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Induction of Phenylalanine Ammonia-lyase in Xanthium Leaf Disks. Photosynthetic Requirement and Effect of Daylength[1]

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Abstract. A cycloheximide-sensitive increase in the activity of phenylalanine ammonia-lyase (EC 4.3.1.5) occurs in *Xanthium* leaf disks exposed to light. Radioactive ammonia-lyase has been isolated by means of sucrose density gradient centrifugation and starch gel electrophoresis from disks fed L-isoleucine-U-^{14}C or L-arginine-U-^{14}C. The incorporation of radioactive amino acids into phenylalanine ammonia-lyase together with the inhibitory effects of cycloheximide indicate that the observed increase in enzyme activity involves the induction of lyase synthesis.

The light-dependent synthesis of the ammonia-lyase is completely inhibited by 50 μM 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) indicating that photosynthesis is involved. Only a trace quantity of some photosynthetic product must be needed because half light saturation occurs at very low intensity (*ca.* 30 ft-c). Exogenous carbohydrate is also required for continuing enzyme synthesis over a 72 hr period. But carbohydrate does not replace the photosynthetic requirement in darkness.

Enzyme formed in light disappears rapidly from disks placed in the dark. The decay of ammonia-lyase activity follows first order kinetics. The half-life of the lyase ranged from 6 to 15 hr in leaf material used. Cycloheximide inhibits the decay of lyase activity. Thus the maintenance of turnover in *Xanthium* leaf disks requires *de novo* synthesis of protein. That turnover, *i.e.*, degradation as well as synthesis of lyase protein occurs is suggested by the apparent loss of radioactive ammonia-lyase from leaf disks placed in darkness. Light-induced synthesis coupled with rapid turnover can produce a diurnal fluctuation of ammonia-lyase activity in *Xanthium* leaf disks. Alternating periods of enzyme synthesis and degradation were observed in disks exposed to 24 hr cycles of light and dark. The average level of enzyme activity maintained in the tissue was directly related to the length of the light period. Induction of lyase synthesis was also observed in excised leaves and to a lesser extent in leaves of whole plants.

Phenylalanine ammonia-lyase catalyzes the deamination of L-phenylalanine to trans-cinnamic acid. In turn, the carbon skeleton of cinnamic acid serves as a major substrate for many pathways of phenolic biosynthesis in plants.

Phenylalanine ammonia-lyase was first isolated and described by Koukol and Conn (18). Since that time, several factors known to stimulate the production of phenolic compounds in plants have been shown to induce the synthesis of the enzyme. Stimulating effects of light on phenolic biosynthesis in several tissues have now been ascribed to a light-induced synthesis of the ammonia-lyase (1, 7, 8, 22, 27, 36). Stimulation of lyase synthesis by exogenous carbohydrate (4) and temperature effects on lyase synthesis (11) have been cited as the mechanism whereby these factors influence phenolic production. Lignification is also correlated with appearance of ammonia-lyase activity (2, 16, 24). In these situations plant tissues appear to regulate the flow of carbon into phenolic pathways by controlling the synthesis of this enzyme.

A study of the induction of phenylalanine ammonia-lyase was begun in *Xanthium* to determine whether synthesis of the lyase is light-dependent in this photoperiodically sensitive plant and to determine whether induction of the enzyme is related to photoperiodic reactions. Some characteristics of the regulatory system controlling ammonia-lyase synthesis in *Xanthium* leaf tissue are presented below.

Materials and Methods

Plant Material. Plants of *Xanthium pennsylvanicum* (Wall) were grown from seed in a greenhouse. Most plants used were 30 to 60 days old. Effects recorded below were observed in leaf tissue from both vegetative and flowering plants.

Experimental Conditions. Experiments were carried out with leaf disks 1.6 cm in diameter which were cut from the lamina of the most recently matured, fully expanded leaves on a plant. Leaf disks from a number of leaves were pooled in each

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experiment. Randomly chosen sets of 15 disks moistened with 3 ml of fluid were cultured on filter paper in disposable petri dishes. Loose fitting lids allowed a continuous supply of CO_2 and O_2 to reach the tissue. Except where indicated, cultures were maintained at 19 to 20° under 350 to 400 ft-c of light from cool white and white fluorescent tubes. Light intensity was increased by placing the dishes closer to the light source, or it was decreased by covering the dishes with neutral density filters. A Weston light meter was used to measure the intensities obtained. Dishes were wrapped in aluminum foil for culture in darkness. Contamination of leaf disks by microorganisms was decreased by changing the bathing medium after 48 hr, before visible growth was evident. Heavy contamination, which occurred if a nitrogen source was added to the culture fluid, appeared to have little effect on expected levels of enzyme activity.

