Effects of Protein Synthesis Inhibitors on ent-Kaurene Biosynthesis during Photomorphogenesis of Etiolated Pea Seedlings

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Abstract
Excised shoot tips from 10-day-old etiolated pea (Pisum sativum L. cv. Alaska) seedlings were incubated in solutions of chloramphenicol, cycloheximide, and lincomycin at different concentrations during periods of 0, 4, 8, and 12 hours of irradiation with high intensity white light. Enzyme extracts were prepared from the whole shoot tips and compared with extracts from nontreated shoot tips for their capacity to synthesize ent-kaurene from mevalonate. In control samples, kaurene synthesis increased during the first 8 hours of irradiation and decreased after 12 hours. Chlorophyll content increased steadily up to 12 hours of irradiation. Chloramphenicol and cycloheximide reduced both kaurene synthesis and chlorophyll formation to a similar extent during all periods of irradiation, the reduction being greatest after 8 hours of irradiation. Lincomycin, a specific inhibitor of the formation of chloroplast ribosomes in detached pea shoot tips, did not significantly affect kaurene synthesis activity but strongly inhibited chlorophyll formation. It is tentatively concluded that the increase in kaurene synthesis activity during normal photomorphogenesis in pea seedlings is due to photoinduction of de novo synthesis of one or more proteins involved in the biosynthetic pathway from mevalonate to kaurene.

It was reported from our laboratory (6) that cell-free extracts from pea shoot tips of light-grown pea seedlings had a 5-fold greater capacity for synthesizing kaurene, and potentially therefore GA, from mevalonate than extracts from shoot tips of etiolated seedlings of the same age. Upon continuous irradiation of 10-day-old etiolated seedlings with high intensity white light, an exponential increase in kaurene synthesis activity was observed over a period of approximately 12 hr, attaining a level equal to that of light-grown plants of the same age. Although the light-stimulated increase in kaurene synthesis and chloroplast development occurred concurrently, there was no evidence at that time as to whether the two processes are causally related. The possibility that a direct cause and effect relationship between chloroplast development and light-stimulation of kaurene biosynthesis does exist is indicated by numerous reports that: (a) GAs occur in chloroplasts and other plastids (3, 4, 22); (b) at least some GA biosynthesis occurs in those organelles (18, 21, 23); and (c) light affects GA metabolism in a complex way, including apparent effects on biosynthesis (19), interconversions (20), apparent release from membrane-bound forms (3), and phytochrome-mediated efflux from plastids (4, 11, 13, 25).

These investigations were undertaken to determine whether the observed increase in kaurene synthesis activity caused by irradiation with white light is associated with de novo enzyme synthesis and, if so, whether that essential protein synthesis is localized in chloroplasts. Use was made of selective inhibitors of protein synthesis, namely, the d-threo isomer of chloramphenicol, cycloheximide, and lincomycin. On the basis of all available data, it appears that photoinduction of de novo synthesis of one or more proteins essential for kaurene, hence GA, biosynthesis does occur during de- etiolation of pea seedling shoots.

Materials and Methods
Culture and Sampling of Plants. Peas (Pisum sativum L. cv. Alaska), from W. Atlee Burpee Company, Riverside, Calif., were grown in darkness in growth chambers under a regimen consisting of 16 hr at 22 ± 1 C alternating with 8 hr at 17 ± 1 C for 10 days. The Vermiculite rooting medium was irrigated with distilled H2O every other day. Plants used for standardizing conditions for assaying kaurene synthesis in cell-free extracts of shoot tips were exposed to continuous high intensity light (1,000–1,300 ft-c; 1.8 × 10−4 to 2.4 × 10−4 erg cm−2 sec−1) for periods of 4, 8, or 12 hr. Shoot tips were harvested by excising them below the fourth node of the seedling axes (numbering from the cotyledonal node). All tissues above the fourth node were included in the samples, which were frozen and stored in liquid N2.

