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Initiation of Terpenoid Synthesis in Osmophores of *Stanhopea anfracta* (Orchidaceae): A Cytochemical Study

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INITIATION OF TERPENOID SYNTHESIS IN OSMOPHORES OF STANHOPEA ANFRACTA (ORCHIDACEAE): A CYTOCHEMICAL STUDY[1]

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ABSTRACT

The floral scent glands (osmophores) of *Stanhopea* produce a fragrance composed of terpenoids and aromatics which attract pollinators. The terpenoid component is composed of isoprene units synthesized via the mevalonic acid pathway. Localization of hydroxymethylglutaryl CoA synthase in the mevalonic acid pathway was achieved using a technique which elicits the formation of an electron-dense precipitate of uranyl ferrocyanide at the point in the mevalonic acid pathway where an acetyl group from acetyl CoA is transferred to acetoacetyl CoA, releasing free CoA-SH. Applying this technique to cells of the osmophore of *S. anfracta* resulted in a precipitate between the inner and outer mitochondrial membranes, in the smooth endoplasmic reticulum, and, to a lesser extent, on the outer surface of the tonoplast membranes and between the inner and outer membranes and granal membranes of amyloplasts indicating multiple, subcellular locations for at least some enzymes in the mevalonic acid pathway. Localization of carnitine acetyltransferase by a similar technique resulted in a precipitate between the inner and outer mitochondrial membranes and in the smooth endoplasmic reticulum indicating multiple, subcellular locations for this enzyme also.

TERPENOIDS have been cited as the most diverse group of plant products known (Goodwin and Mercer, 1983). Many of these products have functions known to be essential to plant life (e.g., carotenoids, chlorophyll side chains, and some hormones), whereas the function of other terpenoids is unknown. Various studies of terpenoid biochemistry have been published including several that address the subcellular location of terpenoid biosynthesis (Loomis, 1967; Croteau and Loomis, 1973; Loomis and Croteau, 1973; Brooker and Russell, 1975; Goodwin, 1977; Bernard-Dagan et al., 1980; Grumbach and Forn, 1980).

The cytochemical technique used here was introduced originally for the fine-structural localization of carnitine acetyltransferase in animal tissue (Higgins and Barrnett, 1970). Carnitine is hypothesized to facilitate the transport of acyl groups both into and out of mitochondria (Higgins and Barrnett, 1970). Carnitine acetyltransferase catalyzes the reaction, carnitine + acetyl CoA → acetylcarnitine + CoA-SH. The SH group of the free CoA-SH from this reaction can be used to reduce potassium ferricyanide to ferrocyanide which will form a stable, electron-dense precipitate under appropriate conditions (Higgins, 1974).

The technique has been presented for general use in localizing any of several acyltransferase enzymes with specific procedures for particular enzymes (Higgins, 1974). Malate synthase, which catalyzes the reaction acetyl CoA + glyoxylate → malate + CoA-SH in the glyoxysomes of plants, has been localized in cucumber and sunflower cotyledons (Trelease, Becker, and Burke, 1974) by a modification of the procedure for animals (Higgins, 1974).

The present study is part of an investigation into the ultrastructural development of osmophores (floral scent glands) in a number of orchids, some of which produce a fragrance composed of various terpenoids and aromatics (Williams, 1982). A technique that would give an indication of the subcellular location of fragrance synthesis would be valuable as part of the overall examination of the mechanisms of fragrance production in orchids. The general procedures for cytochemical localization of an acyltransferase seemed applicable to HMG-CoA synthase in the mevalonic acid pathway

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Abbreviations: CoA, CoA-SH—coenzyme A; MVA—mevalonic acid; HMG-CoA—hydroxymethylglutaryl coenzyme A; ER—endoplasmic reticulum; SER—smooth endoplasmic reticulum; RER—rough endoplasmic reticulum.

