AN ABSTRACT OF THE THESIS OF

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Title: PHOTOINDUCTION OF KAURENE SYNTHETASE, AN ENZYME OF THE GIBBERELLIN BIOSYNTHETIC PATHWAY, DURING DE-ETIOLATION OF PEA SEEDLINGS

Abstract approved: Redacted for Privacy
Thomas C. Moore

Previous investigations have shown that the enzymes catalyzing the biosynthesis of the gibberellin-type of hormones from mevalonic acid in plants are compartmentalized in plastids. Moreover, the activity of one of the key enzymes in the biosynthetic sequence, kaurene synthetase, exhibits markedly enhanced activity when a dark-grown pea seedling undergoes de-etiolation in high-intensity white light. Kaurene synthetase catalyzes the two-step cyclization reaction by which the diterpene ent-kaurene is formed from geranygeranyl pyrophosphate.

The purposes of these investigations were to determine whether the effect of light on kaurene synthetase activity is to induce de novo synthesis of enzyme or to activate enzyme already present, and to contribute more detailed

information regarding compartmentation of kaurene synthetase in chloroplasts.

Excised shoot tips from 10-day old etiolated pea (Pisum sativum 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 non-treated shoot tips for kaurene synthetase activity. The rate of chlorophyll formation was used as a measure of de-etiolation. It was found that, in control samples, kaurene synthetase activity increased 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 synthetase activity and chlorophyll content to a similar magnitude during all periods of irradiation, the reduction being greatest after 8 hours of irradiation. Lincomycin, which has been shown to be a specific inhibitor of the formation of chloroplast ribosomes in detached pea shoot tips, hardly affected kaurene synthetase activity but strongly inhibited chlorophyll formation. On the basis of the combined effects of the three antibiotics tested, it is tentatively concluded that: (a) the increase in kaurene synthetase activity during de-etiolation is due to photoinduction of de novo synthesis of the enzyme;

and (b) kaurene synthetase or its precursor is synthesized on cytoplasmic ribosomes but that the functional enzyme is compartmentalized in chloroplasts.

In advancing this tentative interpretation as a working hypothesis, it is acknowledged that other proteins than kaurene synthetase may be found in further research to be involved in the observations here reported.

Photoinduction of Kaurene Synthetase, an Enzyme of the Gibberellin Biosynthetic Pathway, during De-etiolation of Pea Seedlings

by

Gladys Gomez-Navarrete

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Redacted for Privacy

Professor of Botany
in charge of major

Redacted for Privacy

Chairman of the Department of Botany and
Plant Pathology

Redacted for Privacy

Dean of the Graduate School

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Typed by Deanna L. Cramer for Gladys Gomez-Navarrete

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INTRODUCTION

Evidence has been accumulating since 1968 that at least some GA[1] biosynthesis is compartmentalized in chloroplasts and other plastids. Stoddart (1968) reported that extracts of chloroplast fractions of leaves of Brassica oleracea and Hordeum vulgare had biological activity resembling that of GA's. Subsequently, he showed that preparations of B. oleracea converted ent-kaurenoic acid to a GA-like substance (Stoddart, 1969). Later, Murphy and Briggs (1975) showed that ent-kaurenol and ent-kaurenoic acid were converted to ent-kaurenal and ent-hydroxykaurenoic acid, respectively, in preparations of barley leaf chloroplasts. Extracts of proplastids from wild cucumber (Marah marcocarpus) endosperm were reported to readily convert geranylgeranyl pyrophosphate or copalyl pyrophosphate to ent-kaurene; extracts from etiolated pea (Pisum sativum) shoot tips and developing castor bean (Ricinus communis) endosperm also converted copalyl pyrophosphate to kaurene, but showed

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[1]Abbreviations: ent-kaurene, also known as ent-kaur-16 ene and (-)-kaur-16 ene, referred to elsewhere in the text simply as kaurene; kaurenoic acid: ent-kaur-16-ene-19-oic acid; GA: gibberellins; MVA: mevalonic acid; PVP: polyvinylpyroolidone; ATP: adenosine 5-triphosphate; RNA: ribonucleic acid.

little or no activity for synthesizing kaurene from geranylgeranyl pyrophosphate (Simcox et al.,1975). At about the same time, Moore and Coolbaugh (1976) independently presented evidence that kaurene synthetase is present in pea chloroplasts, since chloroplast extracts were able to convert geranylgeranyl pyrophosphate to ent-kaurene and also enhanced kaurene production from mevalonic acid-(2-^{14}C) in extracts of pea shoot tips.

Numerous reports indicate that light induces alterations in GA metabolism. GA biosynthesis, RNA and protein synthesis appeared to be implicated in an observed increase in GA-like substances which occurred after 30 minutes of red light treatment (Reid et al., 1968). In etiolated barley (Hordeum vulgare) leaf sections the increase in GA-like substances also appeared to require GA biosynthesis (Reid and Clements, 1968) and RNA and protein synthesis. Homogenates of etiolated barley leaves exposed to brief periods of red irradiation, displayed an immediate increase in the levels of GA-like substances, probably due to a greater conversion of ^{3}H-GA_9 into other GA-like substances (Railton and Reid, 1974b). This possibility is supported by the fact that six GA-like substances have been detected in light-grown shoots of pea seedlings. However, only one such substance was detected in chloroplasts isolated from young leaves (Railton and Reid, 1974a). In etiolated wheat (Triticum aestivum) leaf sections, an increase in GA-like substances

occurred after 5 minutes of red light treatment, which appeared to be the result of a release of GA from bound forms (Loveys and Wareing, 1971).

The involvement of plastids in the rapid effect of red light on GA metabolism was based initially on the response of etioplasts *in vitro* and *in situ* to red and far red light, suggesting the presence of a phytochrome mechanism inside or on the plastid envelope (Wellburn and Wellburn, 1973). This has been confirmed by Cooke and Saunders (1975) who demonstrated a phytochrome dependent increase in extractable GA-like activity in plastid preparations from etiolated wheat leaves. Later Cooke and Kendrick (1976) reported that phytochrome and GA-like substances were associated with etioplast envelopes. Smith and Elliot (1975) isolated a bound phytochrome from maize (*Zea mays*) seedlings, and Evans and Smith (1976a) presented spectrophotometric evidence for the presence of phytochrome in the envelope membrane of barley etioplasts.

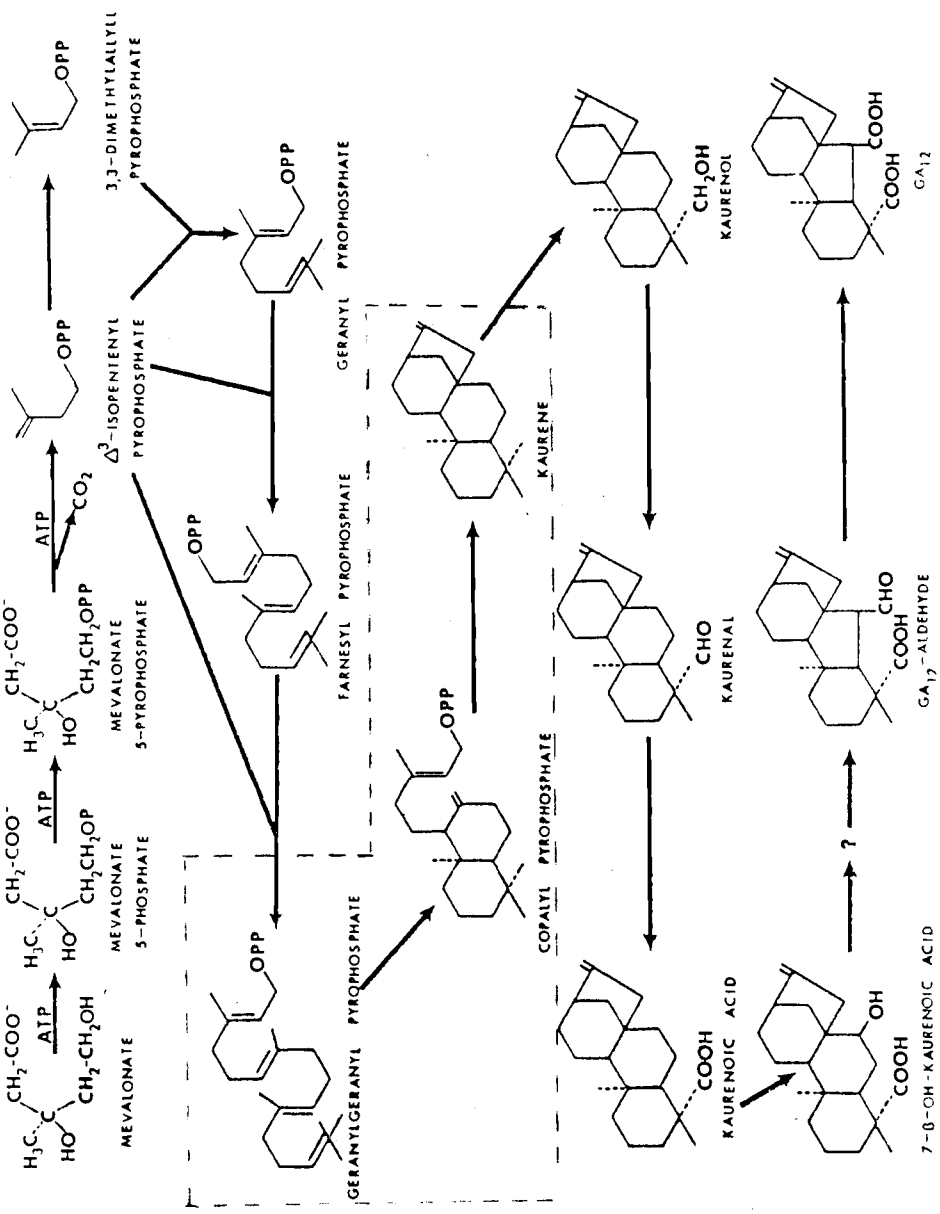
Wellburn and Wellburn (1973) suggested that the enhancement of plastid development by red light was due to an effect of phytochrome on the plastid envelope, thereby modifying the permeability of the envelope. This suggestion has been supported by Evans and Smith (1976b), who reported that the enhanced level of GA-like substances extractable from a suspension of intact etioplasts after five minutes of irradiation, was a phytochrome effect causing

the transport of GA from inside the etioplasts to the surrounding medium. Wellburn and Hampp (1976) reported changes in the envelope penetrability of Avena sativa plastids associated with fluxes of GA and abscisic acid during plastid development. Hampp and Schmidt (1977) presented evidence that phytochrome controls the permeability of membranes of mitochondria and plastids. When dark-grown laminae of Avena sativa were irradiated with continuous far-red light or red light pulses prior to the isolation of the organelles and incubation of them in a solution containing δ-aminolevulinate and succinate, both types of organelles exhibited a sharp rise in the internal concentrations of both metabolites. The same effects were observed after in vitro irradiation of isolated mitochondria and etioplasts (Schmidt and Hampp, 1977). Finally, Browning and Saunders (1977) reported that gibberellins A_9 and A_4 are in intimate association with the chloroplast membranes because they are released only by detergent treatment and not by sonication.