Enzyme Extraction and Assay. Phenylalanine ammonia-lyase was extracted from washed samples of 3 leaf disks weighing 150 mg by homogenizing them in a glass homogenizer with 5 ml of cold 25 mM borate-HCl buffer, pH 8.8, containing 2 mM sodium bisulfite. Clear yellow-green supernatants were obtained by centrifugation of the homogenates at 12,000*g* for 10 min. At least 90 % of the lyase activity in the homogenate was recovered in the supernatant, even if the original homogenization was carried out at slightly acid instead of alkaline pH. The clarified extracts could be stored frozen overnight without loss of activity.

The enzyme was assayed by a spectrophotometric method previously described (36). The reaction mixture contained 100 μmoles of borate at pH 8.8 (pH optimum between 8.8 and 10), 15 μmoles of L-phenylalanine (K*m* near saturation = 75 μM) and 0.2 ml of enzyme extract (about 700 μg of protein) in a volume of 3 ml. The mixture was preincubated at 30° for 15 min to allow for an initial non-enzymatic decrease in absorbance which could usually be detected in control reactions containing boiled enzyme or no substrate. The rate of the enzymatic reaction was measured at 30° in a Guilford recording spectrophotometer by following the linear increase in absorbance at 290 mμ over a period of 15 to 20 min. After the initial pre-incubation, the rate was linear for at least 2 hr and was directly proportional to the amount of enzyme added. No evidence for activators or inhibitors of the ammonia-lyase was obtained by mixing active and inactive extracts, purification of the enzyme, or heating extracts for 5 min at temperatures between 50° (enzyme stable) and 70° (enzyme denatured).

One unit of activity represents that amount of enzyme which converts 1 μmole of phenylalanine to cinnamic acid in 1 min at 30°. The formation of 1 μmole of cinnamic acid would produce an increase in absorbance at 290 mμ of 3.0 in the reaction mixture. Most data are reported as milli-units (mU) of enzyme per 100 mg original fresh weight of leaf disks. One mU/100 mg fresh weight could readily be determined quantitatively and as little as 0.2 mU could be detected with the assay used. Values listed represent the average of data obtained from duplicate sets of disks extracted for each assay. Characteristic variations between duplicate samples in an experiment are shown in Fig. 3. All experiments were repeated several times.

Labeling Experiments. Radioactivity of samples dried in an infinitely thin layer was measured in a gas flow counter with an efficiency of approximately 25 %. To obtain a radioactive enzyme fraction, leaf disks were cultured in the light on 0.1 M sucrose for 24 hr. Thirty disks (1.5 g fresh wt) were then blotted dry, remoistened with 2 ml of sucrose solution containing L-isoleucine-U-^{14}C (70 mμmoles, 12.8 × 10^6 cpm) or L-arginine-U-^{14}C (75 mμmoles, 15 × 10^6 cpm), and returned to the light for an additional 16 hr. During this time 80 % of the radioactive amino acid was absorbed and enzyme activity increased 30 to 40 % or about 200 mU. The disks were then washed free of unabsorbed amino acid, and phenylalanine ammonia-lyase was extracted from 15 of the disks as described below. The remaining disks were transferred to water and placed in the dark for an additional 24 hr before extraction.

The enzyme was extracted by grinding the disks in 20 ml of cold 25 mM borate buffer, pH 8.8, and 5 mM bisulfite. An insoluble residue containing 50 to 60 % of the total radioactivity absorbed was removed from the extract by centrifugation. Ammonium sulfate was added to the supernatant solution. The enzyme was precipitated almost quantitatively between 30 and 50 % saturation if enough bisulfite had been added to prevent browning. The precipitate, collected by centrifugation, was dissolved in a small volume of extracting medium. The resulting solution contained 15 to 25 % of the radioactivity in the original extract. Cold ethanol (−15°) was added slowly to a concentration of 20 %. After 2 hr at −15°, the alcoholic solution was centrifuged and the precipitate was discarded. This procedure removed the high molecular weight protein fraction I which interfered with isolation of the ammonia-lyase by sucrose density centrifugation. The colorless alcoholic supernatant was dialyzed several hr in the cold against 10 mM borate buffer. The ammonia-lyase was precipitated from the dialyzed solution by adding ammonium-sulfate to 50 % saturation. The precipitate was dissolved in 1 ml of 10 mM borate buffer and was stored frozen until it was used for sucrose density gradient centrifugation. This purified fraction contained about 5 % of the radioactivity present in the original homogenate and up to 50 % of the phenylalanine ammonia-lyase activity.