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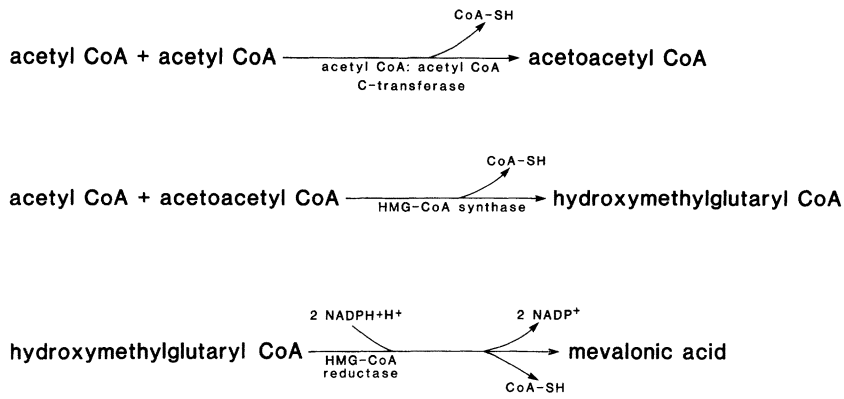

Fig. 1. Mevalonic acid pathway. Hydroxymethylglutaryl CoA synthase is targeted for localization by adding acetyl CoA and acetoacetyl CoA substrates to the tissue. Localization of HMG-CoA synthase depends on precipitation of uranyl ferrocyanide by free CoA-SH.

(Fig. 1) leading to terpenoid production. The plant chosen for this current study is *Stanhopea anfracta* Rolfe because the two major components of its floral aroma are monoterpenes, myrcene (37%) and ipsdienol (40.3%; Williams and Whitten, 1983; N. H. Williams, personal communication), while only a small percent is a mixture of aromatics.

MATERIALS AND METHODS—A flower from *S. anfracta* was collected at midday and at 2100 hr on the first day of anthesis. The morphology of similar flowers and their osmophores has been reported (Stern, Curry, and Pridgeon, 1987). The odor is strongest at midday and weakest at night based on chromatographic data (H. Hills, personal communication, using techniques from Williams and Whitten, 1983) so, presumably, terpene biosynthesis is active during the day and inactive at night. Freehand sections about 1 mm thick were made through the osmophore (secretory tissue) of the flower for fixation and cytochemistry. Localization of the mevalonic acid pathway is based on adding acetyl CoA and acetoacetyl CoA to the tissue; these are catalyzed by HMG-CoA synthase to HMG-CoA (Fig. 1). This reaction yields free CoA-SH. When potassium ferricyanide is added to the tissue, CoA-SH reduces it to ferrocyanide. The ferrocyanide, in turn, reacts with added uranyl acetate to precipitate as uranyl ferrocyanide.

A similar protocol for localization of carnitine acetyltransferase was included in the experimental design, since it was through the use of this cytochemical technique that the localization of HMG-CoA synthase is based. Carnitine is known to be present in some plant tissue (Goodwin, 1977).

Protocol—1) Fixation: Tissue was fixed for 10 to 30 min. Fixation of all tissue was carried out at 12 C in 4% (w/v) formaldehyde (freshly prepared from paraformaldehyde) and 1% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer made to pH 7.0 and followed by a buffer rinse (0.05 M cacodylate, pH 7.0).

2) Preincubation: Tissue was preincubated at ambient temperature for 20 min in 3 mM potassium ferricyanide in 0.05 M cacodylate buffer (pH 7.0) followed by a buffer rinse. Preincubation minimizes the effect of endogenous electron donors (e.g., electron transport system) on potassium ferricyanide and uranyl acetate used later during the incubation step.

