22–26 at 90 min was 23 480 dpm.

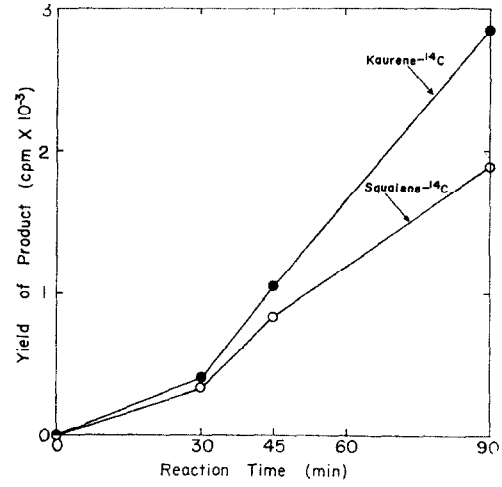

FIG. 3. TIME COURSES OF NET SYNTHESIS OF KAURENE-^{14}C AND SQUALENE-^{14}C FROM MVA-2-^{14}C IN STANDARD REACTION MIXTURES CONTAINING 100 000 *g* SUPERNATANT OF EXTRACTS OF IMMATURE PEA COTYLEDONS.

Standard reaction mixtures containing MVA-2-^{14}C, ATP, Mg^{2+} Mn^{2+} and S_{100} enzyme extract (see Experimental) were incubated for the indicated times. Yields of kaurene-^{14}C and squalene-^{14}C were determined by acetone–benzene extraction, TLC, radiochromatogram scanning, elution and liquid scintillation counting.

Kinetics of ^{14}C-KP Formation

The time course of ^{14}C-KP formation was investigated by synthesizing kaurene-^{14}C from MVA-2-^{14}C in reaction mixtures containing S_{100} enzyme extract for various times and

analyzing the yields of kaurene-^{14}C and protein in ^{14}C-KP isolated on a column of Bio-Gel A-5m (Fig. 2). There was a direct correlation between the kinetics of accumulation of both kaurene-^{14}C and protein in ^{14}C-KP, indicating that the formation of KP results directly from the binding of kaurene to a relatively low MW protein fraction, which is thereby caused to aggregate. It was repeatedly determined in other experiments that a kaurene-protein complex indistinguishable from the KP generated endogenously readily is formed by incubating exogenous kaurene-^{14}C in S_{100}.

The kinetics of accumulation of squalene-^{14}C in the ^{14}C-KP fractions isolated in the experiment summarized in Fig. 2 were not precisely determined. However, the total extractable squalene-^{14}C in the ^{14}C-KP fractions at 90 min was 23 480 dpm, or about 68% of the amount of radioactivity present in kaurene.

The time courses of both kaurene-^{14}C and squalene-^{14}C synthesis in reaction mixtures containing S_{100} of extracts of pea cotyledons were determined in other experiments where the yields of both products were measured by direct acetone–benzene extraction, TLC and liquid scintillation counting. The results of a representative experiment (Fig. 3) show a marked resemblance between the kinetics of kaurene-^{14}C synthesis (Fig. 3) and kaurene accumulation in ^{14}C-KP (Fig. 2). Probably the same correlation pertains to squalene, although this was not precisely ascertained.

Identification of Squalene

Previously it was stated that the fractions of protein containing kaurene-^{14}C which were isolated by gel filtration chromatography also contained squalene-^{14}C. The product identified as squalene-^{14}C exhibited identical mobility to authentic squalene on TLC in four solvents.[17] Squalene-^{14}C was identified also by mixing the radioactive product eluted from TLC plates with authentic unlabeled squalene, forming the hexahydrochloride isomerides, and recrystallizing the hot acetone-soluble isomerides to constant specific radioactivity.[6,18,19] Radioactive material (149 700 cpm) was mixed with unlabeled squalene (250 mg) in acetone, which was maintained saturated with gaseous HCl at −5° for 1 hr, to give a specific activity of 599 cpm/mg. After preparation of the hexahydrochloride mixture, four recrystallizations of the hot acetone-soluble isomerides from fresh acetone yielded a constant specific activity of 353 cpm/mg, indicating that a minimum of 59% of the radioactive product was squalene-^{14}C. These results, combined with the report by Graebe[6] that squalene was formed from MVA-2-^{14}C in particle-free enzyme extracts of young pea fruits and seeds, are taken as reasonable identification of squalene.