Previously it was reported by Moore and Ecklund (1974) and Ecklund and Moore (1974) that cell-free extracts from pea shoot tips of light-grown pea seedlings had a five-fold greater capacity for synthesizing kaurene from mevalonate (Figure 1) 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 synthetase

Figure 1. Partial pathway of GA biosynthesis from mevalonate. The two-reaction cyclization of geranylgeranyl pyrophosphate to kaurene, catalyzed by kaurene synthetase, is enclosed by a dashed line.

Figure 1


MEVALONATE
ATP
MEVALONATE 5-PHOSPHATE
ATP
MEVALONATE 5-PYROPHOSPHATE
ATP
CO_2
Δ^3-ISOPENTENYL PYROPHOSPHATE
3,3-DIMETHYLALLYL PYROPHOSPHATE
GERANYL PYROPHOSPHATE
FARNESYL PYROPHOSPHATE
GERANYLGERANYL PYROPHOSPHATE
COPALYL PYROPHOSPHATE
KAURENE
KAURENOL
KAURENAL
KAURENOIC ACID
7-β-OH-KAURENOIC ACID
GA_{12}-ALDEHYDE
GA_{12}
?

activity was observed over a period of approximately 12 hours, attaining a level equal to that of light-grown plants of the same age. Although the increase in capacity for kaurene synthesis and chloroplast development occurred concurrently, there was no evidence presented concerning whether the two processes are causally related (Moore and Ecklund, 1974; Ecklund and Moore, 1974).

The purposes of these investigations were to determine whether the observed increase in kaurene synthetase activity caused by irradiation with white light is associated with de novo enzyme synthesis, and whether that activity is localized in chloroplasts. Extensive use was made of selective inhibitors, namely, the D-threo isomer of chloramphenicol, which inhibits chloroplastic protein synthesis (Anderson and Smillie, 1966; Ellis, 1969; Ingle, 1968; Levine and Goodenough, 1970; Margulies and Brubaker, 1970; Margulies, 1971; and Parthier et al., 1964); cycloheximide, which inhibits cytoplasmic protein synthesis (Cocucci and Marré, 1973; Ellis and MacDonald, 1970; Lüttge et al., 1974); and lincomycin, which inhibits chloroplastic protein synthesis (Celma et al., 1970; Hoober et al., 1969; Igarashi et al., 1969; Vasquez and Monro, 1969). Incidental to the investigations was improvement of conditions for assaying kaurene synthesis in cell-free enzyme extracts of pea shoot tips.

MATERIALS AND METHODS

Culture and Sampling of Plants

Peas (Pisum sativum L. cv Alaska; from W. Atlee Burpee Company, Riverside, California) were grown in darkness, in growth chambers in 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 water every other day.

Plants used for standardizing conditions for assaying kaurene synthesis in cell-free extracts of shoot tips were grown in darkness for 10 days and then were exposed to continuous high-intensity light (1000-1300 ft-c; 1.8×10^4 to 2.4×10^4 erg cm^{-2} sec^{-1}) for periods of 4, 8, or 12 hr. After that, shoot tips were harvested by excising them below the fourth node of the seedling axes (node numbers begin with the cotyledonary node). All tissues above the fourth node were included in the samples, which routinely were frozen and stored in liquid nitrogen.

Plants used for investigating the effects of chloramphenicol, cycloheximide and lincomycin were grown in darkness during 10 days under the same conditions just described. The etiolated shoot tips were excised as before and placed in sterile petri dishes (approximately 1.2 g, 40 shoot tips, per petri dish), each of which contained a disc

of filter paper and 10 ml of one of the following solutions: water, 0.1 mg/ml, 1.0 mg/ml, 10 mg/ml and 33 mg/ml of chloramphenicol or cycloheximide or 0.01 µg/ml; 0.10 µg/ml, 1.0 µg/ml, 10 µg/ml and 100 µg/ml of lincomycin. After incubation for one hour in darkness (Margulies, 1962; Reid et al., 1968), the petri dishes were transferred to the irradiation chamber and illuminated for periods of 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 always was handled under a green safelight (General Electric 15W 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 nitrogen.

Preparation of Enzyme Extracts and Reaction Conditions

General procedures were as described previously (Coolbaugh et al., 1973). Excised frozen shoot tips were ground in a chilled mortar with pestle to a fine powder. As the tissue thawed, insoluble polyvinylpyrrolidone (0.5 g wet PVP g^{-1} fr/wt of tissue) and 0.1 M KH_2PO_4 buffer (pH 7.1; 1.0 ml g^{-1} fr/wt of tissue) containing 20 mM dithiothreitol 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 10,000 g for 10 min. The 10,000 g supernatant was centrifuged at 100,000 g for 90 min, and the resulting supernatant was the enzyme source. Both centrifugations were done at 0 to 4 C.

Following optimization of the reaction conditions, reaction mixtures routinely contained 19.2 μM MVA-2-^{14}C (13 mCi/m mole), 2 mM $MgCl_2$, 2mM $MnCl_2$, 12 mM ATP, 0.70 ml enzyme extract, 75 mM KH_2PO_4-K_2HPO_4, 15 mM dithiothreitol, and 100 μM chloramphenicol in a total volume of 1.0 (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 acetone containing 2.5 μg of non-radioactive kaurene. Acetone containing kaurene was added to controls prior to incubation. Each reaction mixture was extracted once with 1 ml of benzene and twice with 2 ml of benzene-acetone (3:1). The organic extracts from each reaction mixture then were combined and evaporated to dryness under vacuum. The resulting residue was extracted twice with 0.05 ml of acetone, which was transferred to a thin layer of Silica Gel G commercial plate (2.0 x 20 cm) for chromatography. Authentic kaurene was co-chromatographed with each sample extract. Thin-layer chromatograms were developed in n-hexane. The kaurene region of chromatogram was located by staining with iodine vapors and was removed for liquid scintillation spectrometry in a solution

of toluene containing 4 g of Omnifluor per liter. The methods used to identify kaurene have been described in detail previously (Coolbaugh et al., 1973). 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 pre-counted. Routinely, all assays were run in duplicate or triplicate, and all experiments were repeated at least twice.

The chlorophyll content of greening shoot tips was estimated by the procedure of Arnon (1948).

Protein content of 10% trichloroacetic acid-insoluble material in the enzyme extracts was estimated by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Reagents

Mevalonic acid-2-^{14}C (specific radioactivity 13 mCi/mmole) in benzene solution was purchased from Amersham/Searle Corporation, Des Plaines, IL. The benzene was evaporated under nitrogen, and the lactone was hydrolized in 100% excess NaOH for 8 hr. Polyvinylpyrrolidone (PVP) (insoluble Polyclar-AT) was obtained from GAF Corporation, Graselli, NJ. The PVP was purified according to the procedure of Loomis (1974), washed once with 0.1 M KH_2PO_4-K_2HPO_4 buffer, pH 7.1, and then suspended in 0.1 M KH_2PO_4-K_2HPO_4, 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 Company. Lincomycin HCl (potency 860 mcg/mg) was a gift from Dr. George B. Withfield, Jr. of the Upjohn Company, Kalamazoo, MI. Omnifluor was purchased from New England Nuclear Company. Kaurene was a gift from Dr. Robert K. Clark, Jr. of Abbott Laboratories, North Chicago, IL.

RESULTS

Partial Optimization of Assay Conditions

The preparation and assay conditions which yielded maximum activity for kaurene synthesis are described in detail in Materials and Methods. The most critical conditions were: a) 0.1 M KH_2PO_4-K_2HPO_4 buffer, pH 7.1, containing 20 mM dithiothreitol for enzyme extraction; increase of dithiothreitol concentration from 2 mM to 20 mM resulted in a four-fold increment in activity; b) ATP at concentrations from 12 to 15 mM in incubation mixture yielded maximal activity; higher concentrations were inhibitory (Figures 2 and 3); c) final pH of 6.1 in reaction mixture (Figure 3); d) Mg^{++} and Mn^{++} each at concentration of 2 mM; and 3) an MVA concentration between 18 to 20 µM, which was saturating.

Effect of Light on Kaurene Synthetase Activity in Shoot Tips During De-etiolation of Whole Seedlings

Irradiation of 10-day old etiolated seedlings with high-intensity white light induced an approximately exponential increase in kaurene synthetase activity from a very low activity at 0 hr to a maximum activity at 12 hr. Thereafter, kaurene synthesizing activity remained constant

Figure 2. Effect of ATP concentration on kaurene synthesizing activity of cell-free extracts from etiolated 10-day-old Alaska pea shoot tips irradiated during 4, 8 and 12 hr with high-intensity white light. Reaction mixtures each contained 0.70 ml enzyme extract (pH 7.1), 75 mM KH_2PO_4-K_2HPO_4, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 15 mM dithiothreitol, 100 µM chloramphenicol, 19.2 µM MVA-2-^{14}C (13 m Ci/mmole), ATP concentration ranging from 3 mM to 30 mM and pH values varying between 6.6 to 5.5 in a total volume of 1.0 ml. The data represent the results of a single experiment, which were confirmed in two additional experiments.