Sucrose Density Gradient Centrifugation. Gradients of 5 to 20 % sucrose (Mann density gradient grade) in 0.1 M bicine buffer [*N,N-bis*(2-hydroxyethyl) glycine], pH 8.8 were made according to the procedure of Martin and Ames (20). The tubes

containing 4.6 ml of gradient were equilibrated several hr at 2 to 4°. Just before centrifugation, 0.2 ml of enzyme sample was placed on top of the gradient. Centrifugation was carried out at 2° in a Spinco SW-50L rotor for 10 to 12 hr at 39,000 rpm. At the end of the run, each gradient tube was punctured from the bottom and fractions of approximately 0.1 ml were collected.

Phenylalanine ammonia-lyase activity was located in the gradient as follows: 3 ml of 10 mM borate buffer, pH 8.8, containing 10 μmoles of L-phenylalanine were added to each fraction and the absorbance at 290 mμ was recorded. After an incubation of 16 to 20 hr at room temperature, the absorbance was measured again. An increase in absorbance indicated the presence of enzyme. The change was proportional to the amount of enzyme present as measured by determination of initial rates in a standard assay. The long period of incubation increased sensitivity greatly and facilitated the handling of a large number of fractions.

The radioactivity of each fraction was then measured by plating out aliquots of each reaction mixture and counting the dried samples in a gas flow counter. The distribution of protein along the gradient was estimated from the absorbance at 230 mμ of a duplicate gradient which was passed through the flow cells of the Guilford spectrophotometer before being collected.

Starch Gel Electrophoresis. Horizontal slabs of 11 % starch gel (4 × 25 × 0.5 cm) in 10 mM borate buffer, pH 8.8 were prepared according to Smithies (30). The sample was placed in a slot 2 mm in width cut perpendicular to the long axis of the gel. Electrophoresis was carried out for 20 hr at 4° using 10 mM borate buffer, pH 8.8, in the electrode compartments, a constant voltage of 25 V/cm and an applied current of 3 to 5 mA.

Phenylalanine ammonia-lyase activity was located in the gel by slicing it into 1 cm sections. Each section was placed in 3 ml of 10 mM borate buffer containing 10 μmoles of L-phenylalanine and allowed to elute and react overnight at room temperature. Eluates from those sections containing enzyme showed a pronounced absorbance at 290 mμ.

The radioactivity eluted from each section was measured by plating out an aliquot of the reaction mixture. Since some of the starch gel dissolved in the eluting borate buffer, the dried samples formed a hard thin film over the surface of the planchettes. Addition of an internal standard indicated the dried starch gel films quenched the samples about 40 %. If we correct for this extent of quenching, then the elution procedure appears to have recovered at least 85 % of the radioactivity initially applied to the gel.

Results

Induction of Phenylalanine Ammonia-lyase. A significant increase in the activity of phenylalanine ammonia-lyase occurs in *Xanthium* leaf disks maintained for 24 hr in the light. However, little change is noted in the low endogenous activity if disks are kept in darkness (table I, expt. I).

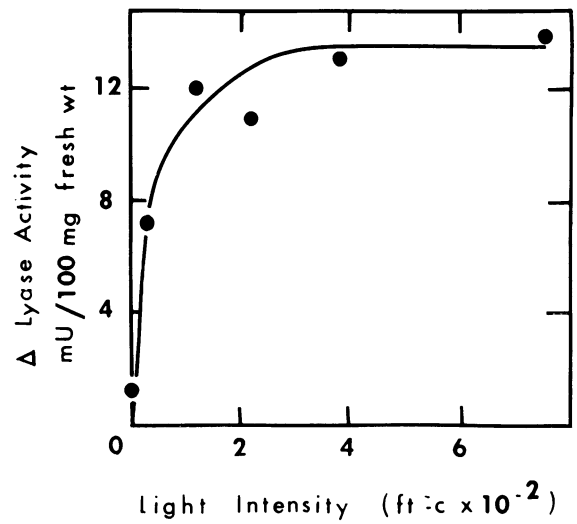

FIG. 1. Effect of light intensity on the synthesis of phenylalanine ammonia-lyase in *Xanthium* leaf disks. Disks moistened with 0.1 M sucrose solution were maintained at 19 to 20° under the appropriate light intensities. After 24 hr duplicate samples of disks cultured at each intensity were extracted and assayed for ammonia-lyase activity as described under Methods. All values have been corrected for the initial endogenous activity of 2.0 mU/100 mg fresh weight of tissue.

The change in lyase activity is inhibited by 10 μg/ml of cycloheximide (table I, expt. I). Thus, as in other tissues (1, 4, 7, 8, 21, 27, 34, 36, 37), the increased activity appears to result from the induction of phenylalanine ammonia-lyase synthesis in the leaf disks. Direct evidence for *de novo* synthesis of the lyase is presented below.