Enzymic Oxidation of Kaurene-^{14}C

It was reported previously[1] that kaurene-^{14}C did not undergo enzymic oxidation when free exogenous kaurene-^{14}C was added to enzyme preparations. This result was readily confirmed in the present investigations when exogenous kaurene-^{14}C was added, in *para*-dioxane solution, to a preparation of microsomes in the presence of molecular oxygen, NADPH and Mg^{2+} (Table 2). On several occasions as much as 20% or more exogenous kaurene-^{14}C was transformed to unidentified products, but this was not oxidation by mixed function oxidases since the loss was neither inhibited by CO nor dependent upon heat-labile protein.

[17] R. C. Coolbaugh, Ph.D. Thesis, Oregon State University, Corvallis (1970).
[18] I. M. Heilbron, E. D. Kamm and W. M. Owens, *J. Chem. Soc.* 1630 (1926).
[19] E. Capstack, Jr., D. J. Baisted, W. W. Newschwander, G. Blondin, N. L. Rosin and W. R. Nes, *Biochemistry* **1**, 1178 (1962).

In contrast to the results with exogenous kaurene-^{14}C, kaurene-^{14}C as endogenously generated ^{14}C-KP did serve as a substrate for CO-sensitive enzymic oxidation in microsomal preparations (Table 2). The percentage oxidation did not exceed approximately 30% and the percentage loss which was CO-sensitive rarely exceeded 20%. However, the result was seen consistently in several experiments, and the percentages oxidation observed were not greatly different from those reported for exogenous kaurene-^{14}C in microsomal preparations from *Echinocystis macrocarpa* endosperm.[9,10] Quite probably the reactions were limited by rapid inactivation of the microsomal oxidases at the reaction temperature. The products of CO-sensitive kaurene oxidation were not identified in these experiments, but on the basis of previous results,[1] it is reasonable to conclude that kaurenol was the first product.

TABLE 2. MICROSOMAL OXIDATION OF KAURENE-^{14}C *in vitro*

Form and amount of kaurene-^{14}C	Experimental conditions	Kaurene-^{14}C recovered (dpm)	% Decrease
Endogenous KP, 1110 dpm	Complete	760	32
	Complete + CO	970	13
	Complete, boiled enzymes	1095	1
Endogenous KP, 3505 dpm	Complete	2580	27
	Complete, zero time	3300	6
	Complete, boiled enzymes	3320	5
Boiled endogenous KP, 1175 dpm	Complete	685	42
	Complete + CO	970	18
	Complete, boiled enzymes	1055	10
Boiled endogenous KP, 1155 dpm	Complete	905	22
	Complete + CO	1020	12
	Complete, boiled enzymes	1070	7
Exogenous KP, 1065 dpm	Complete	710	33
	Complete + CO	970	18
	Complete, boiled enzymes	870	18
Exogenous K, 3410 dpm	Complete	2705	21
	Complete + CO	2685	21
	Complete, boiled enzymes	2705	21

A typical complete reaction mixture utilizing ^{14}C-KP contained 0·5 ml kaurene-^{14}C-protein complex, 1 μmol $MgCl_2$, 0·5 μmol NADPH and 0·5 ml microsomal suspension in a total vol. of 1·1 ml 0·1 M KH_2PO_4–K_2HPO_4 buffer (pH 7·1). Exogenous kaurene-^{14}C was added, in 0·05 ml *para*-dioxane, to a 1·0 ml reaction mixture containing 0·75 ml microsomal suspension, 1 μmol $MgCl_2$ and 0·5 μmol NADPH. Reaction mixtures were incubated 60–90 min at 34°.

Kaurene-^{14}C supplied as ^{14}C-KP which was formed by the direct incubation of kaurene-^{14}C with 100 000 *g* supernatant also served as a substrate for a small percentage of apparent enzymic oxidation (Table 2). And kaurene-^{14}C supplied as boiled, endogenously generated ^{14}C-KP also was a utilizable form in which to supply the substrate to microsomal preparations. The latter result is consistent with the absence of any detectable catalytic activity of KP for either kaurene synthesis or oxidation.

Preliminary experiments have indicated that squalene-^{14}C associated with endogenously generated ^{14}C-KP also undergoes enzymic oxidation in preparations of microsomes from the cotyledons of immature pea seeds. This oxidation also appears to be at least partially sensitive to inhibition by CO and is not precluded by boiling the ^{14}C-KP.