3) Incubation: Tissue was incubated for 45 min at ambient temperature to allow enzyme catalysis and precipitation to occur. Complete media, one including acetoacetyl CoA and the other including carnitine, were used along with several controls.

a) Complete acetoacetyl CoA medium:
2.0 mg/ml potassium ferricyanide
1.0 mg/ml uranyl acetate
0.8 mg/ml acetyl CoA, sodium salt
1.6 mg/ml acetoacetyl CoA, sodium salt
0.05 M sodium cacodylate buffer, pH 7.0
Acetyl CoA and acetoacetyl CoA were from Sigma Chemical Company, St. Louis, Missouri.

b) Complete carnitine medium:
As "a" above, but 1.6 mg/ml DL carnitine (from Sigma Chemical Company) substituted for acetoacetyl CoA.

c) Substrate control medium for acetoacetyl CoA:
As "a" above, but acetyl CoA substrate omitted.

d) Substrate control medium for carnitine:
As "b" above, but acetyl CoA substrate omitted.

e) Primary reaction product control medium:

As "a" or "b" above, but acetoacetyl CoA and carnitine omitted.

f) Final reaction product control medium: As "a" or "b" above, but acetyl CoA, acetoacetyl CoA, and carnitine omitted.

4) Postfixation: Tissue was postfixed for one hr at ambient temperature in 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.0).

Osmophores were also prepared without cytochemical testing to provide comparative tissue for assessing the effects of aldehyde and osmium fixatives, buffer, and lead stain. This tissue was fixed for two hr in 2% (w/v) formaldehyde (freshly prepared from paraformaldehyde) and 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (made to pH 7.0), buffer rinsed (0.05 M cacodylate, pH 7.0), and postfixed for 45 min in 1% (w/v) osmium tetroxide in 0.05 M cacodylate buffer, pH 7.0. All tissue, whether for cytochemical examination or not, was dehydrated through an ethanol series (50, 70, 85, 95, 100%) and 100% acetone and embedded in epoxy resin ERL4206 (Spurr, 1969). Thin-sectioned material was stained 10 min in Sato's lead citrate (Hayat, 1981).

RESULTS—The osmophore cells of *S. anfracta* show the typically dense cytoplasm and slight vacuolation reported in other plant secretory cells (Schnepf, 1969), and they are essentially the same as the osmophore cells of other species of *Stanhopea* (Stern et al., 1987). Mitochondria, SER, and amyloplasts with prominent plastoglobuli typically associated with the grana are common (Fig. 2, 3, 6); dictyosomes and RER are uncommon. Lipid globules and present in the cytoplasm (Fig. 2, 5, 6, 13), and there are usually several small vacuoles in the plane of each section (Fig. 4, 5, 8–11). Amorphous, electron-dense material, suggested as the fragrance produced by these cells in other species of *Stanhopea* (Stern et al., 1987), occurs between the plasmalemma and the cell wall of *S. anfracta.*

Although different fixation times were executed, there were no obvious differences in the degree of tissue preservation or in the quantity or distribution of precipitate in any of the tissue. Most of the tissue was adequately preserved, but examples of poorly fixed cells could be found in all experimental and control groups at all fixation times. Precipitate in poorly preserved cells was much heavier than in well-preserved cells, whether in experimental or control tissue, and always occurred immediately outside the plasmalemma in these cells as well as being associated with mitochondria, SER, and plastid membranes. No distinction will be made among the three fixation times, and the following descriptions are based on well-preserved areas of tissue.

Osmophore tissue collected at midday and incubated in the complete acetoacetyl CoA medium commonly had precipitate distributed between the inner and outer mitochondrial membranes (Fig. 4), and the intermembrane area of SER (Fig. 4–6). Precipitate was seen infrequently in the intermembrane spaces of the grana (Fig. 6) and between the inner and outer membranes of the amyloplasts. Precipitate immediately outside the tonoplast was rare (Fig. 5), and no precipitate was seen associated with mitochondrial cristae, RER, the nucleus, the cytosol, or within or without the cell wall.

Areas of poorly fixed cells could be found in the otherwise adequately fixed material for all experimental and control tissues. Precipitate occurred immediately outside the plasmalemma in the cells of these various tissues (Fig. 7), and it was also associated with the inner and outer mitochondrial membranes, SER, amyloplast membranes, and tonoplast. The precipitate was generally heavier and more widespread in the poorly fixed cells than in the adequately preserved areas.

