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ENT-KAURENE SYNTHESIS IN CHLOROPLASTS FROM HIGHER PLANTS

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Key Word Index—*Pisum sativum*, Leguminosae, *ent*-kaurene biosynthesis; chloroplasts, kaurene synthetase, membrane activation

Abstract—Lysed chloroplasts from several higher plants synthesized *ent*-kaurene from copalyl pyrophosphate but not from geranylgeranyl pyrophosphate. The copalyl pyrophosphate transforming activity (so-called *B* activity of kaurene synthetase) was relatively stable in plastid lysates from *Pisum sativum* but remarkably unstable in similar preparations of *Hordeum vulgare*. The bulk of the *B* activity of kaurene synthetase appeared to reside in the stroma of plastids from *P. sativum* but required the presence of plastid membranes for maximum activity.

INTRODUCTION

The biosynthetic route to the gibberellins (GAs) in higher plants has been investigated primarily in developing seeds [1–4], and consequently knowledge of the pathway in vegetative tissues is only limited [3–5]. Since GAs exert their most dramatic effects on vegetative growth and development [6], demonstration of the biosynthetic steps to GAs in vegetative tissues is essential if we are to understand GA-regulated plant development.

Studies in dicotyledons and monocotyledons have shown that GA-like substances occur both in chloroplasts [7–12] and in red light-irradiated etioplasts [13–15] and thus these organelles could represent an important subcellular site of GA biosynthesis in vegetative tissues. However, only fragmentary biochemical evidence is available to support this possibility. Thus, isolated chloroplasts from *Brassica oleracea*, disrupted by sonication, metabolized *ent*-[17-^{14}C]kaurenoic acid to products which exhibit low GA-like biological activity [16] and sonicated plastids from *Hordeum distichon* were reported to oxidize *ent*-kaurenol to *ent*-kaurenoic acid and *ent*-7α-hydroxykaurenoic acid but were unable to metabolize *ent*-kaurene [17]. Attempts to study the later stages of GA biosynthesis in chloroplasts of *Pisum sativum* showed that GA_{20} was metabolized to an unidentified polar, acidic product and GA_9 was oxidized to GA_{10} and a dihydro, dihydroxy GA_9 derivative [18–20], neither of which occur naturally in seeds [21] or shoots [22] of *P. sativum* or form part of the biosynthetic pathway in this species [23–27]. Other biochemical evidence implicating plastids in GA biosynthesis has been the demonstration that the enzyme, kaurene synthetase, occurs in these organelles [28, 29]. Kaurene synthetase catalyses the first committed step in GA biosynthesis (Fig. 1), namely the conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP) (so-called *A* activity of kaurene synthetase) and then a further conversion of CPP to *ent*-kaurene (*B* activity of kaurene synthetase). It is thus a branch-point enzyme with potential regulatory properties for GA biosynthesis in that it diverts GGPP, which would otherwise be used for carotenoid and chlorophyll biosynthesis, into *ent*-kauranes and eventually GAs [1, 30].

The results in which kaurene synthetase activity was investigated in etioplast preparations of *P. sativum* [28] were anomalous in that these preparations were able to catalyse efficiently the conversion of CPP to *ent*-kaurene whereas GGPP was not utilised as a substrate. Moore and Coolbaugh [29], however, detected a very low level of GGPP conversion to *ent*-kaurene in chloroplast preparations of *P. sativum* but did not investigate the relative conversion of CPP to *ent*-kaurene in the same preparations. The studies reported in this paper were undertaken to further elucidate the types of kaurene synthetase activities in chloroplasts and to determine the site of *ent*-kaurene biosynthesis within this organelle.

RESULTS AND DISCUSSION

The results of a representative experiment to measure the metabolism of [1-^{3}H]GGPP and [1-^{3}H]CPP to *ent*-kaurene by chloroplast lysates are shown in Table 1. All chloroplast preparations were able to synthesize *ent*-

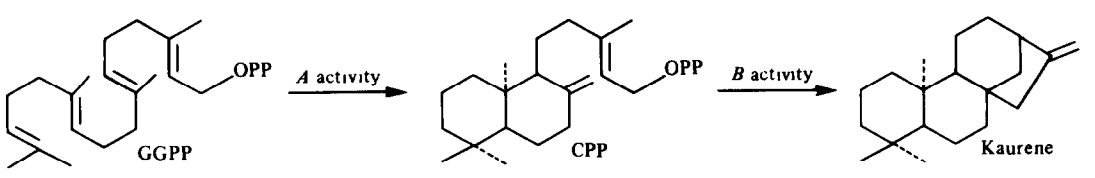

Fig 1. Kaurene synthetase catalysed conversion of GGPP → *ent*-kaurene *via* CPP.