Δ-Δ 4 hr of irradiation

□ □ 8 hr irradiation

o-o 12 hr irradiation

Figure 2

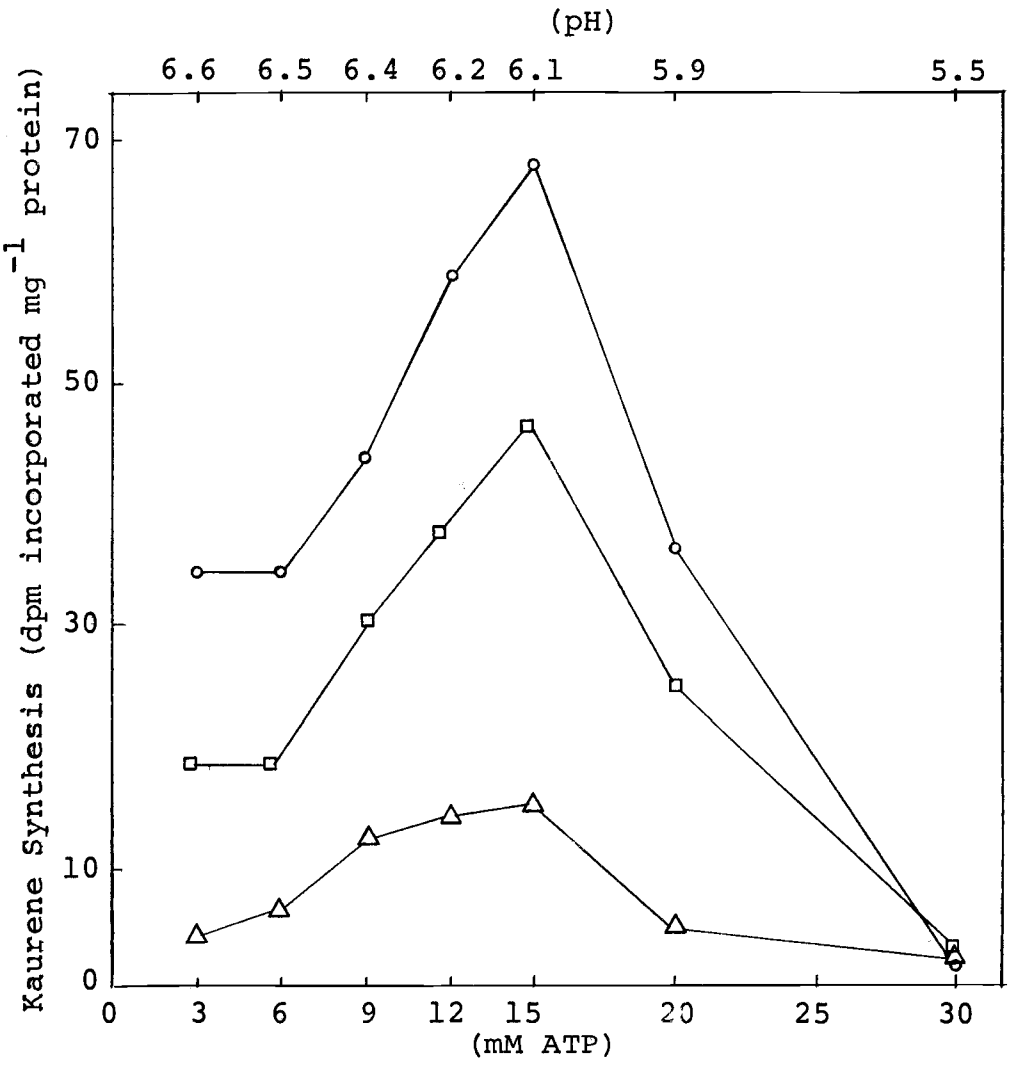

(pH)
6.6
6.5
6.4
6.2
6.1
5.9
5.5
Kaurene Synthesis (dpm incorporated mg-1 protein)
70
50
30
10
0
0
3
6
9
12
15
20
25
30
(mM ATP)

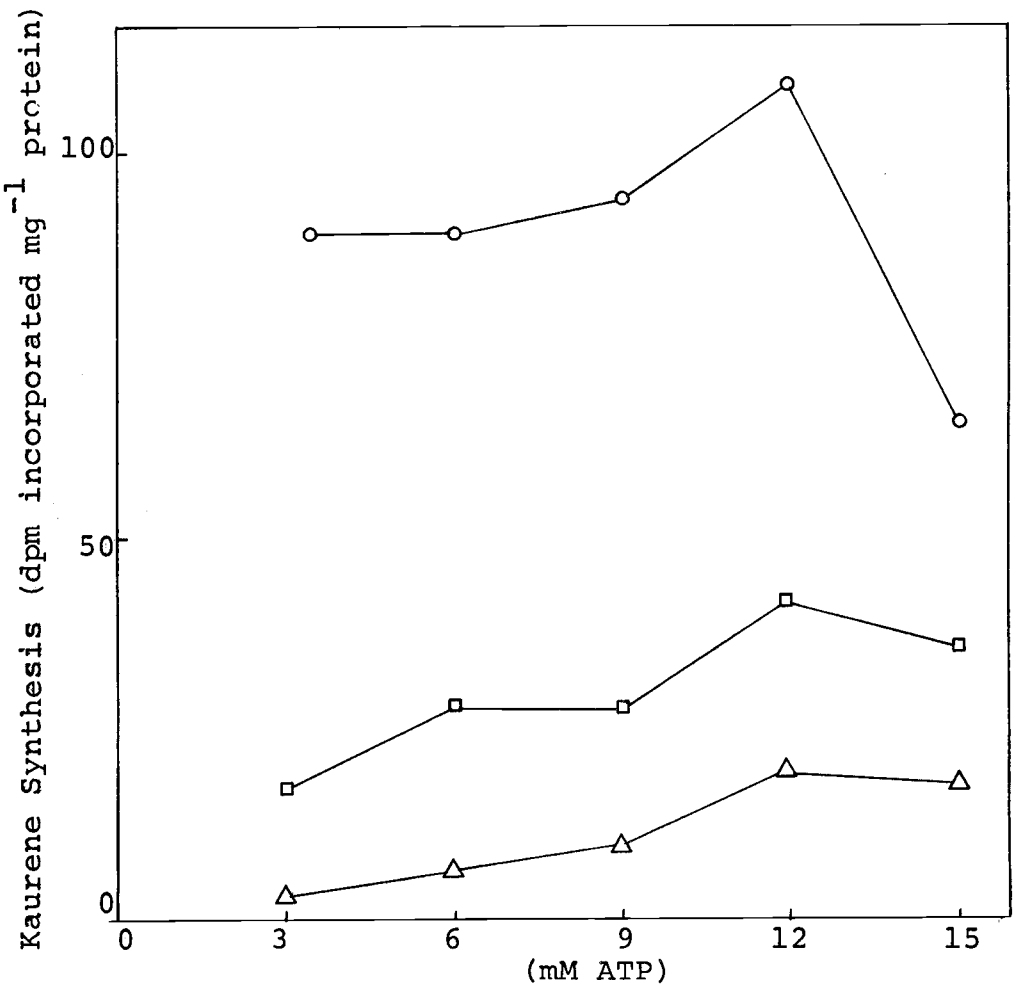
Figure 3. Effect of ATP concentration at pH 6.1 on kaurene synthesizing activity of cell-free extracts from etiolated 10-day old Alaska pea shoot tips irradiated during 4, 8 and 12 hr with high intensity white light. Reaction mixtures each contained 0.70 ml enzyme extract (pH 7.1), 75 mM KH_2PO_4-K_2HPO_4, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 15 mM dithiothreitol, 100 µM chloramphenicol, 19.2 µM MVA-2-^{14}C (13 mCi/mmole), ATP concentration ranging from 3 mM to 15 mM, final pH 6.1 adjusted with 0.1 N H_3PO_4 in a total volume of 1.0 ml. The data represent the results from a single experiment, which were confirmed in one additional experiment.

Δ-Δ 4 hr irradiation

□-□ 8 hr irradiation

o-o 12 hr irradiation

Figure 3


Kaurene Synthesis (dpm incorporated mg-1 protein)
100
50
0
0
3
6
9
12
15
(mM ATP)

through 16 hr of irradiation. Chlorophyll content increased at a fairly constant rate from 0 hr to 12 hr (Figure 4).

Effect of Light on Detached Etiolated Shoot Tips

In enzyme extracts prepared from detached shoot tips of irradiated 10-day-old etiolated seedlings kaurene synthetase activity consistently increased between 0 hr and the 8th hr of irradiation, and then decreased from the 8th to the 12th hr, again attaining a level comparable to that observed after 4 hr of irradiation. In contrast, chlorophyll content continued to increase with 12 hr of irradiation (Figure 5). The results obtained both with whole etiolated pea seedlings and with excised etiolated shoot tips, showed concurrent increases in the capacities for kaurene synthesis and chloroplast development during the first several hours of de-etiolation, confirming the results obtained by Moore and Ecklund (1974) and Ecklund and Moore (1974) on etiolated seedlings. By comparison with results reported here and elsewhere (Moore and Ecklund 1974; Ecklund and Moore, 1974) 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 and responded to photoinduction by a lower magnitude of change.

Figure 4. Change in chlorophyll content and in kaurene-synthesizing capacity of cell-free extracts from etiolated 10-day-old Alaska pea shoot tips during 12-hr irradiation of pea seedlings. Reaction mixtures each contained 0.70 ml enzyme extract, 75 mM KH_2PO_4-K_2HPO_4 (pH 6.1), 2 mM $MnCl_2$, 2 mM $MnCl_2$, 12 mM ATP, 15 mM dithiothreitol, 100 µM chloramphenicol, and 19.2 m MVA-2-^{14}C (13 mCi/mmole) in a total volume of 1.0 ml. The data represent the results of a single experiment, which was confirmed in two additional experiments. Chlorophyll content was determined according to the procedure of Arnon (1949).