Osmophore tissue collected at midday and incubated with the complete carnitine medium was similar to tissue incubated with complete acetoacetyl CoA (Fig. 8, 9). Precipitate was found between the inner and outer mitochondrial membranes and the intermembrane area of SER. Precipitate was seen infrequently associated with amyloplast membranes, and no precipitate was associated with tonoplast.

In osmophore tissue collected at midday, representing acetoacetyl CoA substrate control (Fig. 10), carnitine substrate control (Fig. 11), and primary reaction product control (Fig. 12), cells infrequently exhibited precipitate between inner and outer mitochondrial membranes only. Most of this control tissue was similar to that control tissue on which no cytochemical enzyme localization tests had been attempted. Tissue representing a control for the final reaction product showed no precipitate whatsoever (Fig. 13). Osmophore tissue collected at 2100 hr showed no precipitate whether experimental or control.

DISCUSSION—*HMG-CoA synthase localization*—The principal enzyme sought for localization was HMG-CoA synthase which catalyzed the reaction between the acetyl CoA and acetoacetyl CoA added to the tissue to yield HMG-CoA and the free CoA-SH (Fig. 1) which,

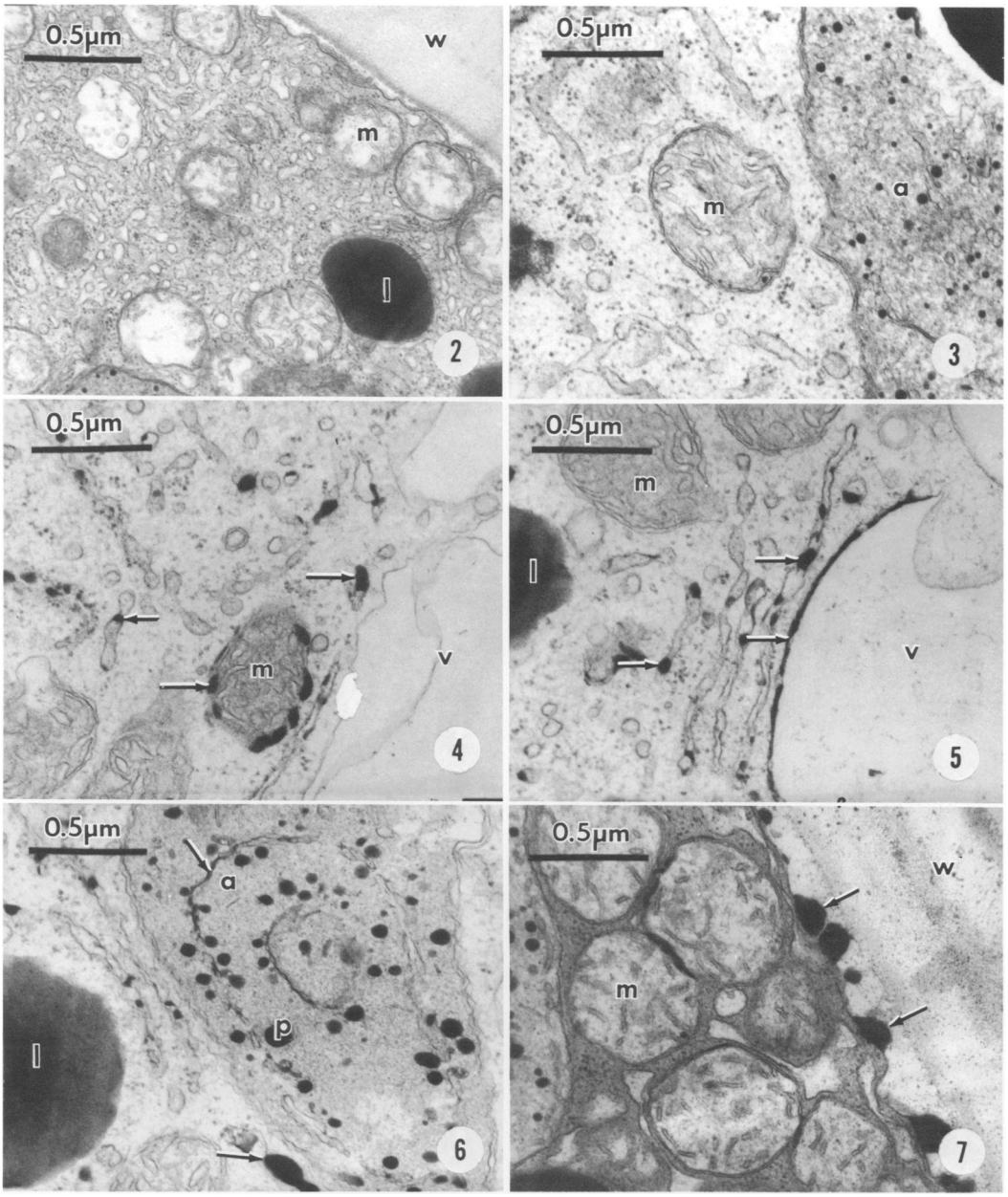

Fig. 2–7. Osmophore cells of *Stanhopea anfracta*. a = amyloplast, w = cell wall, l = lipid globule, m = mitochondrion, p = plastoglobulus, v = vacuole. **2, 3.** Cells representing standard fixation; no cytochemical enzyme localization. **4–7.** Cells representing complete acetoacetyl CoA medium. Arrows indicate some intermembrane precipitates in mitochondria, SER, and granal membranes of amyloplast. **7.** Poorly fixed tissue representing complete acetoacetyl CoA medium. Precipitate outside the plasmalemma (arrows) is considered an artifact.

in turn, led to a uranyl ferrocyanide precipitate. The HMG-CoA subsequently is reduced to mevalonic acid (Fig. 1) which is expected in the osmophore of *S. anfracta* to be channeled mainly into the terpenoid component of its fragrance. Actually, any point in the mevalonic acid pathway where free CoA-SH is generated will yield the uranyl ferrocyanide precipitate, so localization of acetyl CoA : acetyl CoA C-transferase and HMG-CoA reductase are inseparable from HMG-CoA synthase localization where the complete incubation medium is used. The highest level of precipitation is expected to be associated with HMG-CoA synthase because of the quantity of substrate presented to this enzyme.