Table 1. Kaurene synthetase activity in chloroplast lysates from leaves of higher plants

Species	Radioactivity in *ent*-kaurene (10^{-3} × dpm/mg chlorophyll)	
	*AB**	*B*
P. sativum	0	485
Helianthus annuus	0	297
Phaseolus coccineus	0	244
H. vulgare	0	46.4
Zea mays Tall	0	8.1
d_5	0	7.7

**AB*, *ent*-kaurene formed from [1-^{3}H]GGPP; *B*, *ent*-kaurene formed from [1-^{3}H]CPP.

Chloroplast enzyme equivalent to 50 μg chlorophyll was used in all assays except *Z. mays* where 100 μg chlorophyll was used.

kaurene from [1-^{3}H]CPP. *ent*-Isokaurene, which had been reported to be a product of [1-^{3}H]CPP metabolism in cell-free extracts of etiolated dwarf-5-maize seedlings was not detected in the present study when plastid-generated radioactive *ent*-kaurene was rechromatographed on silver nitrate-impregnated silica gel developed with benzene [31].

The reasons for the absence of the *AB* activity* in chloroplast lysates are unclear but, not withstanding the possibility that CPP is derived from a precursor other than GGPP in chloroplasts, suggests that instability during organelle isolation and lysis or the presence of inhibitors of *A* activity could be contributory factors. Naturally occurring inhibitors of the *A* activity of kaurene synthetase have been described from castor bean cell suspensions [32] and have been implicated in other studies in vegetative tissues [33]. Even so, Moore and Coolbaugh [29] reported femtomole conversions of GGPP into *ent*-kaurene in sonicated chloroplasts from *P. sativum* cv. Alaska and Moore and coworkers have consistently observed low conversions of MVA into *ent*-kaurene by excised shoot tips of the same species [34, 35].

Initially we concentrated on the *B* enzyme from chloroplasts of *Hordeum* where the metabolism of *ent*-kaurene had been investigated previously [17], but large losses in activity were noted during subsequent manipulations following plastid isolation. Comparisons of the stability of the *B* activities from lysed chloroplasts of *Hordeum* and *Pisum* (Fig. 2a,b) revealed a marked difference between these two species. Whereas the enzyme from *Pisum* retained much of its activity over a 24 hr period, that from chloroplasts of *Hordeum* was remarkably unstable and lost almost all its activity within 5 hr of standing on ice. Attempts to increase enzyme stability with leupeptin (Fig. 2b) were unsuccessful suggesting that released leupeptin-sensitive proteases were not responsible for loss of synthetase activity in *Hordeum* as has been demonstrated for some plant enzymes in other tissues [36]. Likewise, buffered glycerol or added thiols were unable to prevent loss of synthetase activity (data not shown). Thus

*A activity, GGPP → CPP; *B* activity, CPP → *ent*-kaurene; *AB* activity, GGPP → *ent*-kaurene.