Plants used for investigating the effects of chloramphenicol, cycloheximide, and lincomycin were grown in darkness for 10 days. Then, etiolated shoot tips were excited as before and placed in sterile Petri dishes (approximately 1.2 g, 40 shoot tips/Petri dish), each of which contained a disc of filter paper and 10 ml of one of the following solutions: 0.1 mg/ml of water, 1 mg/ml, 10 mg/ml, and 33 mg/ml of chloramphenicol or cycloheximide or 0.01 µg/ml, 0.10 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml of lincomycin. After incubation for 1 hr in darkness (17), the Petri dishes were transferred to the irradiation chamber and illuminated for 4, 8, or 12 hr as described before. Shoot tips in Petri dishes were in contact with the test solution throughout the irradiation period. Except during irradiation with white light, live plant material was always handled under a green safelight (General Electric 15-W green fluorescent lamp covered with eight layers of amber and three layers of green cellophane). After the irradiation period, shoot tips were removed from the Petri dishes, blotted,
frozen, and stored in liquid N₂.

Preparation of Enzyme Extracts and Reaction Conditions. General procedures were as described previously (5, 6, 18). Excised frozen shoot tips were ground in a chilled mortar with pestle to a fine powder. As the tissue thawed, insoluble PVP (0.5 g wet PVP g⁻¹ fresh wt of tissue) and 0.1 M KH₂PO₄-K₂HPO₄ buffer (pH 7.1; 1 ml g⁻¹ fresh wt of tissue) containing 20 M MDT and 133 µM chloramphenicol were quickly added, and the mixture was homogenized immediately. The homogenate was filtered once through four layers of cheesecloth, and the filtrate was centrifuged at 10,000g for 10 min at 0 to 4°C. The 10,000g supernatant was centrifuged at 100,000g for 90 min at 0 to 4°C, and the resulting supernatant was the enzyme extract. Average protein concentrations (mg/ml) in the enzyme extracts were as follows: etiolated shoot tips, 20.3; shoot tips excised from whole seedlings after irradiation for 4, 8, and 12 hr, 22.6, 18.6, and 16, respectively; and shoot tips excised prior to irradiation for 4, 8, and 12 hr, 16.1, 14.6, and 13.6, respectively.

Following optimization of the reaction conditions, reaction mixtures routinely contained 19.2 µM [2-¹⁴C]MVA (13 mCi/mmol), 2 mM MgCl₂, 2 mM MnCl₂, 12 mM ATP, 0.70 ml of enzyme extract, 75 mM KH₂PO₄-K₂HPO₄, 15 mM DTT, and 100 µM chloramphenicol in a total volume of 1 ml (pH 6.1). Reaction mixtures were incubated at 30°C for 2 hr in an aerobic atmosphere. Each reaction was stopped by the addition of 2 ml of acetic acid containing 2.5 µg of nonradioactive kaurene. The yield of radioactive kaurene then was measured as described previously (5, 6, 18). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, model 2425. Counting rates were converted to dpm by internal standardization. All vials were counted prior to addition of radioactive samples to obtain a background value for each sample. Routinely, all assays were run in duplicate or triplicate, and all experiments were repeated at least twice.

The Chl content of greening shoot tips was estimated by the procedure of Arnon (2).

The protein content of 10% trichloroacetic acid-insoluble material in the enzyme extracts was estimated by the method of Lowry et al. (16), using BSA as a standard.

Reagents. [2-¹⁴C]MVA (13 mCi/mmol) in benzene solution was purchased from Amersham/Searle. The benzene was evaporated under nitrogen, and the lactone was hydrolyzed in 100% excess NaOH for 8 hr. PVP (insoluble Polyclar-AT) was obtained from GAF Corporation, Grasselli, N.J. The PVP was purified according to the procedure of Loomis (15), washed once with 0.1 M KH₂PO₄-K₂HPO₄ buffer (pH 7.1), and then suspended in 0.1 M KH₂PO₄-K₂HPO₄ (pH 7.1), containing 100 µM chloramphenicol. After sedimentation, the excess buffer was removed and the wet PVP was stored at 4°C. Chloramphenicol and cycloheximide were purchased from Sigma Chemical Co. Lincomycin HCl (potency 860 µg/mg) was a gift of the Upjohn Company, Kalamazoo, Mich. Omnifluor was purchased from New England Nuclear. Kaurene was a gift from Abbott Laboratories, North Chicago, Ill.

RESULTS

Partial Optimization of Assay Conditions. The preparation and assay conditions which yielded maximum activity for kaurene synthesis are described in detail under "Materials and Methods." The most critical conditions (cf. 6) were: (a) 0.1 M KH₂PO₄-K₂HPO₄ buffer (pH 7.1) containing 20 M MDT for enzyme extraction; (b) ATP at concentrations from 12 to 15 mM in incubation mixture; (c) final pH of 6.1 in reaction mixture; (d) Mg²⁺ and Mn²⁺ each at concentration of 2 mM; and (e) MVA concentration between 18 and 20 µM.