The light requirement for enzyme synthesis is apparently linked to photosynthesis. CMU, a specific inhibitor of photosystem II (28, 35) effectively blocks the action of light (table I, expt. I). Far red light does not induce enzyme synthesis in contrast to phytochrome controlled induction (7). The chlorophylls, not phytochromes, appear to be the photoreceptors in this system.

The light saturation curve for enzyme induction indicates that only a low rate of photosynthesis is required. Fig. 1 shows that maximal rates of lyase synthesis over a 24 hr period occur in disks exposed to just a few hundred ft-c of light. This intensity is at least an order of magnitude lower than that required for maximal rates of photosynthesis in the disks as measured by fixation of $^{14}CO_2$. Only 30 ft-c are needed to obtain a half-maximal effect on lyase synthesis.

Photosynthesis is not the only requirement for continuing rapid synthesis of the ammonia-lyase. Disks maintained on water alone for longer than

Table I. *Factors Affecting Phenylalanine Ammonia-Lyase Activity in* Xanthium *Leaf Disks*

Leaf disks were moistened with culture fluid and maintained at 19 to 20° under 350 to 400 ft-c of light from cool white and white fluorescent tubes (standard conditions of culture), under far-red light obtained by passing the beam of a 150 w spotlight through water and a Schott RG 8 filter, or in darkness. Phenylalanine ammonia-lyase was extracted from duplicate sets of randomly chosen leaf disks and assayed as described under Methods. Enzyme activity is expressed on the basis of original fresh weight. The protein concentration of enzyme extracts remained unchanged or decreased somewhat during culture.

Expt	Time interval	Culture condition during interval	Ammonia-lyase activity at end of interval
			mU/100 mg fresh wt
	Initial		3
	First 24 hr	Light - water	13
I	First 24 hr	Dark - water	2
	First 24 hr	Light - cycloheximide (10 μg/ml)	2
	First 24 hr	Light - CMU (10 μM)	8
		(50 μM)	2
	First 24 hr	Far red - water	2
		Disk in light and water transferred to:	
	24 to 48 hr	Light - water	5
	24 to 48 hr	Light - sucrose (0.1 M)	15
	Initial		1.2
	First 48 hr	Dark - sucrose (0.1 M)	2.2
II	First 48 hr	Dark - water	1
		Disks transferred to Light - water	
	48 to 72 hr	Previously in sucrose	9.3
	48 to 72 hr	Previously in water	1.1

24 to 48 hr begin to lose enzyme activity even though exposed to light. However, if the disks are transferred from water to sucrose at the end of 24 hr, the increase in enzyme activity continues (table I, expt. I). Glucose, fructose, mannose, maltose, and to a lesser extent, galactose, ribose, and sorbose can replace sucrose as the carbohydrate source. At least 0.1 M concentrations are required for maximal effectiveness.

The initial light induction of lyase synthesis is not stimulated by carbohydrate except in disks from very old leaves or from leaves of plants grown during winter months. Exogenous carbohydrate cannot replace the photosynthetic requirement because sucrose alone does not support enzyme synthesis in darkness (table I, expt. II). Although light stimulates the uptake of carbohydrate by *Xanthium* leaf disks, sugar is readily taken up in darkness. Enough carbohydrate can be absorbed by the disks in darkness to allow for induction in a subsequent light period even though exogenous sugar is removed (table I, Expt. II). Therefore the photosynthetic effect is not merely one involving uptake of carbohydrate. Photosynthesis and exogenous carbohydrate appear to represent distinct requirements.

Inactivation of Phenylalanine Ammonia-lyase. The decay of enzyme activity in disks maintained for long periods on water alone suggests that inactivation as well as synthesis of enzyme occurs. The loss of enzyme activity can be clearly demonstrated in leaf disks transferred from sucrose in the light to water in the dark. Disks supplied with