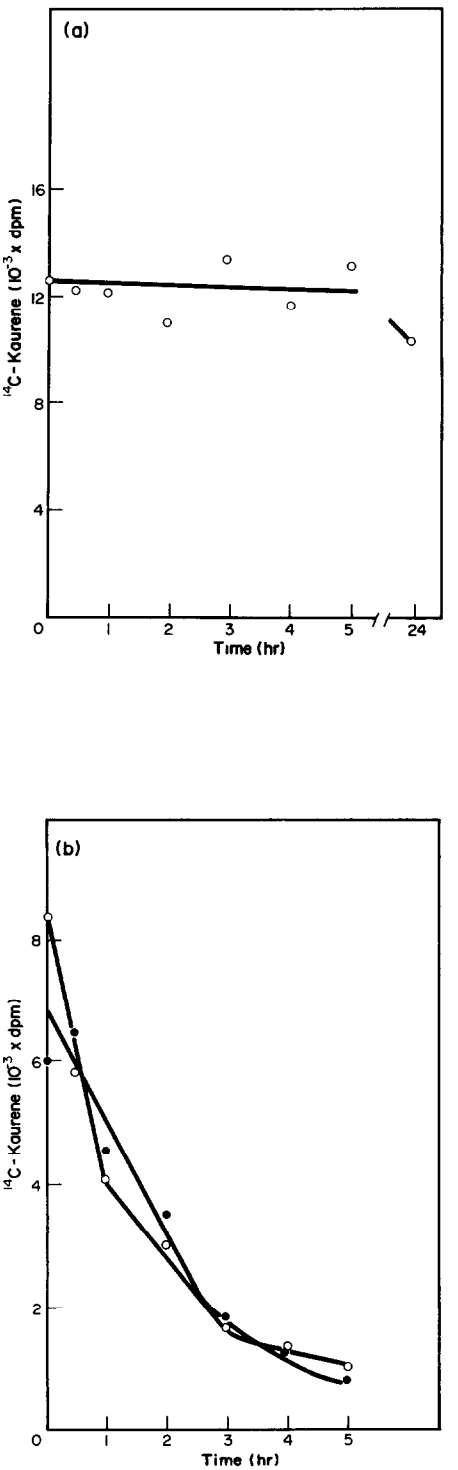

Fig. 2. The stability of the *B* activity of kaurene synthetase in chloroplast lysates of (a) *P. sativum* and (b) *H. vulgare* with (●——●) and without (o——o) 10 μM leupeptin. Chloroplasts were prepared as described under 'Experimental' using grinding medium with or without 10 μM leupeptin. Intact chloroplasts were lysed for 25 min in distilled H_2O containing 10 μM leupeptin and aliquots (≡ 200 μg chlorophyll) assayed for kaurene synthetase.

Table 2. Relationship between leaf age and kaurene synthetase *B* activity in chloroplasts from 10 day old plants of *P. sativum*

Leaf	Radioactivity in *ent*-kaurene (10^{-3} × dpm/mg chlorophyll)
Apical bud	89.45
Node 3	1.3
Node 2	0.84
Node 1	0.16

Chloroplasts isolated as described under 'Experimental' were purified on Percoll. 50 μg chlorophyll used per assay.

all subsequent work was carried out with the enzyme from *Pisum* chloroplasts.

The most active chloroplast preparations were obtained from the apical buds of *Pisum* (Table 2) and this tissue was used as a source of the *B* activity of kaurene synthetase throughout. Chloroplasts of *P. sativum* which were further purified on Percoll showed no loss of associated synthetase *B* activity (Fig. 3) nor any significant enhancement of activity at the enzyme concentrations used. This implies synthetase localization within the organelle rather than non-specific adsorption to the outside of plastid envelope membranes and also shows that Percoll treatment did not remove substances which might interfere with synthetase activity. Other detailed studies reported elsewhere using sucrose gradients and enzyme markers have further characterized these plastid-associated activities [37].

In order to localize kaurene synthetase in chloroplasts, the capacity of chloroplast subfractions from *Pisum* to

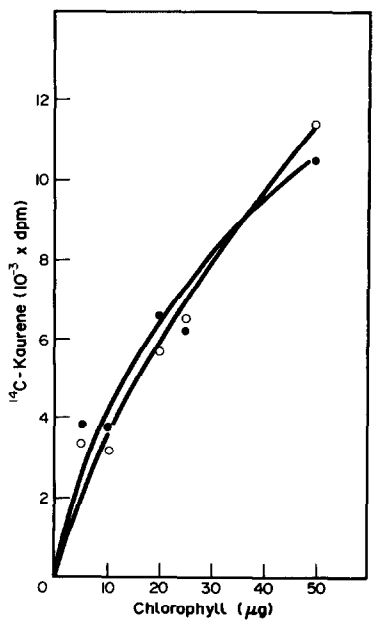

Fig. 3. Comparison of kaurene synthetase *B* activity in lysates (0–50 μg chlorophyll) of chloroplasts from *P. sativum* purified with (o——o) and without Percoll (●——●).

biosynthesize *ent*-kaurene was determined by incubation with [1-^{3}H]GGPP and [1-^{3}H]CPP. Neither membranes (thylakoids and envelope membranes) nor stromal fractions were able to synthesize *ent*-kaurene from GGPP (Table 3). However, both membranes and stroma converted CPP into *ent*-kaurene (Table 3) although the total activity was significantly less than that in an equivalent amount of lysed, non-fractionated plastids. Recombination of membranes and stromal fractions resulted in full restoration of *B* activity, suggesting close co-operation between membranes and stroma in maintaining maximal activity of the *B* enzyme of kaurene synthetase in chloroplasts. Although significant synthetase activity was associated with plastid membranes (Table 3), studies with the enzyme from endosperm of immature seed of *Marah macrocarpus* have shown that it is a soluble protein [1, 30] and therefore such membrane associated activity might be expected to represent stromal contamination. This was further implied following recentrifugation of the membrane fraction, when a decrease in associated synthetase activity was observed (Table 3). Such 'washed' membranes still retained the ability to enhance kaurene synthetase activity in stromal fractions, resulting in even higher activities than in non-fractionated plastid preparations or in stromal recombinations with non-washed membranes (Table 3).