DISCUSSION

The collective results of these experiments show that kaurene-^{14}C synthesized from MVA-2-^{14}C in reaction mixtures containing 100 000 *g* supernatant of enzyme extracts from immature pea seed cotyledons becomes bound in a large proteinaceous complex (KP). Kaurene, supplied either as undenatured or boiled KP, undergoes CO-sensitive oxidation in heat-labile preparations of microsomes in the presence of molecular oxygen and NADPH. Although exogenous kaurene-^{14}C can readily be demonstrated to bind to the microsomal fraction, exogenous kaurene does not undergo CO-sensitive, enzymic oxidation. Thus a soluble protein fraction, present in 100 000 *g* supernatant of pea seed extracts, appears to function as a noncatalytic 'carrier' which binds kaurene, thereby making the diterpene substrate reactive to mixed function oxidases in the microsomal fraction. Minimally, the role of KP would appear specifically to be to facilitate the transfer of the water-insoluble kaurene, as a water-soluble complex, to active sites on the microsomal oxidases, but a more complicated role cannot at present be excluded.

Several questions immediately arise concerning the relationship of KP in the pea seed system to kaurene metabolism in other systems,[9,10] and the relationship of the kaurene carrier protein to the sterol carrier protein which has been investigated extensively by others.[11–16]

Exogenous kaurene-^{14}C was reported to be readily oxidized enzymically in microsomal preparations of liquid endosperm of immature *Echinocystis macrocarpa* seeds,[9,10] and no dependence on carrier protein was apparent. Interestingly, Dennis and West[9] found that a boiled S_{105} fraction effectively substituted for NADPH in stimulating oxidation of exogenous kaurene-^{14}C. However, there is no evidence that microsomal oxidation of exogenous kaurene-^{14}C in the *E. macrocarpa* system was actually dependent upon the supernatant fraction. Therefore there appears to be a more probable explanation for the different results obtained with exogenous kaurene in microsomal preparations from *E. macrocarpa* and pea seeds. That is, that the kaurene-binding protein fraction of pea seeds was inadvertently liberated from the microsomal fraction during preparation, whereas the binding protein fraction was not extensively solubilized in preparing microsomes from the liquid endosperm of *E. macrocarpa* seeds. If this tentative explanation is correct, the kaurene-protein complex may be an artifact of biochemical procedures, albeit one which nevertheless should be useful in understanding kaurene metabolism.

The kaurene carrier protein fraction is analogous, to the extent that it has been investigated, to the sterol carrier protein which has been described extensively by others.[11–16] It is even conceivable that the KP complex which was isolated in the present investigations is equivalent to, or else contained in addition, sterol carrier protein and some bound components, since squalene was associated with the protein fractions to which kaurene was bound. Scallen *et al.*[13] proposed that sterol carrier protein of rat liver originates from the endoplasmic reticulum, that is, that it is solubilized during preparation of liver microsomes. These authors[13] also considered the possibility that sterol carrier protein may bind substrates much smaller than squalene and perhaps even as small as mevalonate. It was further

suggested by Ritter and Dempsey[16] that sterol carrier protein and its bound components may form part of the active site of the complete microsomal enzyme. Consistent with that view is the evidence that sterol carrier protein may be lipoprotein, and that the relatively small MW protein ('Apo-SCP') which aggregates upon binding a sterol precursor also binds stoichiometric amounts of NADPH.[16] Whatever the relationship between kaurene and squalene carrier proteins, it seems clear that the oxidation of kaurene in the pea seed system is mechanistically analogous, in its dependence on noncatalytic protein, to the transformation of squalene to cholesterol in preparations of liver microsomes.

EXPERIMENTAL

Source and purity of reagents. Mevalonic acid-2-^{14}C lactone (sp. act. 5·86 mCi/mmol) in benzene solution was purchased from Amersham/Searle Corporation, Des Plaines, Illinois. The benzene was removed under N_2 and the lactone was hydrolysed by treating for several hours with 100% excess NaOH, after which the MVA was diluted with H_2O to a concentration of *ca.* 0·01 μCi/μl. Bio-Gels A-1·5m, A-5m and A-15m were obtained from BioRad Laboratories, Richmond, California. Polyvinylpyrrolidone (PVP) (insoluble Polyclar-AT) was purchased from General Aniline Film Corporation, Graselli, New Jersey, and prepared for use as previously described.[1] All other chemicals were reagent grade, and all organic solvents were redistilled.

Plant material. Pea plants (*Pisum sativum* L. cv. Alaska; W. Atlee Burpee Company, Riverside, California) were cultured in a greenhouse as described previously.[7] Enzyme extracts were prepared from excised cotyledons of freshly harvested seeds which were collected on the 12–15 day after anthesis.