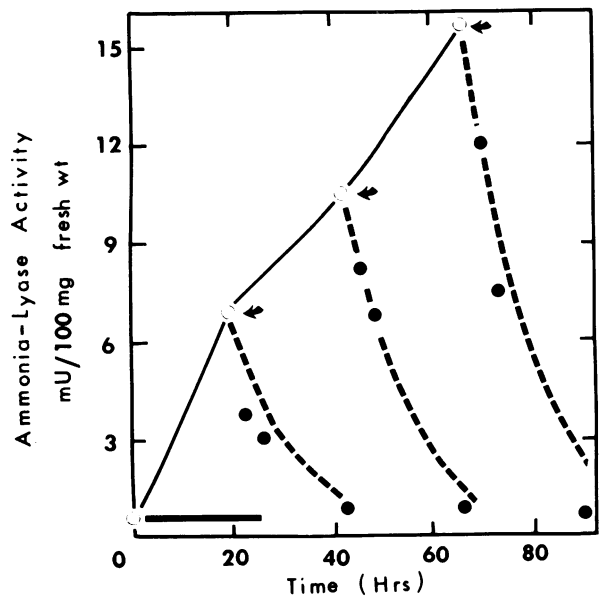

FIG. 2. Decay of ammonia-lyase activity in darkness. *Xanthium* leaf disks were cultured on 0.1 M sucrose under 350 to 400 ft-c of light (○, thin line). At times indicated by arrows, samples of disks were transferred to darkness (●). At appropriate points disks were withdrawn for enzyme assay. Symbols are mean values of duplicate sets of disks. The dashed lines represent the theoretical first-order rate of turnover of a protein with a half-life of 9 hr. The thick, solid line represents activity of disks cultured in darkness.

sucrose and light will continue to increase in ammonia-lyase activity for at least 72 hr as shown in Fig. 2. Some yellowing of the disks occurred under these conditions. Although kinetin at 40 μg/ml prevented loss of chlorophyll, it had no effect on lyase activity. At times indicated by arrows in Fig. 2, disks were transferred to water and placed in darkness. The transfer results in removal of the 2 known factors required for continuing rapid synthesis of the lyase. Under these conditions unfavorable to further synthesis, enzyme activity rapidly disappears from the tissue as indicated by the closed circles in Fig. 2. Within 24 hr lyase activity decays to the low endogenous level maintained in disks floated on sucrose but kept in darkness (thick solid line in Fig. 2). The dashed lines in Fig. 2 represent the theoretical first-order kinetics expected for degradation of a protein with a half-life of 9 hr. That a degradation of lyase protein actually occurs is suggested by data presented below.

The observed rates of enzyme decay in Fig. 2 agree well with the theoretical curves for a constant first-order rate of turnover at all stages of induction. Lyase turnover appears to be unaffected by the extent of lyase induction, in contrast to the sequential induction of a lyase-inactivating system which occurs in potato disks (37) and bean embryoes (34).

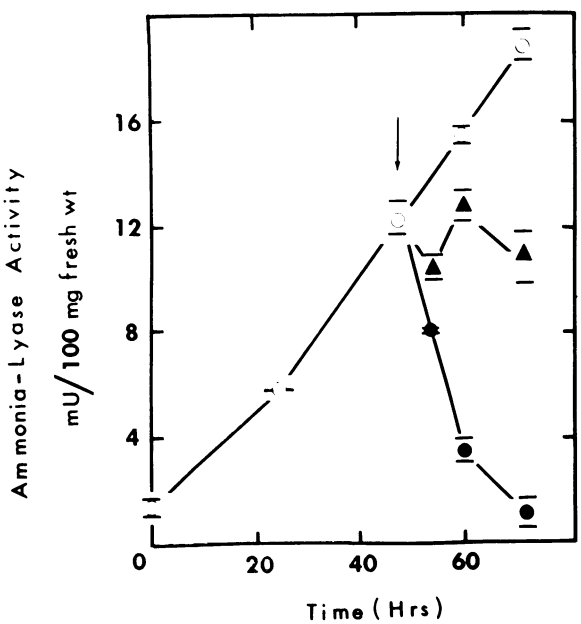

FIG. 3. Effect of cycloheximide on the decay of lyase activity in darkness. Disks were cultured on 0.1 M sucrose in the light (○). See table 1 for details. After 48 hr, some disks were transferred to water (●), or to cycloheximide, 10 μg/ml, (▲) and put in the dark. The arrow indicates the point of transfer. At times shown, duplicate samples were chosen at random from sets of disks exposed to each treatment, and the samples were extracted and assayed as described under Methods. Symbols are mean values of duplicates, and bars represent individual samples.

Neither sucrose nor light affect the decay of enzyme activity in *Xanthium* leaf disks. Approximately the same dark rate of turnover is observed in the presence or absence of carbohydrate (table II). Rapid inactivation also occurs in the light if photosynthesis is inhibited by CMU or if disks are transferred from sucrose to water (table II). Thus only the rate of lyase synthesis is affected by these external factors.

If disks cultured under saturating light conditions described in Fig. 2 are transferred to lower light intensities rather than to darkness, they do not lose activity as rapidly. Presumably the lower intensities support some lyase synthesis which helps to counteract inactivation. The effect of light intensity on continuing enzyme synthesis is similar to that for induction (see Fig. 1). Disks transferred from 350 to 30 ft-c lose only about half as much activity over a 24 hr period as disks transferred to darkness.