Despite the implications that plastid kaurene synthetase might be a soluble, stromal enzyme requiring plastid membranes for activity, it was also possible that the enzyme was membrane bound and required soluble, heat stable, cofactors from the stroma. Results of attempts to distinguish between these alternatives are presented in Table 4.

Heated stroma did not enhance significantly synthetase activity in non-heated membrane fractions, whereas heated membranes were able to stimulate synthetase activity in non-heated stromal fractions. This suggests that kaurene synthetase is a stromal enzyme requiring plastid membranes for activity. However, heated membranes were less able than unheated ones to sustain *ent*-kaurene synthesis in the presence of unheated stromal fractions suggesting that they were not simply supplying a heat stable cofactor for synthetase activity.

Table 3. Kaurene synthetase activity in chloroplast subfractions and reconstituted chloroplasts from *P sativum*

Enzyme source	Radioactivity in *ent*-kaurene (10^{-3} × dpm/mg chlorophyll) *AB*	*B*
Lysed plastids	0	218
Membranes	0	22.7
'Washed' membranes	0	7.3
Stroma (7.8 mg protein)	0	37 6
Membranes + stroma	0	276
'Washed' membranes + stroma	0	338

'Washed' membranes were prepared from plastid membranes after initial centrifugal separation of plastid compartments by resuspending membrane pellets in distilled H_2O and resedimenting them at 100000*g* for 1 hr at 0° in an ultracentrifuge. Chloroplast enzyme equivalent to 50 μg chlorophyll was used in assays of lysed plastids and membranes. Stroma used equivalent to 390 μg protein.

Table 4. Activity of kaurene synthetase in heated chloroplast subfractions and in reconstituted chloroplasts of *P. sativum*

Enzyme source	Radioactivity in *ent*-kaurene from CPP (dpm)
Membranes (25 μg chlorophyll)	202
Heated membranes	0
Stroma (400 μg protein)	84
Heated stroma	25
Stroma + membranes	12 500
Heated stroma + membranes	490
Stroma + heated membranes	4 400

Chloroplast subfractions, separated by ultracentrifugation, were held in a boiling H_2O bath for 2 min and then assayed for kaurene synthetase using CPP as substrate.

Table 5. Kaurene synthetase activity in 'stored' stromal fractions from chloroplasts of *P. sativum*

Enzyme source	Storage time (hr)	
	0	24
	Radioactivity in *ent*-kaurene from CPP (10^{-3} dpm)	
Stroma (450 μg protein)	0.26	4 12
Membranes (25 μg Chl)	1.57	0 54
Stroma + membranes	9.7	11.0

Stromal fractions, separated from lysed chloroplasts by ultracentrifugation, were stored in darkness in a refrigerator at ± 2° for 24 hr and kaurene synthetase assayed as in 'Experimental'.

Since plastid membranes exerted such a marked effect on kaurene synthetase activity, varying amounts of membranes were added to a fixed amount of stroma in order to determine the minimum quantity of membrane required to enhance synthetase activity (Fig. 4). The results demonstrated a linear relationship between the quantity of membrane added to stromal fractions and kaurene synthetase activity, at least over the range tested, and showed the quantities of membranes containing as little as 5 μg chlorophyll could significantly enhance the activity of this enzyme. Since stromal fractions alone usually exhibit some synthetase activity (Tables 3 and 4), clearly, the presence of small amounts of residual membrane material in these fractions could be responsible.

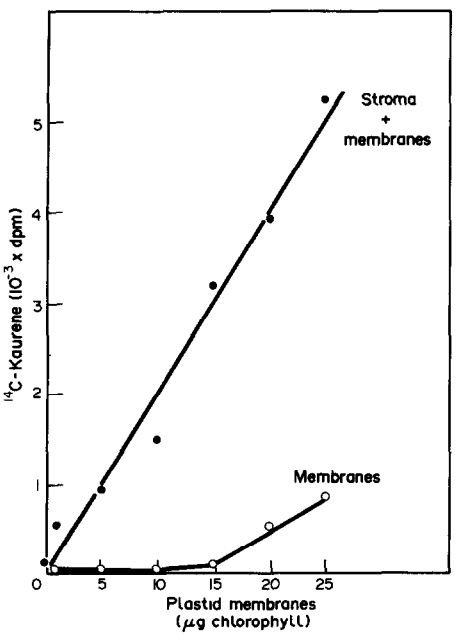

Fig. 4. Effect of plastid membranes on the *B* activity of kaurene synthetase in stromal fractions of *P. sativum*. Total plastid membranes (envelopes and thylakoid membranes) were recombined with stromal fractions (≡ 478 μg protein) and kaurene synthetase assayed as described in 'Experimental'.