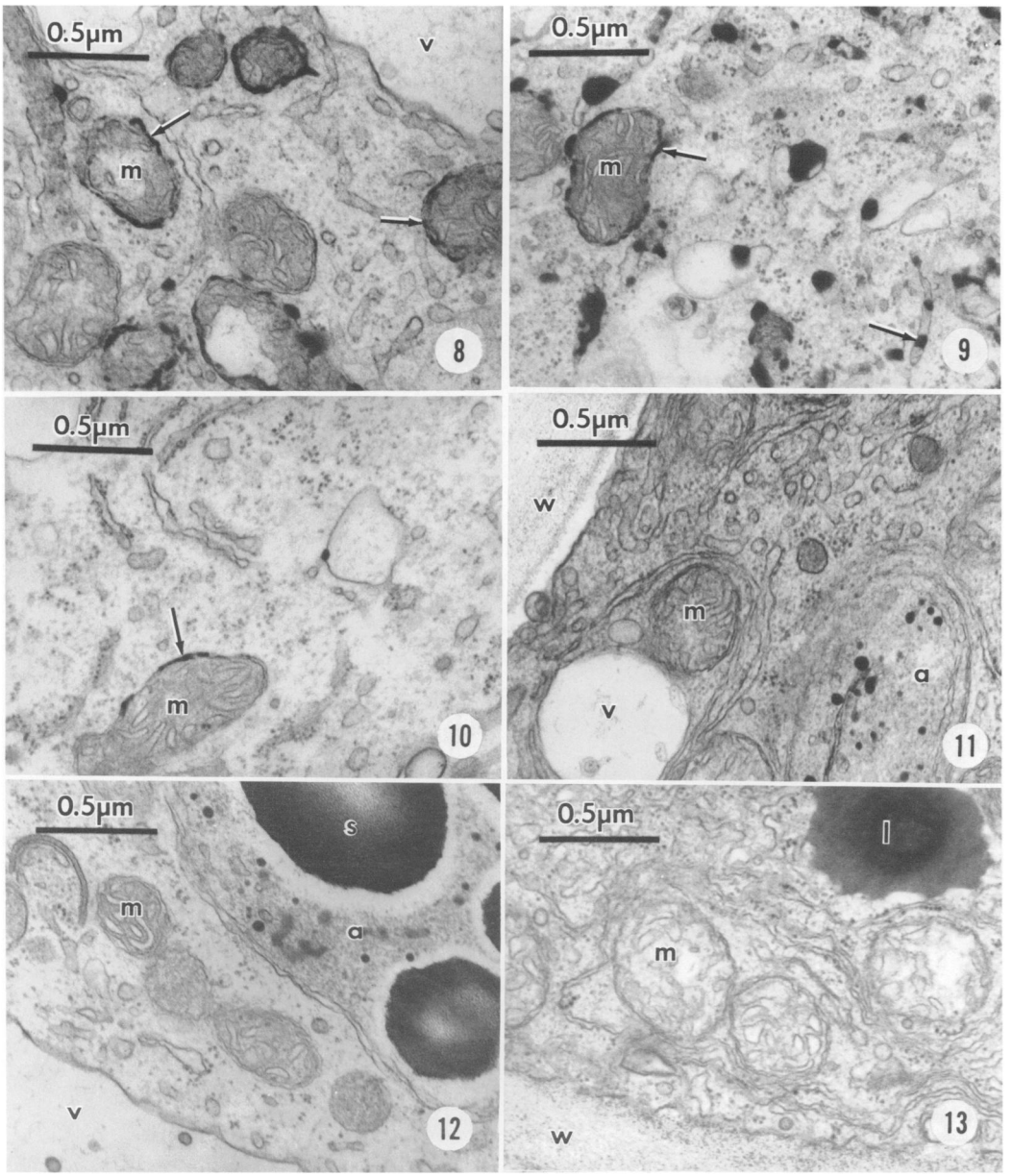

Fig. 8–13. Osmophore cells of *Stanhopea anfracta*. a = amyloplast, w = cell wall, l = lipid globule, m = mitochondrion, s = starch grain, v = vacuole. **8, 9.** Cells representing complete carnitine medium. Arrows indicate some intermembrane precipitate in mitochondria and SER. **10.** Cell representing acetoacetyl CoA substrate control medium (acetyl CoA omitted) showing rare precipitate (arrow). **11.** Cell representing carnitine substrate control medium (acetyl CoA omitted). No precipitate is present. **12.** Cell representing primary reaction product control medium (acetoacetyl CoA and carnitine omitted). No precipitate is present. **13.** Cell representing final reaction product control medium (acetyl CoA, acetoacetyl CoA, and carnitine omitted). There is no precipitate.

In the original carnitine acetyltransferase localization procedure (Higgins and Barrnett, 1970) the control for the primary reaction product, in which carnitine is omitted from the incubation medium, is used to test for the possibility of acetyl CoA undergoing hydrolysis to form free CoA-SH and acetate. This control in the mevalonic acid pathway actually represents a situation in which a large amount of substrate (acetyl CoA) is presented to acetyl CoA : acetyl CoA C-transferase (Fig. 1). Acetoacetyl CoA is produced by combining two molecules of acetyl CoA (Fig. 1) so the control for the primary reaction product should lead to a pre-

cipitate, because it provides substrate to the acetyl CoA : acetyl CoA C-transferase enzyme. The infrequency of the precipitate is difficult to explain.