The decay of lyase activity can be prevented by treating the leaf disks with cycloheximide. Fig. 3 demonstrates that this inhibitor of protein synthesis quickly stops the loss of ammonia-lyase activity in darkness.

Incorporation Experiments. Direct evidence for the synthesis of phenylalanine ammonia-lyase in *Xanthium* leaf disks has been obtained from experiments on the incorporation of radioactive amino acids. Radioactive L-isoleucine and L-arginine were fed to disks under conditions where rapid increase in enzyme activity was occurring. The ammonia-lyase was then isolated by a combination of ammonium sulfate and alcohol precipitation followed by separation in sucrose density gradient centrifugation and finally by starch gel electrophoresis (see Methods for details). Fig. 4 shows the separation of phenylalanine ammonia-lyase from other radioactive leaf proteins by sucrose density gradient centrifugation. During centrifugation, lyase molecules moved more rapidly through the gradient than most other *Xanthium* leaf proteins contained in the purified fraction. The mobility of the *Xanthium* enzyme corresponded closely to that of phenylalanine ammonia-lyase obtained from potato disks. If the *Xanthium* lyase has a similar Stoke's radius, then its molecular weight would be close to that of the potato enzyme which is reported to be 330 000 (15).

At the end of the run, the peak of lyase activity was near the bottom of the tube while most proteins (as indicated by absorption at 230 mμ) were located in the top part of the gradient. Measurement of radioactivity along the gradient (open circles connected by solid lines in Fig. 4) also indicated a major radioactive fraction near the top of the gradient. The peak of radioactivity corresponded both in shape and position to the major protein peak.

A much smaller but distinct peak of radioactivity occurred near the bottom of the gradient. The position of this radioactive peak corresponded to that of lyase activity. Very little protein could be detected in this region of the gradient. To test whether the radioactivity was associated with lyase protein,

Table II. *Effect of Sucrose and Light on the Decay of Phenylalanine Ammonia-Lyase Activity in* Xanthium *Leaf Disks*

Experimental details as in Table I. After 72 hr in light and sucrose solution, disks were transferred to conditions described in the table.

Time interval	Culture conditions during interval	Ammonia-lyase activity at end of interval
		mU/100 mg fresh wt
Initial	...	3.2
First 72 hr	Light - sucrose (0.1 M)	19
72–96 hr	Light - sucrose (0.1 M)	21.5
72–96 hr	Light - sucrose + CMU (50 μM)	2
72–96 hr	Dark - sucrose	2
72–96 hr	Light - water	11.5
72–96 hr	Dark - water	1

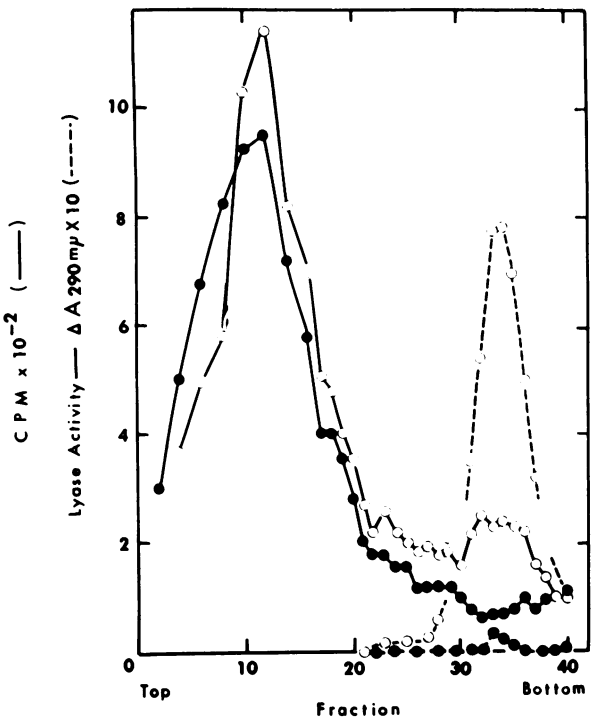

FIG. 4. Sucrose density gradient fractionation of *Xanthium* leaf proteins labeled with L-isoleucine-U-^{14}C. Thirty leaf disks were cultured on 0.1 M sucrose in the light. At 24 hr 70 mμmoles of isoleucine-^{14}C (12.8 × 10^6 cpm) were added, and disks were maintained an additional 16 hr in light. One half of the disks were then extracted. The remaining disks were transferred to water and darkness for 24 hr more before extraction. A purified phenylalanine ammonia-lyase fraction prepared from each extract was centrifuged in a 5 to 20 % sucrose gradient (SW-50 L rotor, 39,000 rpm for 11 hr). Fractions 1/10 ml in size were collected from bottom to top of the gradients. ———, radioactivity; - - - -, phenylalanine ammonia-lyase activity. ○, purified sample from disks extracted immediately after the period of incorporation in light; ●, sample from disks subsequently exposed to darkness before extraction. See Methods for detailed procedures.