Possible support for this suggestion was the finding (Table 5) that when *Pisum* stromal fractions were stored (2° for 24 hr), partial restoration of synthetase activity was observed, even in the absence of added membrane. Although this could be interpreted as slow activation of the enzyme in the presence of minimal amounts of residual membrane, other effects, e.g. a slow conformational change which is non-membrane linked, would seem more likely.

The close co-operation between stromal kaurene synthetase and plastid membranes during *ent*-kaurene synthesis from CPP suggested possible differences in the rates of *ent*-kaurene production between lysed, non-fractionated plastids, where stromal-membrane associations would be already established, and reconstituted plastid systems where such associations would only be initiated after recombination of the separated components. In reconstituted *Pisum* chloroplasts, time course experiments indicated (Fig. 5) the absence of an appreciable lag period in *ent*-kaurene synthesis compared with lysed plastids, suggesting a rapid association of enzyme and membranes following recombination of these fractions.

In order to investigate the membrane effect further, membrane preparations were fractionated and the effect of thylakoids on synthetase activity was examined (Table 6). Combinations of thylakoids and stromal fractions resulted in increases in *ent*-kaurene production similar to those induced by 'washed', non-fractionated membranes.

In view of the apparent membrane involvement in changes in the activity of plastid kaurene synthetase in *Pisum*, it was clearly of interest to examine the specificity of this effect by mixing membranes, thylakoids and stromal fractions from different plant species. The results in Table 7 show that combining either *Hordeum* membranes or thylakoids with stromal material from *Pisum*, increased kaurene synthetase activity. Both membranes and thylakoids from *Hordeum* chloroplasts were generally less efficient at increasing synthetase activity in *Pisum* stromal fractions than were *Pisum* membranes and thylakoids themselves. Varying the amounts of such membranes had little effect on activity (data not shown), although 25 μg chlorophyll additions of both *Hordeum* membranes and thylakoids produced the highest synthetase activities.

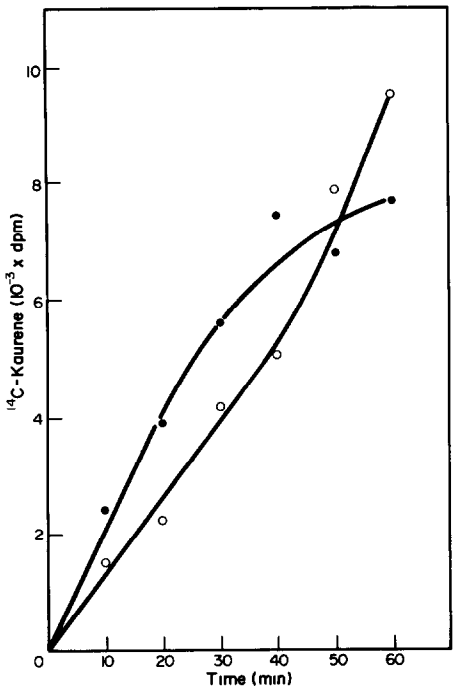

Fig. 5. Kinetics of *ent*-kaurene synthesis from [1-^{3}H]CPP by lysed chloroplasts (●——●) and reconstituted chloroplasts (o——o) of *P. sativum* Aliquots of lysed plastids (22 μg chlorophyll) and recombinations of "washed" membranes (22 μg chlorophyll) and stromal fractions (154 μg protein ≡ 22 μg chlorophyll) were assayed for kaurene synthetase as described under 'Experimental'.