-●--●- chlorophyll content

-o—o- kaurene synthesis

Figure 4

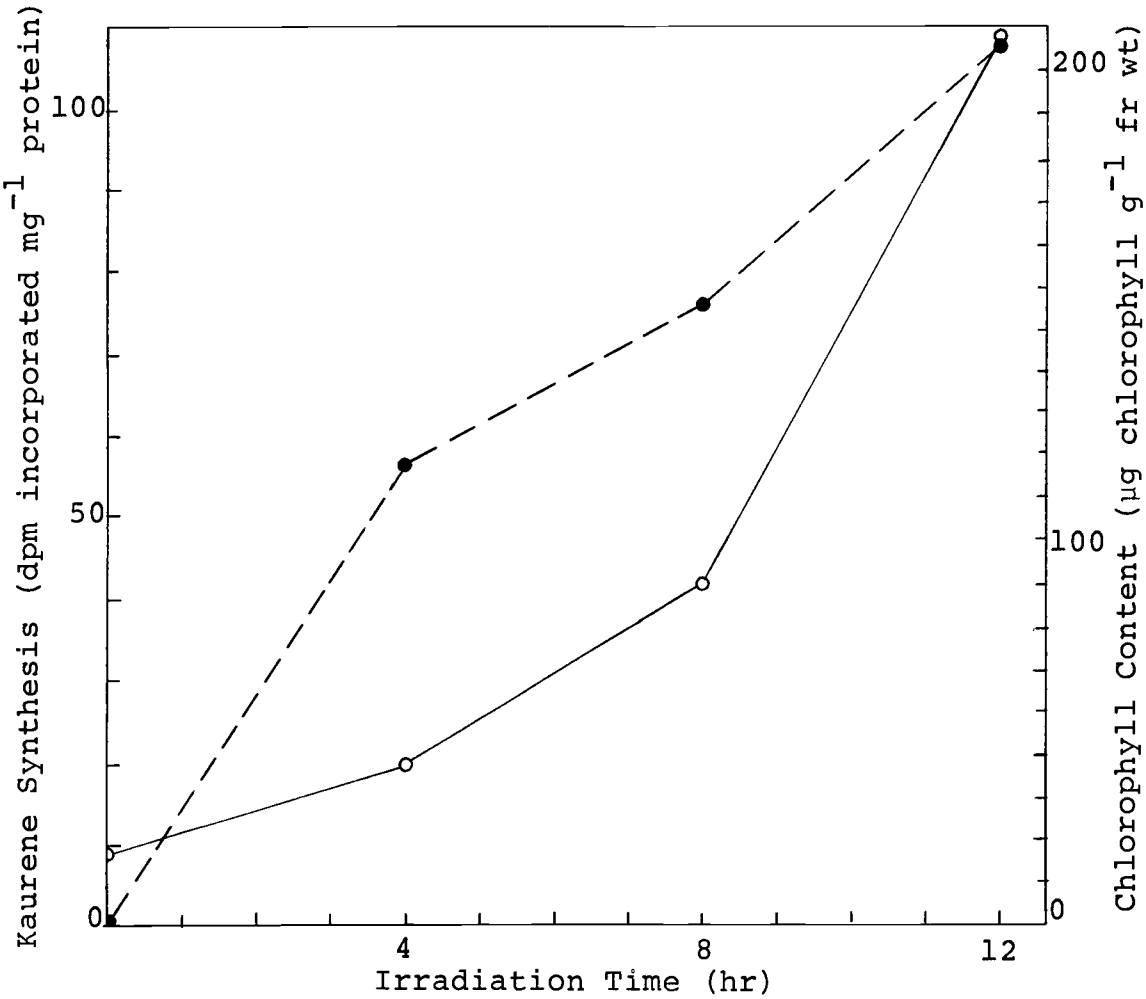
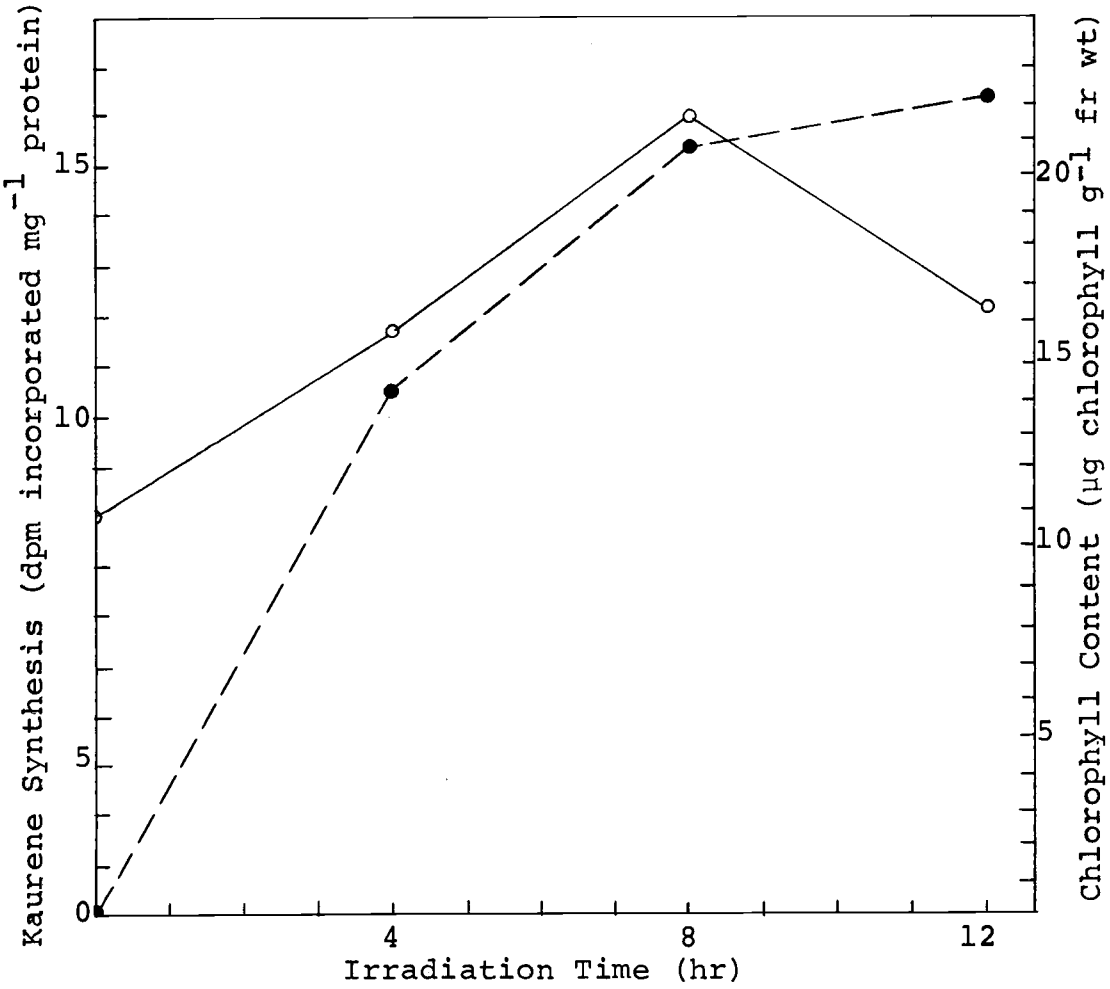

Kaurene Synthesis (dpm incorporated mg^{-1} protein)
100
50
0
4
8
12
Irradiation Time (hr)
200
100
0
Chlorophyll Content (µg chlorophyll g^{-1} fr wt)

Figure 5. Increase in kaurene-synthesizing capacity and chlorophyll content of cell-free extracts from 10-day-old detached etiolated pea shoot tips during 12-hr irradiation with high-intensity white light. Composition of reaction mixtures and chlorophyll determination were as described for Figure 4. The data represent means based on six independent experiments.

o—o— kaurene synthesis

-●--●- chlorophyll content

Figure 5


Kaurene Synthesis (dpm incorporated mg^{-1} protein)
Chlorophyll Content (µg chlorophyll g^{-1} fr wt)
Irradiation Time (hr)
0
5
10
15
20
4
8
12

Effects of Chloramphenicol on Kaurene Synthesis and Chlorophyll Formation

Chloramphenicol reduced development of kaurene synthetase activity at all concentrations tested during all periods of irradiation (Table 1). The percentage of reduction, compared with the water control, was consistently greater at 8 hr (Table 1). The range of reduction was 31 to 81% in samples treated with 0.1 mg/ml to 33 mg/ml of chloramphenicol, respectively, at 4 hr illumination, from 34 to 84% at 8 hr, and from 43 to 84% at 12 hr with the same lowest and highest concentrations, respectively, of chloramphenicol. Chloramphenicol also inhibited chlorophyll formation but to a lesser degree than kaurene synthesis (Figures 6 and 7) at all concentrations assayed. At the same concentrations of 0.1 and 33 mg/ml of antibiotic, the degree of inhibition varied from 23 to 53% after 4 hr, 31 to 78% after 8 hr, and 42 to 68% after 12 hr of illumination (Figure 7).

Maximum reduction of enzyme activity also was consistently at 8 hr of illumination, which corresponded to the period of maximum activity for kaurene synthetase observed in the control samples (Figure 5). At this time during de-etiolation, the enzyme seemed to be more sensitive than chlorophyll formation to chloramphenicol at all concentrations assayed (Figure 6). However, the maximum degree of inhibition for chlorophyll formation was also

Table 1. Reduction of kaurene-synthesizing activity by chloramphenicol, cycloheximide and lincomycin on detached etiolated pea shoot types during irradiation. The data are expressed as percentage of inhibition of the control values (expressed as dpm incorporated in kaurene mg^{-1} protein), and are the averages of three experiments in each of which assays were run in triplicate.

Irradiation time (hr)	Control	Inhibitor concentration*				
		0.10	1.0	10	33	100
		Chloramphenicol				
4	10	31	52	68	81	nd**
8	18	34	60	80	84	nd
12	13	43	69	83	84	nd
		Cycloheximide				
4	11	27	31	57	77	nd
8	14	30	58	68	82	nd
12	10	60	61	75	78	nd
		Lincomycin				
4	15	0	36	15	nd	8
8	19	0	22	15	nd	17
12	14	10	0	31	nd	16

* in mg/ml for chloramphenicol and cycloheximide; in µg/ml for lincomycin

**nd = no determination

Figure 6. Effects of chloramphenicol on kaurene-synthesizing activity and chlorophyll content in detached etiolated pea shoot tips during 8 hr irradiation. Composition of reaction mixtures and chlorophyll determination were as described for Figure 4. The data represent means based on three independent experiments.