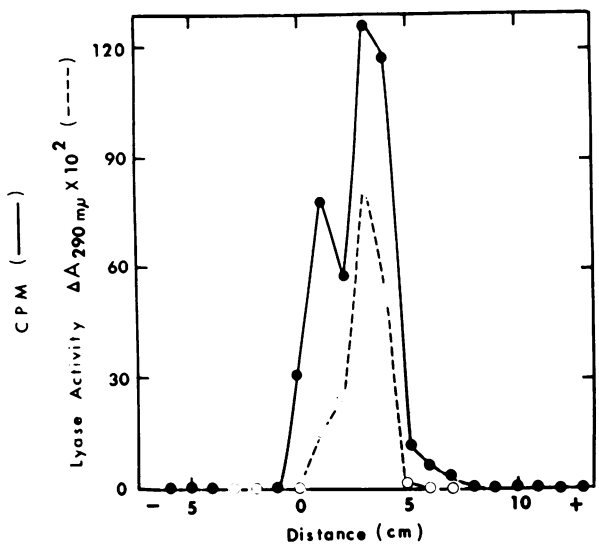

FIG. 5. Horizontal starch gel electrophoresis of radioactive phenylalanine ammonia-lyase fractions from a sucrose density gradient. A purified phenylalanine ammonia-lyase fraction was prepared from disks labeled with L-arginine-U-^{14}C (75 mμmoles, 15× 10^6 cpm) and was isolated by sucrose gradient centrifugation. Conditions were identical to those described in Fig. 4. The most active fractions from the side of the ammonia-lyase peak nearer the top of the gradient were pooled and electrophorized for 20 hr at 4° using a constant voltage of 25 V/cm and 3 to 5 mA of current. Electrode chambers and the starch gel contained 10 mM borate buffer, pH 8.8. After electrophoresis, gels were cut into 1 cm sections. The phenylalanine ammonia-lyase activity (○ - - -) and the radioactivity (● ———) of each section was determined according to procedures described under Methods. The original is at 0 cm. Pooled samples from the bottom side of the lyase peak (see Fig. 4) showed only a single radioactive band between +3 and +4 cm which corresponded to the band of ammonia-lyase activity.

fractions from each side of the lyase peak were pooled and subjected to electrophoresis on starch gel. Samples from pooled fractions of the heavier side of the lyase peak contained only a single radioactive substance which moved toward the anode. Its electrophoretic mobility corresponded to that of the lyase. Pooled fractions from the lighter side of the lyase peak also showed a major band of radioactivity on starch gel electrophoresis which corresponded to the band of lyase activity. However, minor radioactive contaminants also appeared to be present (see Fig. 5).

On the basis of the electrophoretic analyses of sucrose density fractions, it seems fair to conclude that the major portion of radioactivity in the lyase region of Fig. 4 represents radioactive phenylalanine ammonia-lyase.

The closed circles in Fig. 4 indicate enzyme activity and radioactivity in the purified fraction of a duplicate set of disks placed in darkness for 24 hr before extraction (see Methods). As expected only a trace of enzyme activity was detected in the lyase region of the gradient. The radioactive peak associated with lyase activity also disappeared from the disks as a result of culture in the dark. Little change in the radioactivity of other regions of the gradient were observed. The ratio of radioactivity in gradient fractions of Fig. 4 from light and dark exposed disks is shown in Fig. 6. Only in the region of lyase activity (indicated by the dashed line superimposed from Fig. 4) did exposure of disks to darkness produce a significant loss of radioactivity. The lack of any general effect of exposure to darkness on the labeling of other proteins was also evident in comparisons of various fractions separated by the preliminary ammonium sulfate and alcohol fractionations.