A precipitate would occur in the substrate control if the added acetoacetyl CoA reacted with endogenous acetyl CoA via HMG-CoA synthase (Fig. 1). Visible precipitate was rare and so indicated that endogenous pools of acetyl CoA were relatively small. The infrequency of precipitate in both the primary reaction product control and the substrate control leads to the conclusion that HMG-CoA synthase is the principal enzyme being localized by the procedure used here.

Brooker and Russell (1975) suggested the existence of three biosynthetic pathways for isoprenoid synthesis, respectively distributed in the microsomal fraction (mostly ER with tonoplast and plasmalemma), the mitochondria, and the plastids, based on assays for HMG-CoA reductase in pea seedlings. Apparently the mevalonic acid pathway is associated with several subcellular locations in *S. anfracta* as indicated from the different points of precipitation. Each organelle involved may be responsible for the synthesis of one or more specific terpenoids or may be involved in only the early stages of isoprenoid synthesis with the final products being completed elsewhere in the cell.

The precipitate in *S. anfracta* was most common between the inner and outer mitochondrial membranes and in the SER. These same locations of the mevalonic acid pathway have been reported by Brooker and Russell (1975) in *Pisum sativum*. The tonoplast, an infrequent site of precipitate in *S. anfracta,* is part of the microsomal fraction containing HMG-CoA reductase (Brooker and Russell, 1975), but I know of no report actually specifying the tonoplast as a site of mevalonic acid synthesis.

No precipitate was seen in the dictyosomes or RER. These organelles are usually associated with protein and carbohydrate synthesis, so enzymes of the mevalonic acid pathway would not be expected there. However, it should be noted that dictyosomes are uncommon in the osmophores of *S. anfracta,* so any rare precipitate in this organelle could be overlooked.

Precipitate occurring in the poorly fixed areas of the control and experimental osmophore tissues is considered artifactitious in both the HMG-CoA synthase and carnitine acetyltransferase localization experiments. Areas of plasmalemma and other membranes in poorly fixed locations may inhibit one or more of the incubation products, e.g., acetyl CoA, from crossing that area of the membrane possibly resulting in a precipitate there from the increased local concentration.

Material collected at 2100 hr of the first day of anthesis was not expected to show any precipitate in the experimental sample, since the fragrance is weakest at this time. No precipitate was seen, indicating that the mevalonic acid pathway was not at all active or was active at a level too low to produce a visible precipitate.

Carnitine acetyltransferase localization—Several different carnitine acyltransferases have been identified from chloroplasts and mitochondria in barley and pea (Thomas et al., 1982, 1983; McLaren et al., 1985), and tentative evidence for their presence in peroxisomes and in the cytosol has been published (Thomas et al., 1983; McLaren et al., 1985). A precipitate between the inner and outer mitochondrial membranes in osmophore cells corresponds to the subcellular location reported in animal cells (Higgins and Barrnett, 1970) and indicates the presence of carnitine acetyltransferase on the inner and/or outer mitochondrial membranes. The precipitate on the outer, inner, and granal amyloplast membranes, although infrequent, suggests the presence of carnitine acetyltransferase at these locations.

The absence of precipitate in the material collected at 2100 hr indicates that carnitine acetyltransferase is inactive or barely active at night. This suggests some direct or indirect relationship between carnitine acetyltransferase and the mevalonic acid pathway.

Conclusion—The principal enzyme of the mevalonic acid pathway, localized by a technique described here, is HMG-CoA synthase. This enzyme and carnitine acetyltransferase are located primarily in the SER and between the inner and outer mitochondrial membranes and are associated secondarily with plastid membranes. The multiplicity of compartmented subcellular sites for the mevalonic pathway may be necessary for the synthesis of a variety of different terpenoid end products. The cyclic fragrance production by the flowers of *S. anfracta* apparently is controlled at the level of the mevalonic acid pathway rather than at a later step in terpenoid synthesis. The apparent cyclic activity of carnitine acetyltransferase suggests that this enzyme is directly or indirectly linked to isoprenoid production.

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