-o--o- kaurene synthesis

-●--●- chlorophyll content

Figure 6

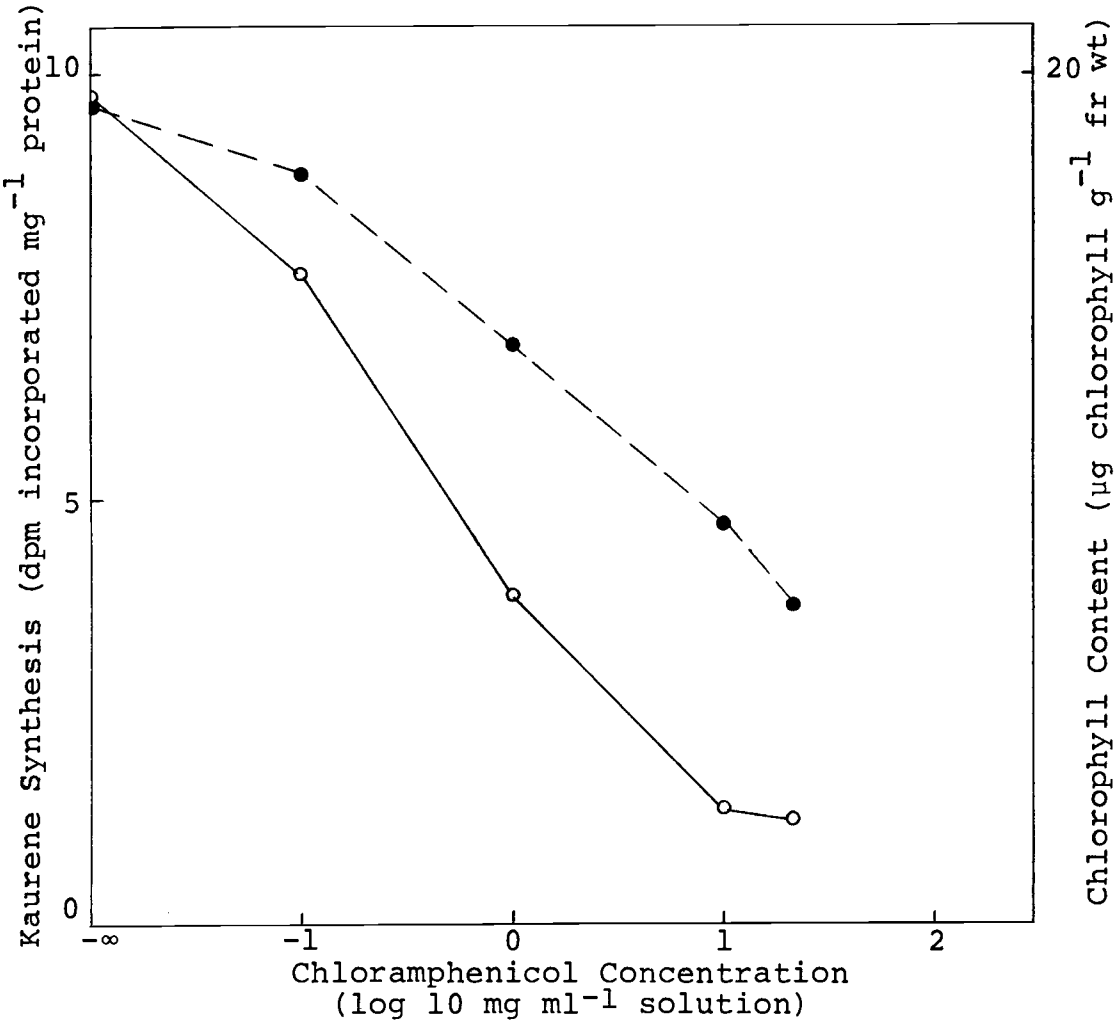
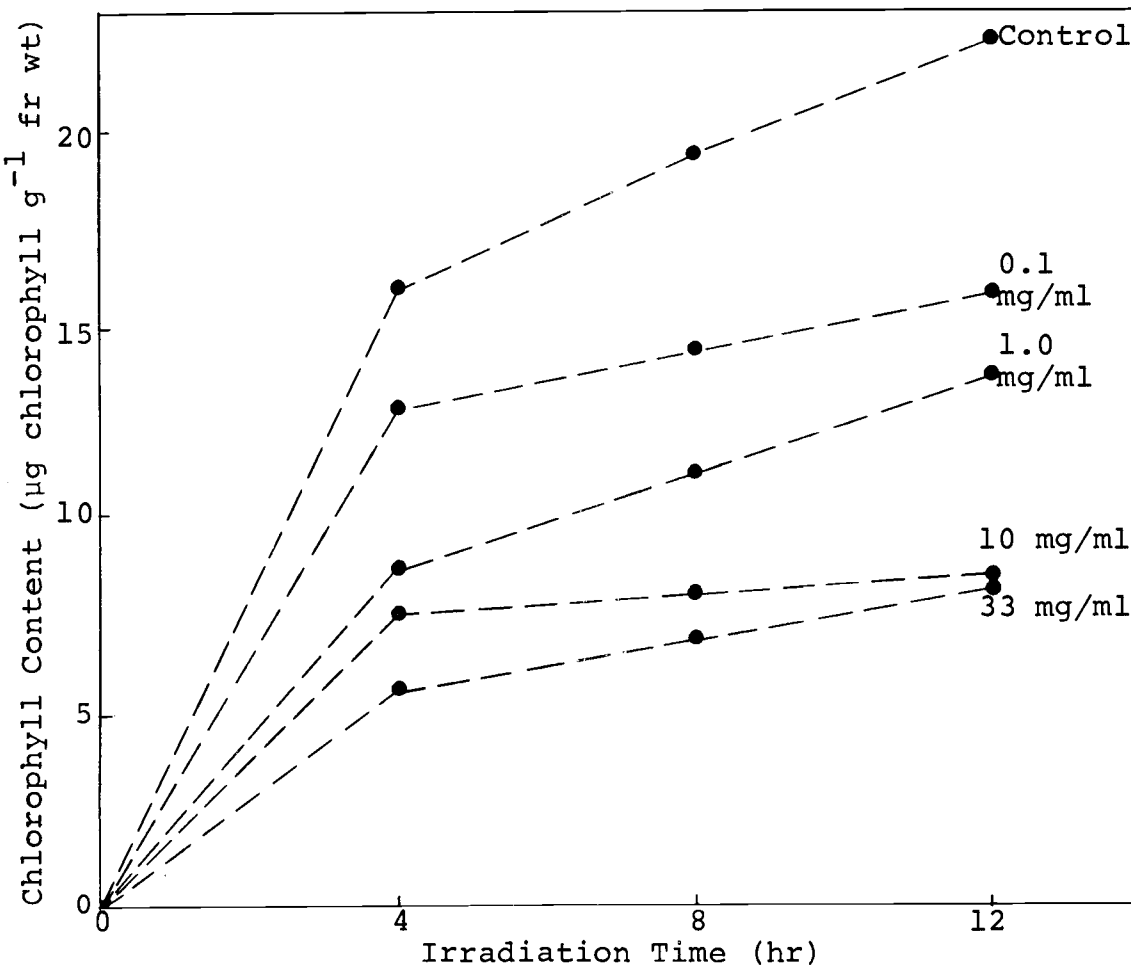

Kaurene Synthesis (dpm incorporated mg^{-1} protein)
Chlorophyll Content (µg chlorophyll g^{-1} fr wt)
10
5
0
20
-∞
-1
0
1
2
Chloramphenicol Concentration
(log 10 mg ml^{-1} solution)

Figure 7. Inhibition of chlorophyll formation by chloramphenicol in detached etiolated pea shoot tips during 4, 8 and 12 hr irradiation with high-intensity white light. The data represent the results of a single experiment which was confirmed in two independent experiments. Chlorophyll determination was as described for Figure 4.

Figure 7


Chlorophyll Content (µg chlorophyll g^{-1} fr wt)
0
5
10
15
20
0
4
8
12
Irradiation Time (hr)
Control
0.1 mg/ml
1.0 mg/ml
10 mg/ml
33 mg/ml

observed at this time, although in control samples the actual maximum chlorophyll content was at 12 hrs (Figures 5 and 7).

Effects of Cycloheximide on Kaurene Synthesis and Chlorophyll Formation

Cycloheximide also reduced kaurene synthesis during all periods of illumination (Table 1) at all concentrations tested (Figure 8). The percentage of reduction always was greatest at 8 hr, ranging from 27 to 77% in samples treated with 0.1 mg/ml to 33 mg/ml cycloheximide, respectively, at 4 hr illumination; 30 to 82% at 8 hr and 60 to 78% at 12 hr in samples treated with the same concentrations of cycloheximide. Cycloheximide also inhibited chlorophyll formation from 28 to 60% at 4 hr, 32 to 79% at 8 hr, and 31 to 72% at 12 hrs illumination in solutions containing 0.1 mg/ml to 33 mg/ml cycloheximide (Figure 9).

It was observed that cycloheximide also affected chlorophyll content at all concentrations assayed, but the highest inhibition was observed after 8 hr of illumination (Figure 9), resembling the effect of the inhibitor on enzyme activity. The degree of inhibition of greening was very similar at 8 and 12 hr irradiation.

In general, the results with cycloheximide revealed a close relationship between reduction in enzyme activity and chlorophyll inhibition at 4, 8, and 12 hr irradiation and at all concentrations used (Figure 8).

Figure 8. Effects of cycloheximide on kaurene synthesizing activity and chlorophyll content of cell-free extracts from detached etiolated pea shoot tips during 8 hr irradiation with high-intensity white light. Reaction mixtures and chlorophyll determination were as described for Figure 4. The data represent means based on three independent experiments.

o-o-o kaurene synthesis

-●--● chlorophyll content

Figure 8

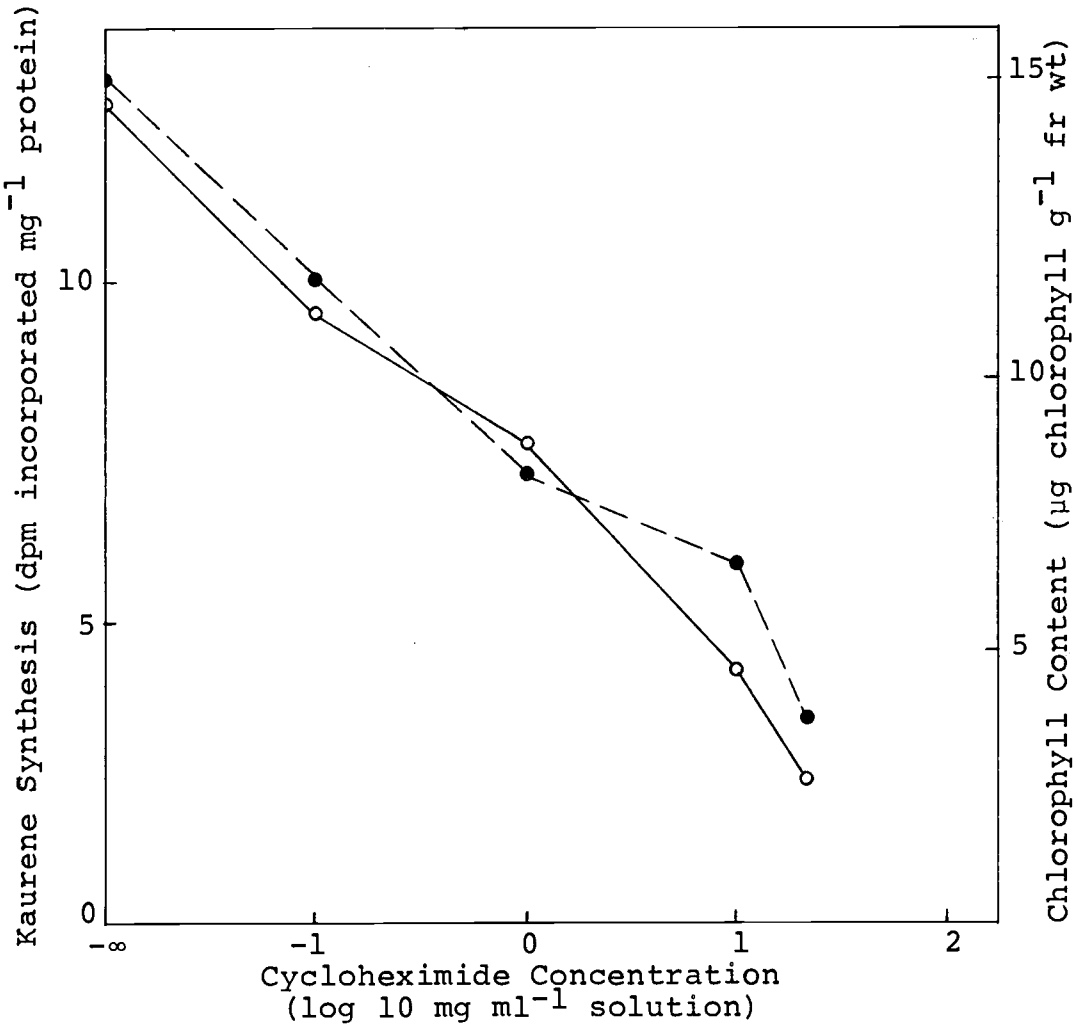
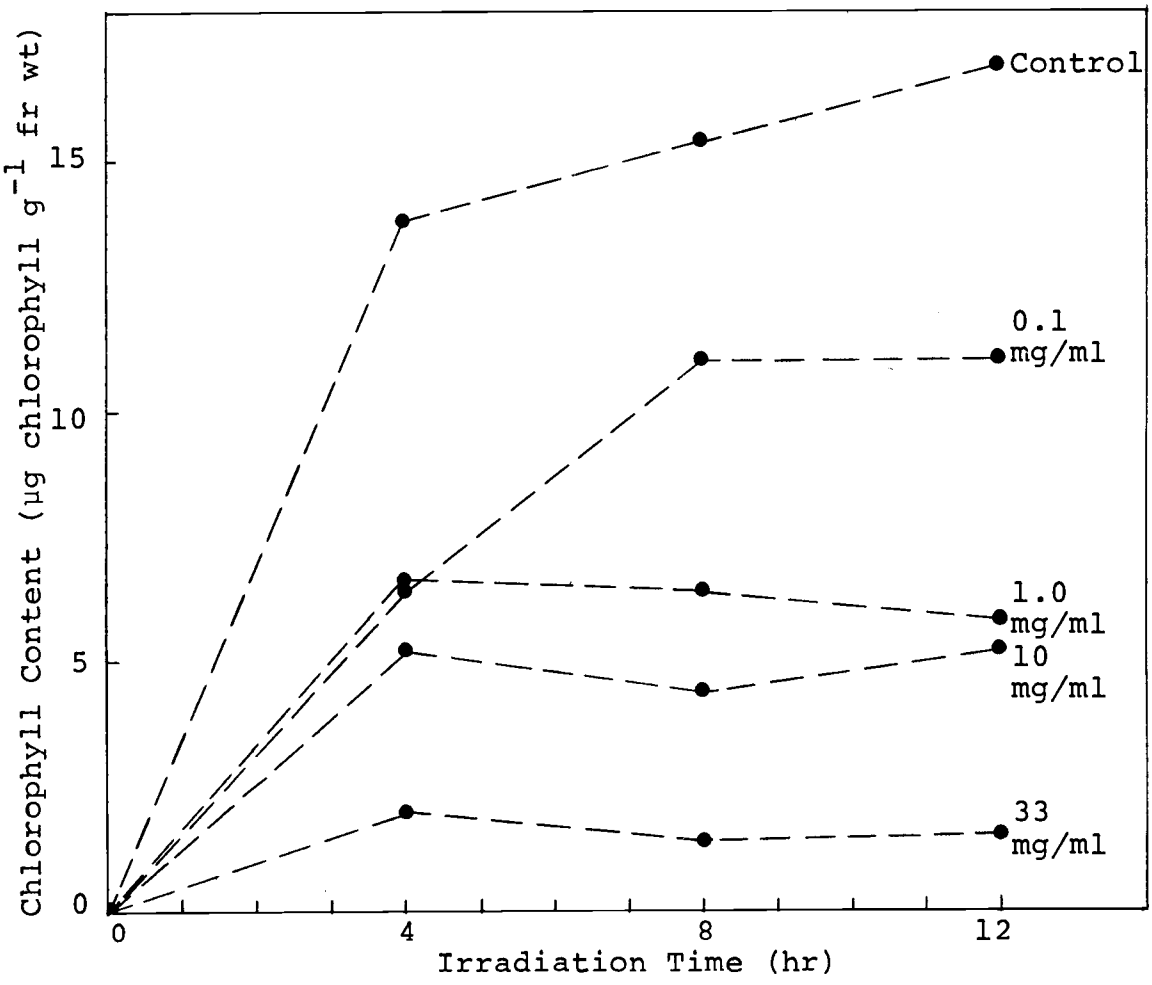