Preparation of enzyme extracts. Excised cotyledons were homogenized in a chilled mortar and pestle with 0·1 M KH_2PO_4–K_2HPO_4 buffer (pH 7·1, 1 ml: g fr. wt) containing 0·1 mM chloramphenicol and with the addition of 0·5 g insoluble PVP per g fr. wt of tissue. The crude homogenate was centrifuged at 10 000–12 000 *g* for 15 min at 0–4°, and the resulting supernatant was used as S_{10} enzyme extract. To prepare S_{100} extract, S_{10} was centrifuged at 100 000 *g* for 60 min or 78 000 *g* for 75 min in a No. 40 angle head rotor of a Spinco Model L preparative ultracentrifuge. The pellet resulting from this ultracentrifugation was utilized as a microsomal preparation. Microsomal pellets were homogenized with a glass mortar and teflon pestle (10 up and down strokes at 400 rpm) in a volume of phosphate buffer (pH 7·1, 0·1 M) equal to the volume of supernatant from which the pellet was isolated. Microsomes were always prepared immediately before use, and held in an ice bath during preparative procedures.

Reaction conditions for producing endogenous kaurene-^{14}C-protein complex. A typical reaction mixture utilized to produce endogenous kaurene-^{14}C-protein complex contained, in a total vol. of 1 ml of 0·1 M KH_2PO_4–K_2HPO_4 buffer (pH 7·1): 0·75 ml of S_{100} enzyme extract (1·8–3·2 mg protein), 0·05 μmol of ATP, 0·5 μmol of $MgCl_2$ and 0·5 μmol of $MnCl_2$. The reaction mixtures generally were incubated for 75 min at 30°. Exogenous kaurene-^{14}C-protein complex was prepared by incubating exogenous kaurene-^{14}C (added in 0·05 ml of *para*-dioxane to each reaction mixture) with addenda in S_{10} or S_{100} for 15 min at 30° prior to gel filtration chromatography.

Gel filtration chromatography. Gel filtration chromatography was conducted in a cold room (0–5°) using glass columns 3·7–3·9 cm in dia. and packed to bed heights of 29–35 cm with Bio-Gel A-1·5m, A-5m and A-15m agarose gels (200–400 mesh). The columns were equilibrated with eluting buffer (0·1 M KH_2PO_4–K_2HPO_4, pH 7·1), and the void volumes were determined using Blue Dextran. Typically 8–10 reaction mixtures containing a total of 12–15 mg protein were pooled and applied to a column. The flow rate was approx. 1·5 ml/min in each case, and 5-ml fractions were collected. Eluate fractions were analysed for protein, extractable kaurene-^{14}C, and occasionally for squalene-^{14}C. Kaurene-^{14}C-protein complex generally was collected as the 5 fractions eluted immediately after the void volume.

Reaction conditions for microsomal oxidations. A typical reaction mixture utilizing kaurene-^{14}C-protein complex contained, in order of addition, 0·5 ml kaurene-^{14}C-protein complex (eluate from gel filtration column), 1 μmol of $MgCl_2$, 0·5 μmol of NADPH and 0·5 ml microsomal suspension. When exogenous kaurene-^{14}C was utilized, the substrate was isolated from reaction mixtures in which it was produced from MVA-2-^{14}C and added in 0·05 ml *para*-dioxane to a 1·0 ml reaction mixture containing 0·75 ml microsomal suspension, 1 mM $MgCl_2$ and 0·5 mM NADPH. Reaction mixtures were incubated at 34° for 60–90 min. Boiled enzyme controls utilized aliquots of microsomal suspension which were boiled for 5 min and rehomogenized. Carbon monoxide treatment was accomplished by gassing the reaction tube for 30 sec prior to the addition of microsomes, continual gassing during the addition of microsomes, further gassing for 2 min, then sealing the reaction tube with a serum cap and sparging with CO for 1 min.

Analysis of kaurene-^{14}C and squalene-^{14}C. Reaction mixtures were stopped by the addition of 2 vol. of acetone, and the mixtures were extracted twice with 1-ml portions of benzene. Further analysis by TLC, radiochromatogram scanning, elution and liquid scintillation counting was by methods which were previously described in detail.[1,7]

Protein determinations. All protein determinations were made by the method of Lowry *et al.*[20] using bovine serum albumin as a standard.

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[20] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).