Clearly, these results demonstrate a general lack of specificity by the plastid kaurene synthetases for plastid membrane material. Even so, this effect might be organelle specific since attempts to mimic increases in synthetase activity with plant membranes from other sources, e.g. mitochondrial membranes from germinating seeds of *Ricinus communis* were unsuccessful (data not shown),

Table 6. Effect of thylakoid membranes on the *B* activity of kaurene synthetase in stromal fractions from chloroplasts of *P. sativum*

Enzyme source	Radioactivity in *ent*-kaurene (10^{-3} dpm)
Lysed plastids (50 μg chlorophyll)	11.0
Stroma (420 μg protein)	1.9
'Washed' membranes (10 μg chlorophyll)	0 20
Thylakoids (10 μg chlorophyll)	0 03
'Washed' membranes + stroma	15 4
Thylakoids + stroma	14.9

Thylakoids were prepared from lysed plastids as described under 'Experimental', mixed with stromal enzyme and the reaction initiated by adding CPP. Synthetase activity was assayed as in 'Experimental'

Table 7 Activity of kaurene synthetase in mixed subfractions from chloroplasts of *P. sativum* and *H. vulgare*

Addition (25 μg chlorophyll)	Radioactivity in *ent*-kaurene from CPP (10^{-3} x dpm)
None	1.9
Hordeum membrane	6 8
Hordeum thylakoid	11.4
Pisum membrane	14.2
Pisum thylakoid	10.2

Thylakoids and total membrane fractions were prepared as described under 'Experimental' and were mixed with *Pisum* stromal fractions (= 567 μg protein) obtained following ultracentrifugal separation of *Pisum* plastid compartments. Reactions were initiated by the addition of CPP and kaurene synthetase activity (corrected for membrane and thylakoid activities) was assayed as in 'Experimental'.

although more detailed studies will be required to clarify this further.

The results presented in this paper are consistent with the idea that kaurene synthetase in chloroplasts of *Pisum sativum* is a soluble, stromal enzyme which normally requires weak association with plastid membranes, perhaps thylakoids, to stabilize the conformation of the enzyme most active in catalysing the conversion of copalyl pyrophosphate to *ent*-kaurene. However, participation of envelope membranes in kaurene synthetase activation in *Pisum* plastids cannot be discounted until the relevant studies have been carried out. Although conversion of CPP to *ent*-kaurene had been demonstrated previously in lysed chloroplasts of *Pisum sativum* [37] the co-operation between organelle compartments in effecting this step had not been recognized. *ent*-Kaurene is the first, hydrophobic intermediate in GA biosynthesis, and, in endosperm from immature seeds, is sequentially oxidized by microsomal, mixed function oxidases to yield *ent*-7α-hydroxy kaurenoic acid [38, 39]. Similar mixed function oxidases for *ent*-kaurene in chloroplasts could also be membrane localized, in which case an association between kaurene synthetase and plastid membrane proteins could be clearly advantageous. Such an arrangement might facilitate the production of *ent*-kaurene at a membrane site for oxidation by membrane-bound enzymes.

Similar results and interpretations were reported by Block *et al.* [40] for geranylgeraniol and geranylgeranylchlorophyll a biosynthesis in spinach chloroplasts, where close co-operation between stromal, thylakoid and envelope membrane compartments was shown to occur. A remarkably similar system has also been discovered in rat liver microsomes [41, 42] where a soluble, cytoplasmic protein, involved in intermembrane squalene transport, binds to microsomal membranes, thus aiding squalene entry into such membranes for oxidation to 2,3-oxidosqualene. Such soluble protein–membrane interactions could, therefore, be a characteristic feature of terpenoid metabolism in organelles.

Whilst the present results demonstrate that CPP can serve as a precursor of *ent*-kaurene in chloroplasts, conditions under which the *AB* activity is expressed must be identified before the full significance of the membrane activation of kaurene synthetase can be determined.

EXPERIMENTAL

Plant material. Seeds of *Pisum sativum* L cv. Alaska, *Helianthus annuus* L and *Phaseolus coccineus* L. were obtained from Atlee-Burpee Co. Riverside, CA, USA. Seeds of *Hordeum vulgare* L cv. Himalaya were purchased from Washington State University, Pullman, WA, USA and seeds of dwarf (d_5) and tall *Zea mays* were kindly supplied by Prof. B. O. Phinney, UCLA. Seeds were soaked in aerated tap water, sown in vermiculite and grown in a heated greenhouse (20°) under natural daylength conditions. Plants of *P. sativum* were used when 7–10 days old, those of *P. coccineus* when 15 days old and *H. annuus* when the first true leaves had emerged. Seedlings of *Z mays* were harvested when 10 days old and those of *H. vulgare* when 7 days old