Kaurene Synthesis (dpm incorporated mg^{-1} protein)
Chlorophyll Content (µg chlorophyll g^{-1} fr wt)
0
5
10
15
-∞
-1
0
1
2
Cycloheximide Concentration
(log 10 mg ml^{-1} solution)

Figure 9. Effects of cycloheximide on chlorophyll formation in detached etiolated pea shoot tips during 4, 8 and 12 hr irradiation with high-intensity white light. Chlorophyll determination was as described for Figure 4. The data represent the results of a single experiment which were confirmed on two independent experiments.

Figure 9


Chlorophyll Content (µg chlorophyll g^{-1} fr wt)
0
5
10
15
0
4
8
12
Irradiation Time (hr)
Control
0.1 mg/ml
1.0 mg/ml
10 mg/ml
33 mg/ml

Effects of Lincomycin on Kaurene Synthesis and Chlorophyll Formation

Lincomycin did not significantly reduce kaurene synthetase activity at any concentration tested (up to 100 µg/ml) under any irradiation conditions (Table 1). However, chlorophyll formation was inhibited under conditions of 4, 8, and 12 hr irradiation and at all concentrations used (Figures 10 and 11). Concentrations from 0.01 µg/ml to 100 µg/ml gave reductions which varied from 25 to 55% at 4 hr, 33 to 59% at 8 hr and 32 to 56% at 12 hr irradiation. After 8 hr of illumination chloramphenicol was slightly more effective than cycloheximide both in reducing kaurene synthetase activity and inhibiting chlorophyll formation in etiolated detached pea shoot tips (Figures 12 and 13). Lincomycin is approximately 15 times more effective than either of the other two antibiotics in causing 50% inhibition of greening (Figure 13).

It is important to note that the reduction observed for kaurene synthetase activity is not due to an inhibitory effect of the antibiotics on the activity of preformed enzyme *in vitro*. Concentrations of chloramphenicol (0.4 mg/ml) and cycloheximide (0.7 mg/ml) which caused 50% reduction in activity when applied to shoot tips as a pretreatment during 8 hr irradiation inhibited activity only by 5% or less when added directly to enzyme extract. No direct inhibition of enzyme activity was observed with lincomycin up to 10 µg/ml.

Figure 10. Effect of lincomycin on chlorophyll formation in detached etiolated pea shoot tips during 4, 8 and 12 hr irradiation with high-intensity white light. Chlorophyll determination was as described for Figure 4. The data represent the results of a single experiment which was confirmed in two independent experiments.

Figure 10

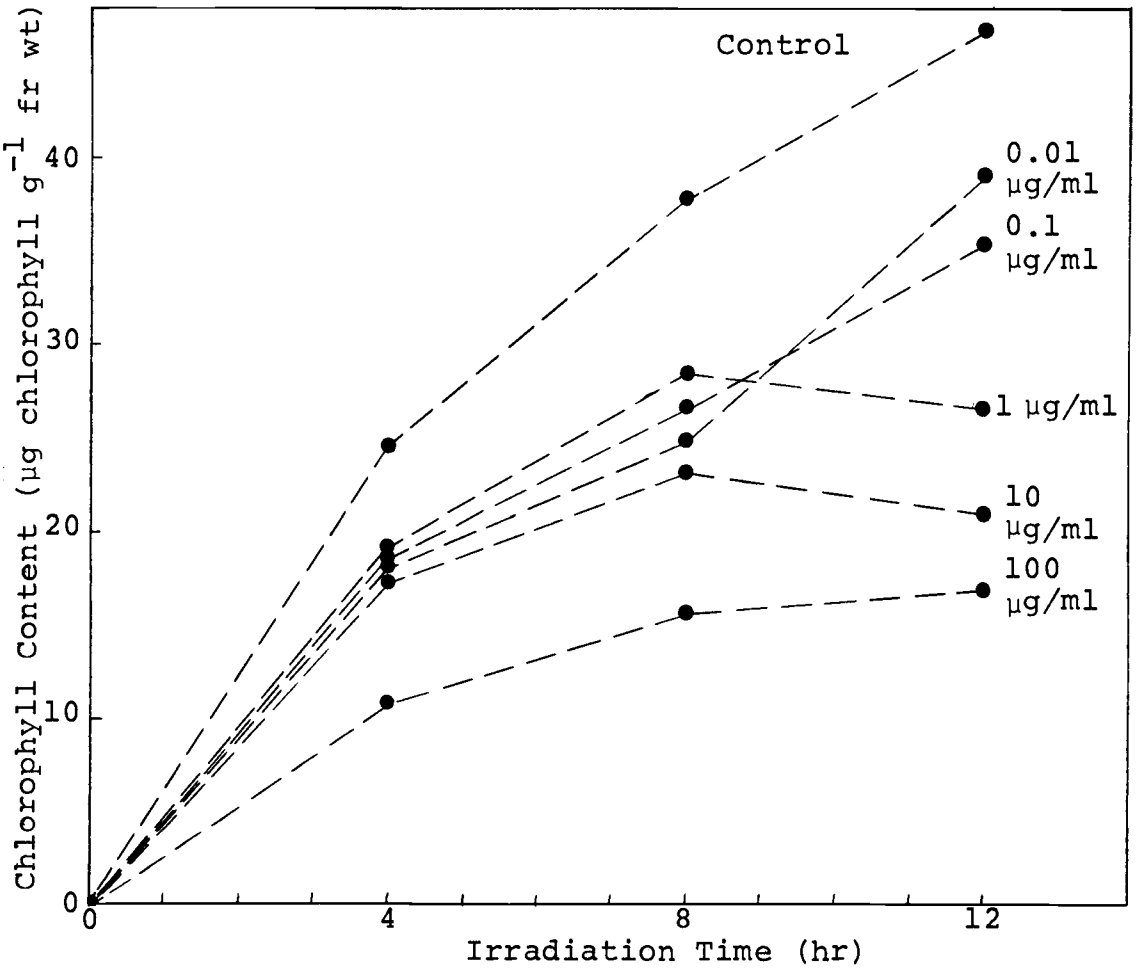

Control
0.01 µg/ml
0.1 µg/ml
1 µg/ml
10 µg/ml
100 µg/ml
Chlorophyll Content (µg chlorophyll g^{-1} fr wt)
0
10
20
30
40
0
4
8
12
Irradiation Time (hr)

Figure 11. Effects of lincomycin on kaurene-synthesizing activity and chlorophyll formation in detached etiolated pea shoot tips during 8 hr irradiation with high-intensity white light. Cell-free extracts were analyzed for kaurene-synthesizing capacity and chlorophyll content as it was described for Figure 4. The data represent means based on three independent experiments.