Effect of Light on Kaurene Synthesis Activity During De- etiolation of Whole Seedlings. Confirming our previous work (6) irradiation of whole 10-day-old etiolated seedlings with high intensity white light induced an approximately exponential increase in kaurene synthesis activity from very low activity (0 dpm incorporated) at 0 hr to a maximum activity at 12 hr. Thereafter, kaurene-synthesizing activity remained constant through 16 hr of irradiation. Chl content increased at a fairly constant rate from 0 hr to 12 hr, also as observed previously (6).

Effect of Light on Detached Etiolated Shoot Tips. In enzyme extracts prepared from detached shoot tips of irradiated 10-day-old etiolated seedlings, kaurene synthesis activity consistently increased between 0 hr and the 8th hr of irradiation, then decreased from the 8th to the 12th hr, again attaining a level comparable to that observed after 4 hr of irradiation. In contrast, Chl content continued to increase with 12 hr of irradiation (Fig. 1). By comparison with results reported here and elsewhere (6) for the change in enzyme activity in extracts prepared from shoot tips of irradiated whole seedlings during de-etiolation, the enzyme activity in excised shoot tips consistently occurred at lower rates, as expected, and responded to irradiation by a lower magnitude of change.

Effects of Chloramphenicol on Kaurene Synthesis and Chl Formation. Chloramphenicol reduced development of kaurene synthesis activity at all concentrations tested during all periods of irradiation. The percentage of reduction, compared with the water control, was consistently greatest at 8 hr, for which data are presented (Fig. 2). Chlor form content also was inhibited but to a lesser degree than kaurene synthesis at all concentrations tested (Fig. 2).

Effects of Cycloheximide. Cycloheximide also reduced kaurene synthesis during all periods of illumination at all concentrations tested. The percentage of reduction always was greatest at 8 hr (Fig. 3) ranging from 27 to 77% in samples treated with 0.1 mg/ml to 33 mg/ml of cycloheximide, respectively, at 4-hr illumination.

![Fig. 1. Increase in kaurene-synthesizing capacity and Chl content of cell-free extracts from 10-day-old detached etiolated pea shoot tips during 12-hr irradiation with high intensity white light. Data represent means based on six independent experiments.](https://via.placeholder.com/150)

![Fig. 2. Effects of chloramphenicol on kaurene-synthesizing activity and Chl content in detached etiolated pea shoot tips during 8-hr irradiation. Data represent means based on three independent experiments.](https://via.placeholder.com/150)
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FIG. 3. Effects of cycloheximide on kaurene-synthesizing activity and Chl content of cell-free extracts from detached etiolated pea shoot tips during 8-hr irradiation with high intensity white light. Data represent means based on three independent experiments.

30 to 82% at 8 hr; and 60 to 78% at 12 hr in samples treated with the same concentrations of cycloheximide. Cycloheximide also affected Chl content at all concentrations assayed (Fig. 3), but the highest inhibition was observed after 8 hr of illumination, resembling the effect of the inhibitor on enzyme activity.

Effects of Lincomycin. Lincomycin did not significantly reduce kaurene synthesis activity at any concentration tested (up to 100 µg/ml) under any irradiation conditions (Fig. 4) in any experiment. Thus, the apparent lower enzyme activity seen at the intermediate concentration in Figure 4 was not a reproducible observation. In contrast, Chl formation was consistently inhibited under conditions of 4-, 8-, (Fig. 4), and 12-hr irradiation and at all concentrations used. Lincomycin was approximately 15 times more effective than either of the other two antibiotics in causing 50% inhibition of greening.

It is important to note that the reduction observed for kaurene synthesis activity was not due to an inhibitory effect of the antibiotics on the activity of preformed enzyme in vitro. Direct addition of chloramphenicol and cycloheximide to reaction mixtures at the same concentrations which caused 50% reduction of enzyme activity when used to treat excised shoot tips inhibited kaurene synthesis by average amounts of only 3 and 7%, respectively. No inhibition was observed with lincomycin added directly to reaction mixtures.

The level of kaurene synthesis activity has been expressed here as dpm incorporated in kaurene per mg of protein. It is noteworthy that similar percentages were obtained when the results were expressed as dpm incorporated in kaurene per g fresh wt of shoot tip tissues.