Isolation of chloroplasts. Chloroplasts were prepared from the apical portion of shoots of *P. coccineus*, *H. annuus* and *P sativum* and from the first true leaves of *H vulgare* and *Z. mays* using similar procedures to those described by Heber and Santarius [43] Excised tissues were pre-chilled on ice or by immersion in ice-cold distilled H_2O and then homogenized in partially thawed, buffered sorbitol (pH 7.6) containing Tricine (50 mM), sorbitol (0.33 M) $MgCl_2$ $6H_2O$ (15 mM), KCl (100 mM), cysteine (1 mM), BSA, Fraction IV (Sigma) (0.2%). After adding 0.4 g ascorbic acid/200 ml of this soln, tissue disintegration was achieved using a Polytron, top drive homogenizer for 7–9 sec at full speed. The homogenates were filtered through eight layers of cheesecloth and were centrifuged at 2000 *g* for 40 sec at 4° in a refrigerated centrifuge The supernatants, and in the case of *P. sativum*, the semi-fluid plastids, were discarded, the pellets resuspended by gentle agitation in fresh grinding medium, and then recentrifuged for a further 40 sec at 2000 *g* to provide the final chloroplast preparation. Such preparations were essentially free from mitochondrial contamination as judged by the results of fumarase assays and the chloroplasts from *P. sativum* have been extensively characterized using sucrose density gradient centrifugation and enzyme markers [37]. Chlorophyll was determined using a nomogram [44].

Percoll centrifugation. This was carried out as described by Mills and Joy [45].

Preparation of chloroplast subfractions. Chloroplasts were lysed in distilled water for 20 min on ice and then centrifuged at 100 000 *g* for 1 hr at 0° in a Beckman Ultracentrifuge, to separate total plastid membranes (thylakoid and envelope membranes) from stroma. 'Washed'-membranes were prepared by recentrifugation at 100 000 *g* of the total plastid membrane pellet following resuspension in distilled water Stromal protein was determined using Bradford reagent [46] Thylakoid membranes, free of envelopes, were prepared using similar procedures to those carried out in refs [47, 48]. Lysed chloroplasts were dispersed using a cooled Thomas homogenizer with 3 up and down strokes of a teflon plunger and were then centrifuged at 3000 *g* for 10 min to pellet thylakoid membranes. The supernatants, containing the envelopes, were discarded and the soft pellets were resuspended in distilled H_2O and recentrifuged at 27 000 *g* for 10 min to yield the final thylakoid membrane fraction.

Assay of kaurene synthetase This was carried out in a similar manner to that described already [30]. [1-^{3}H]GGPP (sp. act. 42 mCi/mmol) and [1-^{3}H]CPP (sp act. 20 mCi/mmol) were prepared as described in ref. [49]. Incubation mixtures contained 40 mM Tes (pH 7 2), 5 mM $MgCl_2$, 10 mM K-Pi and either 2 3 μM [1-^{3}H]GGPP (1.09×10^5 dpm) or 2.3 μM [1-^{3}H]CPP (5.6×10^4 dpm) in a final vol of 0.5 ml. Incubations were carried out at 30° for 1 hr after which they were terminated by the addition of 0.5 ml of ice-cold MeOH.

Such mixtures were extracted (× 3) with 1 ml aliquots of petrol (60–80°)–C_6H_6 (9 1) The combined organic fractions were reduced in vol. under N_2 and the levels of *ent*-kaurene, after $AgNO_3$–silica gel TLC, were monitored in a Beckmann liquid scintillation spectrometer, Model L5-65, using a scintillation cocktail (toluene containing 5% dioxan and Omnifluor, 4 g/l) with a counting efficiency for tritium of 54.5%. *ent*-Kaurene generated from [1-^{3}H]GGPP is referred to as the *AB* activity of kaurene synthetase, whilst *ent*-kaurene produced from [1-^{3}H]CPP is referred to as the *B* activity of this enzyme.