o-o- kaurene synthesis

●--● chlorophyll content

Figure 11

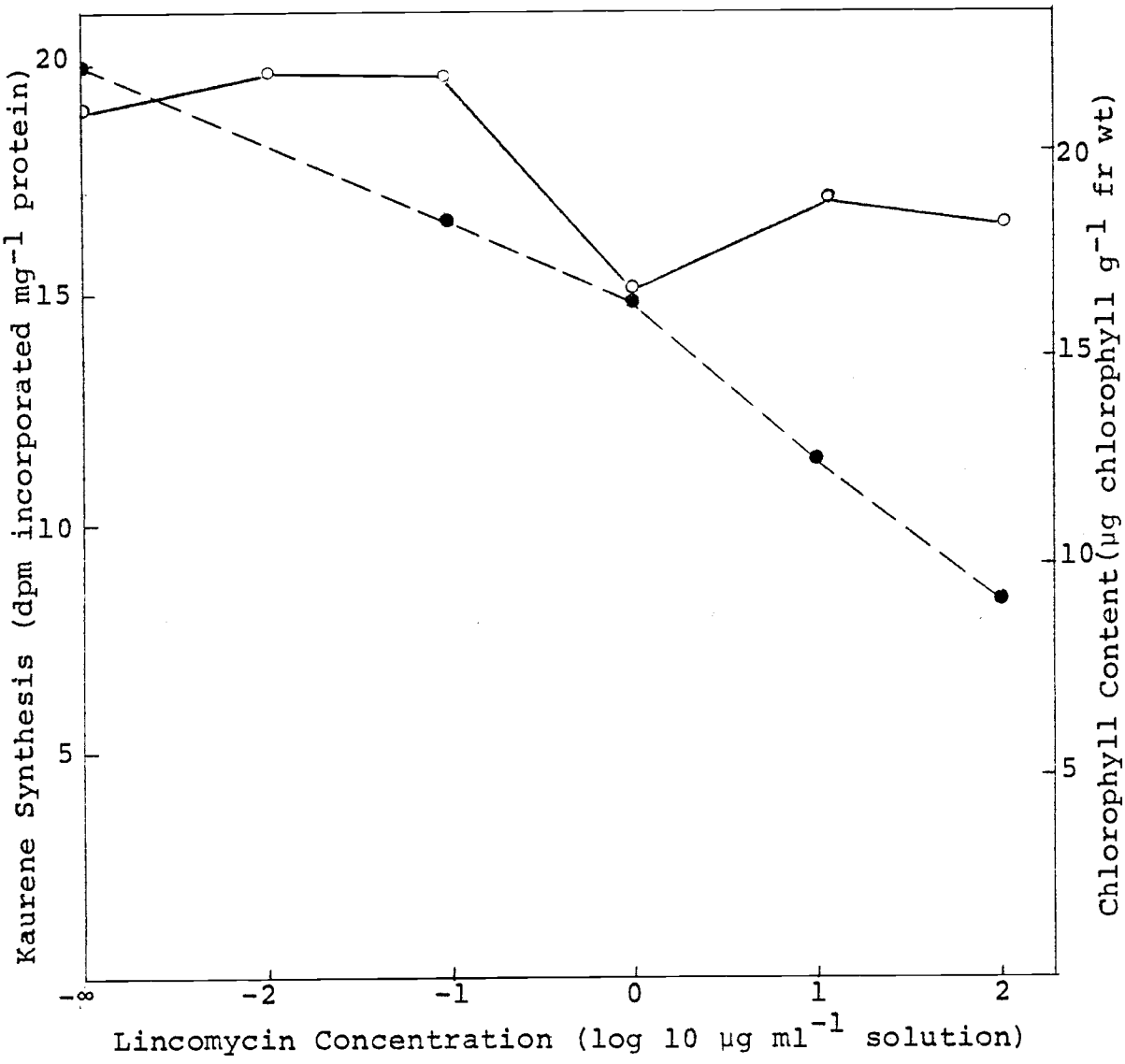

Kaurene Synthesis (dpm incorporated mg-1 protein)
Chlorophyll Content (µg chlorophyll g-1 fr wt)
20
15
10
5
-∞
-2
-1
0
1
2
Lincomycin Concentration (log 10 µg ml-1 solution)

Figure 12. Effects of chloramphenicol, cycloheximide and lincomycin on kaurene synthesizing activity in detached etiolated pea shoot tips during 8 hr irradiation with high intensity white light. Cell-free extracts were analyzed for kaurene synthesizing activity as it was described for Figure 4. Values are expressed as percentages of the control values. The data represent means based on three separated experiments for each inhibitor shown.

Δ-Δ chloramphenicol

□-□ cycloheximide

o-o lincomycin

Figure 12

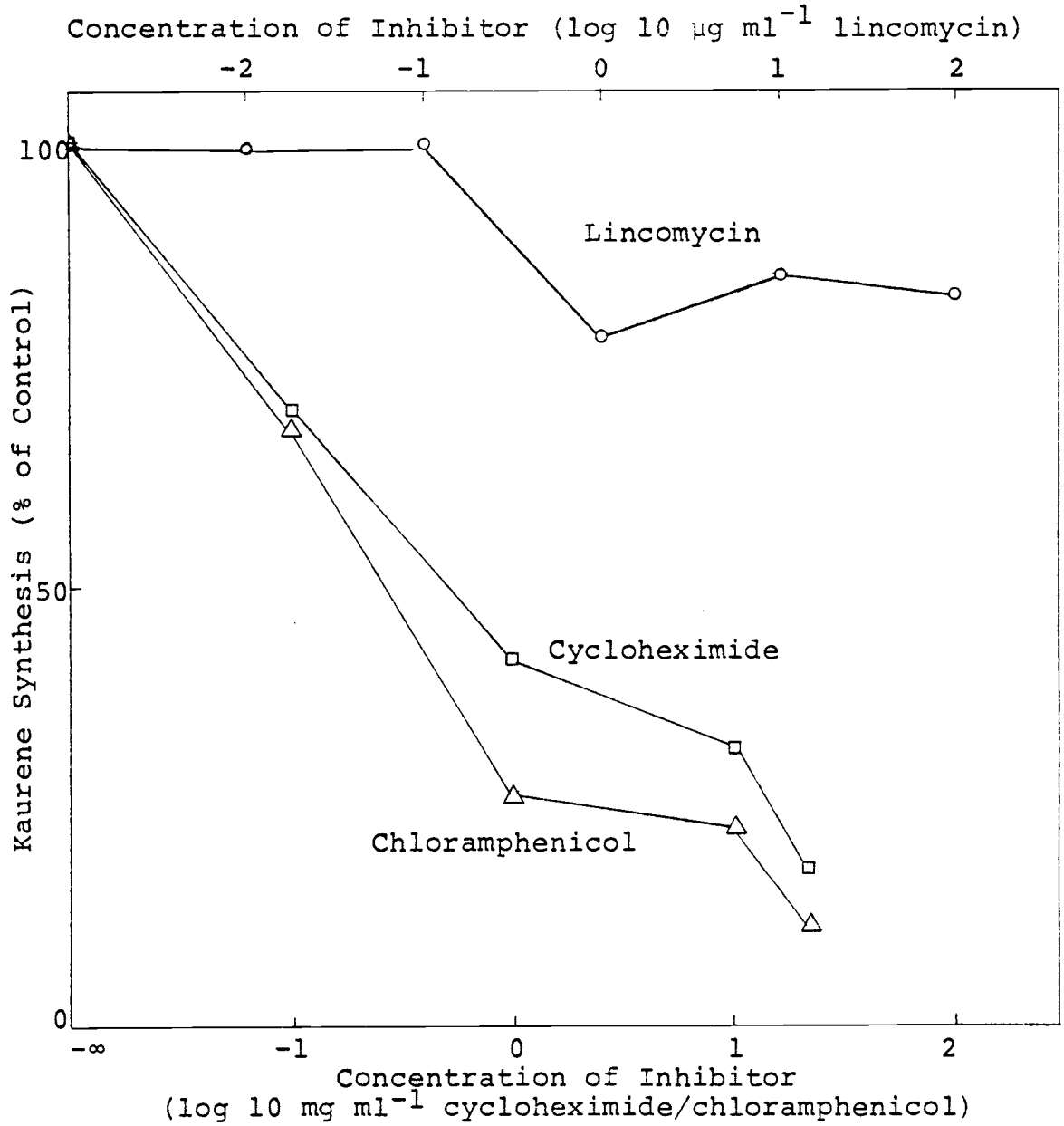

Concentration of Inhibitor (log 10 µg ml^{-1} lincomycin)
-2
-1
0
1
2
100
50
0
Kaurene Synthesis (% of Control)
Lincomycin
Cycloheximide
Chloramphenicol
-∞
-1
0
1
2
Concentration of Inhibitor
(log 10 mg ml^{-1} cycloheximide/chloramphenicol)

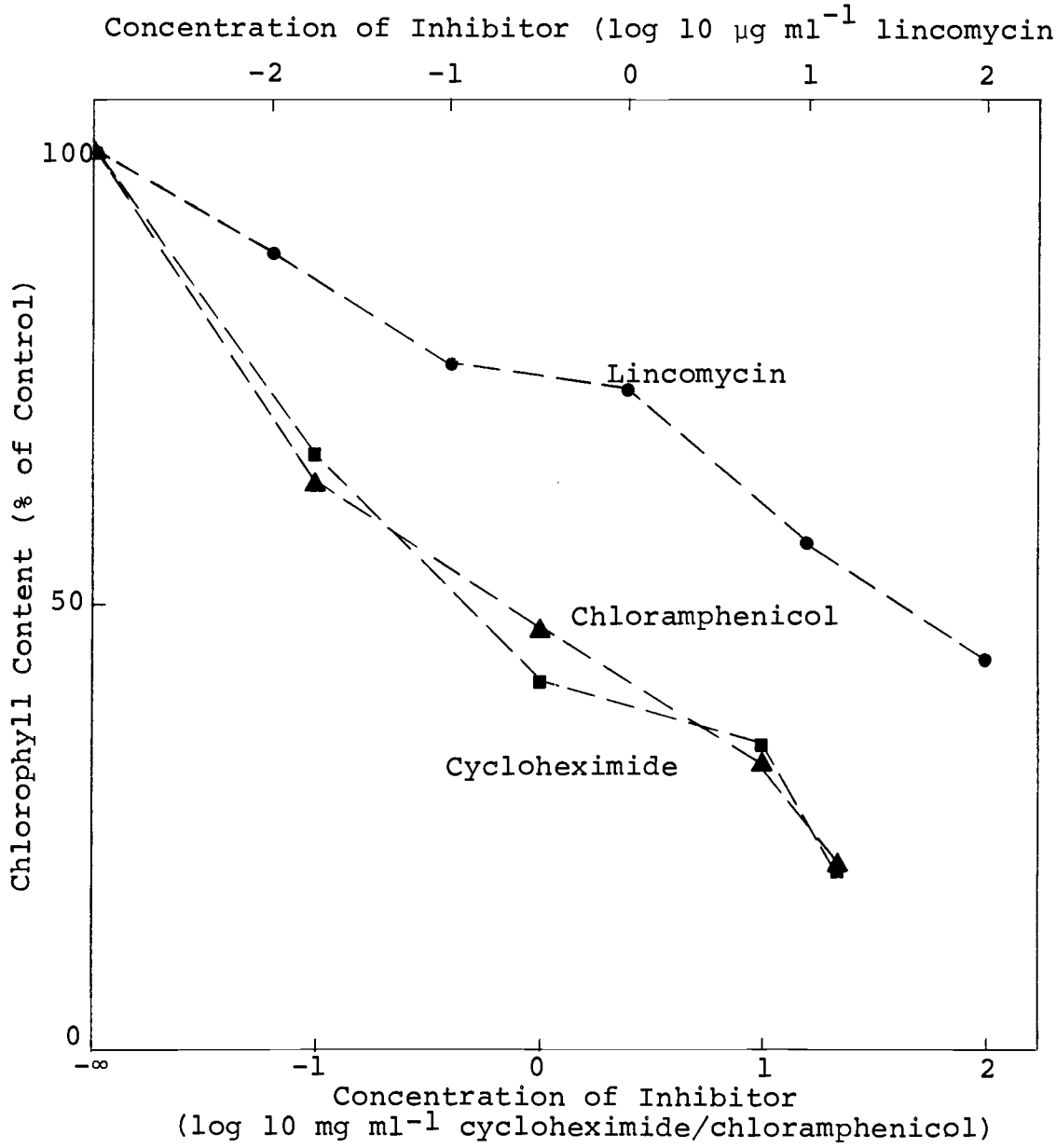
Figure 13. Effects of chloramphenicol, cycloheximide and lincomycin on chlorophyll formation in detached etiolated pea shoot tips during 8 hr irradiation with high-intensity white light. Values are expressed as percentages of the control values. The data represent means based on three separated experiments for each inhibitor shown. Chlorophyll determination was as described for Figure 4.