DISCUSSION

Interpretation of the effects of protein synthesis inhibitors in an intact system or a multiple-step enzymic pathway is difficult at best. Hence, we shall present only a tentative interpretation of the combined effects of chloramphenicol, cycloheximide, and lincomycin on the light-induced increase in kaurene synthesis activity in pea shoot tips. That interpretation is that: (a) the observed light-induced increase in kaurene synthesis activity results from de novo synthesis of an enzyme or other protein which is essential for kaurene synthesis from mevalonate; and (b) although the susceptible protein(s) (hereafter referred to in the singular) may be compartmentalized in chloroplasts, it or its precursor form(s) is (are) synthesized on cytoplasmic ribosomes.

Chloramphenicol reduced both kaurene synthesis activity and Chl formation during irradiation of etiolated detached pea shoot tips. In view of the many reports that chloramphenicol is a selective inhibitor of protein synthesis on chloroplast ribosomes (1, 7), it might be tempting to suggest that the susceptible protein is synthesized de novo on chloroplast ribosomes upon irradiation with white light. This would be consistent with some reports but not others, and on balance would seem to be an erroneous interpretation. Indeed, of the chloroplastic proteins that have been investigated, it appears that only certain membrane proteins required for the immobilization of chloroplast components are synthesized in situ. Most chloroplastic proteins, including the proteins of PSI and PSII of photosynthesis, are synthesized in the cytoplasm (8). Hence, apparently chloramphenicol interferes with development of kaurene synthesis activity only indirectly, as explained later.

Cycloheximide was highly effective also in reducing kaurene synthesis activity and Chl formation, and the effects of this inhibitor on both processes were very similar in extent after 8 hr of illumination. This antibiotic generally has been reported as an inhibitor of cytoplasmic protein synthesis which binds to 80S ribosomes (10). The results reported here for cycloheximide suggest that the susceptible protein is cytoplasmic in origin.

Analyzing the combined results obtained with chloramphenicol and cycloheximide, it appears that the susceptible protein, while ultimately compartmentalized in the chloroplast, actually is synthesized in the cytoplasm. Interference with the increases in kaurene synthesis activity by chloramphenicol can be envisaged to be due to the effect of the antibiotic in inhibiting the development of the membrane system in the chloroplast and in this indirect manner, inhibiting enzyme activity. Fortunately, this idea could be tested by the experiments with lincomycin, which reportedly is a more specific inhibitor than chloramphenicol on chloroplast ribosome activity (14). Of particular relevance is the report by Ellis and Hartley (9), who reported that it was a very effective inhibitor of protein synthesis on chloroplast ribosomes in detached shoot tips and in isolated chloroplasts of P. sativum, and the paper by Thomson and Ellis (24) which showed that lincomycin specifically prevented both the formation of chloroplast membranes and their stacking into grana.

When lincomycin was tested on etiolated detached shoot tips in the present investigations, it did have an inhibitory effect on Chl formation, as has been previously reported (24); but kaurene synthesis activity was not consistently or significantly inhibited under any of the conditions tested, even at concentrations which inhibited Chl formation by 50%. These results seemingly not only ruled out the possibility of the enzyme being synthesized in the chloroplast, but strengthened the possibility that the susceptible protein nevertheless ultimately is localized in the chloroplast, as was first suggested by the results with chloramphenicol. Ellis (8) suggested that one specific role of chloroplast ribosomes is to
synthesize membrane proteins required for the immobilization of some chloroplastic ribosomes, but which also are inhibited by lincomycin. Contrariwise, in Ellis' (8) work, lincomycin hardly affected PSII proteins because they are incorporated into or onto the developing membranes as they enter the chloroplast from the cytoplasm. Hence, so far as site of synthesis and ultimate compartmentation are concerned, the susceptible protein appears to be analogous to the chloroplastic proteins of PSII of photosynthesis.

Preliminary evidence suggests that one enzyme which is susceptible to inhibition by chloramphenicol and cycloheximide may be kaurene synthetase, the enzyme (or enzymes) which catalyzes the two-step cyclization of trans-geranylgeranyl pyrophosphate to kaurene (see 12, 26). Present data do not exclude any of the enzymes in the pathway from mevalonate to kaurene, nor indeed other proteins that may be required in any way for operation of the pathway in situ.

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