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REFERENCES

1. West, C. A. (1973) in *Biosynthesis and its Control in Plants* (Milborrow, B. V., ed.) Ch. 7, p. 143. Academic Press, London.
2 Railton, I. D. (1976) *S. Afr. J. Sci.* **72**, 371.
3. Hedden, P., MacMillan, J. and Phinney, B. O. (1978) *Ann. Rev. Plant Physiol.* **29**, 149
4. Sembdner, G , Gross, D., Liebisch, H-W. and Schneider, G. (1980) in *Encyclopaedia of Plant Physiol.* Vol. 9, p. 281. Springer, Berlin.
5. Railton, I D. (1982) *Cell Biol. Int. Rep.* **6**, 319.
6. Jones, R. L. (1973) *Ann. Rev Plant Physiol.* **24**, 571.
7. Stoddart, J L. (1968) *Planta* **81**, 106.
8. Railton, I. D. and Wareing, P. F (1973) *Physiol. Plant.* **28**, 88.
9. Frydman, V. M. and Wareing, P. F. (1973) *J. Exp. Botany* **83**, 1131.
10 Railton, I D. and Reid, D. M. (1974) *Plant Sci. Letters* **2**, 157.
11 Browning, G. and Saunders, P. F. (1977) *Nature* **265**, 375.
12. Railton, I. D. and Rechav, M. (1979) *Plant Sci. Letters* **14**, 75.
13 Cooke, R. J , Saunders, P. F. and Kendrick, R. E. (1975) *Planta* **124**, 319
14. Evans, A and Smith, H. (1976) *Proc. Natl. Acad. Sci. U.S A.* **73**, 138.
15. Hilton, J. and Smith, H. (1980) *Planta* **148**, 312.
16. Stoddart, J L. (1969) *Phytochemistry* **8**, 831.
17. Murphy, G. J. P and Briggs, D. E. (1975) *Phytochemistry* **14**, 429.
18. Railton, I. D. and Reid, D. M. (1974) *Plant Sci Letters* **3**, 303
19. Railton, I. D. (1977) *S. Afr. J. Sci.* **73**, 22.
20 Railton, I. D. (1977) *Z. Pflanzenphysiol.* **81**, 323.
21. Frydman, V. M , Gaskin, P and MacMillan, J. (1974) *Planta* **118**, 123.
22. Davies, P. J., Emshwiller, E., Gianfagna, T. J., Proebsting, W. M., Noma, M and Pharis, R. P. (1982) *Planta* **154**, 266.
23. Railton, I. D., Murofushi, N., Durley, R. C. and Pharis, R. P. (1974) *Phytochemistry* **13**, 793.
24. Frydman, V. M and MacMillan, J. (1975) *Planta* **125**, 181.
25. Sponsel, V M. and MacMillan, J. (1977) *Planta* **135**, 129.
26. Sponsel, V. M. and MacMillan, J. (1978) *Planta* **144**, 69.
27 Kamiya, Y. and Graebe, J. E. (1983) *Phytochemistry* **22**, 681.
28. Simcox, P. D., Dennis, D. T. and West, C. A (1975) *Biochem. Biophys. Res. Commun.* **66**, 166.
29. Moore, T. C and Coolbaugh, R. C. (1976) *Phytochemistry* **15**, 1241.
30. Frost, R. G. and West, C. A (1977) *Plant Physiol.* **59**, 22.
31 Hedden, P. and Phinney, B. O. (1979) *Phytochemistry* **18**, 1475.
32. Gafni, Y. and Shechter, I. (1981) *Plant Physiol.* **67**, 1169.
33. Shen-Miller, J. and West, C. A. (1982) *Plant Physiol.* **69**, 637.
34. Ecklund, P. R and Moore, T. C. (1974) *Plant Physiol.* **53**, 5.

35. Gomez-Navarette, G and Moore, T. C (1978) *Plant Physiol.* **61**, 889.
36. Alpi, A. and Beevers, H (1981) *Plant Physiol.* **67**, 499.
37. Simcox, P. D. (1976) *Ph.D. diss.* Univ. Calif., Los Angeles.
38. Murphy, P. J. and West, C. A. (1969) *Arch. Biochem. Biophys.* **133**, 395.
39. Lew, F. T. and West, C. A. (1971) *Phytochemistry* **10**, 2065.
40. Block, M. A., Joyard, J. and Douce, R (1980) *Biochim. Biophys. Acta* **631**, 210
41. Friedlander, E. J., Caras, I. W., Hou Lin, L F and Bloch, K. (1980) *J Biol. Chem.* **255**, 8042.
42. Kojima, Y., Friedlander, E. J and Bloch, K. (1981) *J. Biol. Chem.* **256**, 7235.
43. Heber, U. and Santarius, K. A. (1970) *Z. Naturforsch.* **25**, 718
44. Kirk, J. T. O. (1968) *Planta* **78**, 200.
45. Mills, W. R. and Joy, K. W. (1980) *Planta* **148**, 75.
46. Bradford, M. M. (1976) *Analyt. Biochem* **72**, 248.
47. Mackender, R O. and Leech, R M (1974) *Plant Physiol.* **53**, 496.
48. Ashton, A R., Brennan, T. and Anderson, L. E. (1980) *Plant Physiol.* **66**, 605
49 Duncan, J D. and West, C. A. (1981) *Plant Physiol.* **68**, 1128