-▲--▲- chloramphenicol

-■--■- cycloheximide

-●--●- lincomycin

Figure 13


Concentration of Inhibitor (log 10 µg ml^{-1} lincomycin)
-2
-1
0
1
2
100
50
0
Chlorophyll Content (% of Control)
Lincomycin
Chloramphenicol
Cycloheximide
-∞
-1
0
1
2
Concentration of Inhibitor
(log 10 mg ml^{-1} cycloheximide/chloramphenicol)

The level of kaurene synthetase 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 gram of fresh weight of shoot tip tissues.

DISCUSSION

The tentative interpretation of the combined effects of chloramphenicol (D-threo isomer), cycloheximide and lincomycin on the light-induced increase in kaurene synthetase activity in pea shoot tips is that: (a) the light-induced increase in kaurene synthetase activity results from de novo synthesis of kaurene synthetase, its precursor, or some other protein essential for its activity; (b) kaurene synthetase is compartmentalized in chloroplasts, but that the enzyme or its precursor protein(s) is synthesized on cytoplasmic ribosomes. Detailed consideration of the effects of the inhibitors will now be undertaken to show how that interpretation was reached.

Chloramphenicol quite effectively reduced both kaurene synthetase activity and chlorophyll formation during irradiation of etiolated detached pea shoot tips (Figure 7, Table 1). In view of the many reports that chloramphenicol is a selective inhibitor of protein synthesis on chloroplast ribosomes (Anderson and Smillie, 1966; Boulter et al., 1972; Ellis, 1969, Ingle, 1968; Levine and Goodenough, 1970; Margulies and Brubaker, 1970; Margulies, 1971; Parthier et al., 1964), it might be tempting to suggest that kaurene synthetase is synthesized de novo on chloroplast ribosomes upon irradiation with white light. This would be consistent with some observations and

reports but not others, and on balance proves to be an erroneous interpretation. Indeed, of the chloroplastic proteins that have been investigated, it appears as if only certain membrane proteins required for the immobilization of chloroplast components are actually synthesized *in situ*; the proteins of photosystems I and II of photosynthesis are synthesized in the cytoplasm (Ellis, 1975). Moreover, in the present work, when attached etiolated shoot tips were sprayed with chloramphenicol (0.1 to 10 mg/ml) solutions containing Tween 20 at 0.01%, it was observed that the same solution (1.0 mg/ml) which produced 50% inhibition in chlorophyll formation upon irradiation did not affect enzyme activity.

The equivocal results with the D-threo isomer of chloramphenicol are not without precedent, for several workers have reported conflicting results regarding certain enzymes in both algae and higher plants (Ellis, 1970; Graham et al., 1970; Margulies, 1964; Smillie *et al.*, 1967). Ellis and Hartley (1971) suggested that the qualitatively different results might be due to a lack of *in vivo* specificity of this antibiotic. Earlier, Ellis (1969) had in fact suggested that the stereospecific test for chloramphenicol isomers could be used to determine its action at the ribosomal level in *in vivo* systems, although it should be noted that indeed the L-treo isomer also inhibited chloroplast protein synthesis *in vitro*, when it is driven

by light but not by ATP (Ellis and Hartley, 1971). Further complicating interpretations of chloramphenicol effects, Hanson and Hodges (1963) suggested that the inhibitory effect observed on ^{45}Ca-uptake by isolated mitochondria was the consequence of an uncoupling reaction of oxidative phosphorylation. Inhibition of protein synthesis therefore might be an indirect consequence of inhibition of ATP synthesis under some circumstances. More is said later about chloramphenicol.

Cycloheximide was highly effective also in reducing kaurene synthetase activity and chlorophyll formation, and the effects of this inhibitor on both processes were very similar in magnitude after 8 hr illumination (Figure 8). This antibiotic generally has been reported as an inhibitor of cytoplasmic protein synthesis (Boulter et al., 1970; Vasquez and Monro, 1967), which binds to 80S ribosomes. For a while there were some doubts about its specificity on cytoplasmic protein synthesis, because it had been reported to have other effects on green and non-green tissues, such as affecting chloride uptake and oxygen absorption (Cocucci and Marré, 1973; Ellis and MacDonald, 1970). However, more recent work by Lüttge et al. (1974) gave new support for its high specificity on protein synthesis on cytoplasmic ribosomes. And, finally, the issue seems to have been resolved by Wildes et al. (1976), who reported that the inhibitory effect of cycloheximide on ion uptake

by barley roots was due to the inhibitory effect on the synthesis of a specific protein involved in this process. Thus, the results reported here for cycloheximide tentatively suggest that kaurene synthetase may be cytoplasmic in origin.

If now the combined results obtained with chloramphenicol and cycloheximide are analyzed carefully, the intriguing possibility arises that kaurene synthetase, while ultimately compartmentalized in chloroplast, actually is synthesized in the cytoplasm. Interference with the increases in enzyme 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 (Levine and Goodenough, 1970; Ohad et al., 1967a, b), and in this indirect manner, inhibiting enzyme activity. Fortunately, this idea could be tested by using another inhibitor called lincomycin (Hanka et al.,1962; Herr and Bergy,1962; Hoeksemena et al.,1964; Lewis et al.,1962; Mason et al.,1962), which reportedly is a more specific inhibitor than chloramphenicol on chloroplast ribosome activity (Celma et al., 1970; Hoober et al., 1969; Igarashi et al., 1969; Vasquez and Monro, 1967). Ellis and Hartley (1971) reported that it was a very effective inhibitor of protein synthesis on chloroplast ribosomes in detached shoot tips and in isolated chloroplasts from Pisum sativum. Moreover, Thomson and Ellis (1972) showed that lincomycin was a

specific inhibitor of chloroplast ribosomal activity in greening leaves of Pisum sativum, and that it specifically prevented both the formation of chloroplast membranes and their stacking into grama.

When lincomycin was tested on etiolated detached shoot tips in the present investigations, it did have an inhibitory effect on chlorophyll formation, as has been previously reported (Ellis and Hartley, 1971). But kaurene synthetase activity was not significantly inhibited under any of the conditions tested, even at concentrations which inhibited 50% of chlorophyll formation. These results not only ruled out the possibility of the enzyme being synthesized in the chloroplast, but strengthened the possibility that active or functional kaurene synthetase nevertheless ultimately is localized in the chloroplast, as was first suggested by the results with chloramphenicol. An explanation which is accorded in a paper by Ellis (1975) who suggested that one specific role of chloroplast ribosomes is to synthesize membrane proteins required for the immobilization of some chloroplast components, such as photosystem I proteins, synthesis of which is on cytoplasmic ribosomes, but which also are inhibited by lincomycin. Contrariwise, in Ellis' (1975) work, lincomycin hardly affected photosystem II 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, kaurene synthetase appears to be analogous to the chloroplastic proteins of photosystem II of photosynthesis.

In further analogy with photosystem II proteins, it is hypothesized that kaurene synthetase, in its functional state, is intimately associated with a membrane component of chloroplasts. Apparently, the enzyme, or its precursor perhaps, is synthesized de novo in the cytoplasm and is incorporated in, or somehow becomes bound to, the growing membrane system of the chloroplast during illumination. Implicit is the notion that the membrane-associated condition is essential for catalysis of the cyclization of geranylgeranyl pyrophosphate to kaurene. Just as it is known that chloramphenicol inhibits normal membrane organization (Hoober et al., 1969) and synthesis of membrane binding proteins of chloroplasts (Ellis, 1975), so also would kaurene synthetase association with membranes in developing chloroplasts be theoretically inhibited by that antibiotic. The effects of the other antibiotics as well fit logically into the interpretation given for the increase in kaurene synthetase activity during de-etiolation (Ecklund and Moore, 1974; Moore and Ecklund, 1974).

Kaurene synthetase generally has been described as a soluble enzyme (West, 1972), although its extraction has been performed generally under conditions which would release it from organelles. Different reports recently have

shown that it is associated with proplastids (Simcox et al., 1975), and chloroplasts (Moore and Coolbaugh, 1976).

Since total autonomy of chloroplasts has not been shown (Armstrong et al., 1971), Ellis and Hartley (1971) suggested that there must be a specific mechanism for transporting chloroplast proteins made on cytoplasmic ribosomes across the outer membrane of plastids. Recent work suggests that the hypothetical transmembrane transport mechanism may involve phytochrome. Many workers have reported the direct relation between illumination and the level of extractable gibberellin-like activity from leaves of different species (Cooke and Saunders, 1975; Loveys and Wareing, 1971; Railton and Reid, 1974b; Reid and Clements, 1968; Reid et al., 1972). This effect has been demonstrated to be phytochrome dependent (Cooke and Saunders, 1975; Wellburn and Wellburn, 1973). Evans and Smith (1976a, b) demonstrated the presence of phytochrome within the etioplast and its relationship to the increase in extractable gibberellin-like activity upon illumination. They concluded that phytochrome would cause the transport of gibberellin across the etioplast envelope. The transport of different compounds such as gibberellic and abscisic acids and adenosine 3', 5'-cyclic phosphate, across plastid envelopes, has been reported by Wellburn and Hampp (1976) and the regulation of this process by

phytochrome has been confirmed by Hampp and Schmidt (1977) and Schmidt and Hampp (1977).

Theoretically, these findings could explain also the transport of newly synthesized kaurene synthetase from the cytoplasm to chloroplasts, and its increased activity during irradiation of etiolated shoot tips. The reported release of bound GA-like substances upon illumination could lead to an increase in gibberellin biosynthesis, possibly by release of a feedback control of late steps or its biosynthetic pathway, as has been suggested by Evans and Smith (1976b), and hence accord obvious value to transporting kaurene synthetase to the chloroplasts.

By way of summary, it is tentatively concluded that upon continuous irradiation of etiolated pea shoot tips synthesis of kaurene synthetase or its precursor on cytoplasmic ribosomes is induced. Newly synthesized enzyme is transported to the chloroplast, its passage across the plastid envelope being facilitated by the increased permeability of plastid membranes upon illumination due to a phytochrome effect. Once inside the plastid the enzyme is bound to the developing membrane system, thereby becoming functional in kaurene synthesis and also, of course, biosynthesis of gibberellin-type hormones. Not excluded is the possibility that an inactive precursor of kaurene synthetase or a protein essential to its activity, rather than kaurene synthetase <u>per se</u> is the entity the synthesis of

which is susceptible to inhibition by chloramphenicol and cycloheximide. In advancing this tentative interpretation as a working hypothesis, it is acknowledged that other proteins than kaurene synthetase may be found in further research to be involved in the observations here reported.

Finally, it should be stated that this work, like research generally, has both contributed new knowledge and raised further questions. Of most immediate interest would seem to be to: (1) corroborate evidence that kaurene synthetase is induced to be synthesized de novo during deetiolation by a direct method, such as the density labelling technique of Filner and Varner (1967); (2) determine the action spectrum for the light effect on kaurene synthetase, in the hope of elucidating components coinciding predictably both with photosystem II of photosynthesis and, if involved, also the phytochrome system; and (3) elucidate as completely as possible the processes which collectively regulate kaurene synthetase in vivo. The system utilized in these investigations obviously can be utilized further to contribute to our knowledge on these topics and about the larger topic of regulation of biosynthesis of the gibberellin-type hormones in higher plants.

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