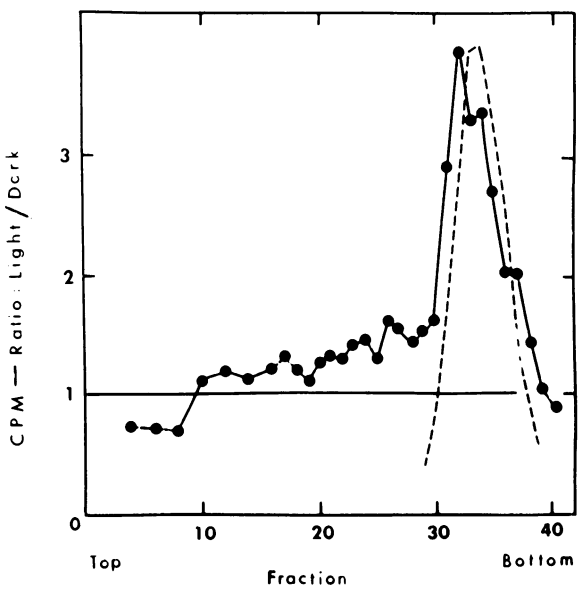

FIG. 6. (Lower left). Effect of darkness on the radioactivity of *Xanthium* leaf proteins separated by sucrose density gradient centrifugation. Gradient fractions in Fig. 4 from a preparation of disks allowed to incorporate L-isoleucine-U-^{14}C for 16 hr in the light are compared to those from similar disks exposed subsequently to 24 hr of darkness before extraction. Dashed line represents the distribution of phenylalanine ammonia-lyase activity along the gradient (superimposed from Fig. 4). Points above the horizontal line indicate a loss of radioactivity in darkness.

The loss of radioactivity from the lyase fraction suggests that the decay of enzyme activity in darkness results from an actual degradation of the enzyme. Since an internal pool of free amino acid containing almost 10^6 cpm existed in the tissue throughout the dark period, the loss of label also suggests that lyase synthesis stopped.

Leaf Disks Vs. Excised and Attached Leaves. All data presented above were obtained using disks of tissue cut from *Xanthium* leaves. Cutting initiates or affects lyase induction in a number of tissues (4, 10, 21, 22, 36). Some question existed, therefore, about the ability of intact leaves to synthesize the enzyme.

Excised leaves maintained with their petioles in 20 mM sucrose solution showed a light-induced increase in ammonia-lyase activity (table III). As in leaf disks, the enzyme disappeared rapidly from the excised leaves when they were transferred to darkness.

The response of whole plants to light was not as striking as that of excised leaves or leaf disks. The level of ammonia-lyase activity was higher in leaves of plants exposed to light than in plants maintained in darkness. However, activity seldom exceeded 2 to 3 mU per 100 mg of leaf tissue. Table III shows an exceptional experiment in which leaves of young plants grown during summer months produced almost as much phenylalanine ammonia-lyase as leaf disks during a period of continuous illumination.

To test whether reported edge effects (12) were involved in lyase induction in *Xanthium* leaf disks, they were induced to synthesize the enzyme. Before extraction, a small circle of tissue was cut from the center of the disks. Lyase activity per unit area was the same in the center section and the outer edge even when synthesis depended on absorption of sugar. Thus induction is not associated specifically with wound tissue near the cut edge but occurs in cells throughout the disks.

Effect of Daylength on Lyase Synthesis. Initially this study was begun to determine whether the light-sensitive synthesis of phenylalanine ammonia-lyase was involved in the phytochrome-controlled, photoperiodic response of *Xanthium* leaf tissue. Although the chlorophylls rather than phytochrome were found to be the photoreceptors, an effect of daylength can be shown on the level of phenylalanine ammonia-lyase in *Xanthium* leaf disks.

Table III. *Synthesis of Phenylalanine Ammonia-lyase in Excised Leaves and in Leaf Tissue of Intact* Xanthium *Plants*

Experimental details are the same as in table I except that excised leaves or whole plants were treated instead of leaf disks. Excised leaves were cultured with their petioles in 20 mM sucrose solution. Higher concentrations of sucrose injured the leaves. No sucrose was applied to leaves of intact plants. At the times indicated, disks were cut from leaves and assayed for activity. Values in parentheses are those for leaves or plants transferred to darkness for an additional 24 hr before being sampled again.

Source of ammonia-lyase	Culture condition	Ammonia-lyase activity
		mU/100 mg fresh wt
Excised leaves	Endogenous	1
	Light, sucrose - 24 hr	4
	48 hr	7 (>1)
	Dark, sucrose - 48 hr	>1
Intact leaves	Endogenous	1
(young plants grown	Light - 24 hr	6 (>1)
during the summer)	Dark - 24 hr	2

Fig. 7 describes an experiment in which leaf disks floated on sucrose were exposed to different 24 hr cycles of light and dark. Those disks receiving 17 hr of light during the cycle showed a marked increase in enzyme activity during the long period of illumination (thin lines, open circles in Fig. 7) and a somewhat less extensive decay of activity during the shorter 7 hr period of darkness (thick lines, closed circles). The opposite effects were observed in disks receiving a short 7 hr exposure to light. Only a small increase in lyase activity occurred during the brief cycle of illumination while most of the enzyme formed was subsequently degraded during the longer period of darkness.

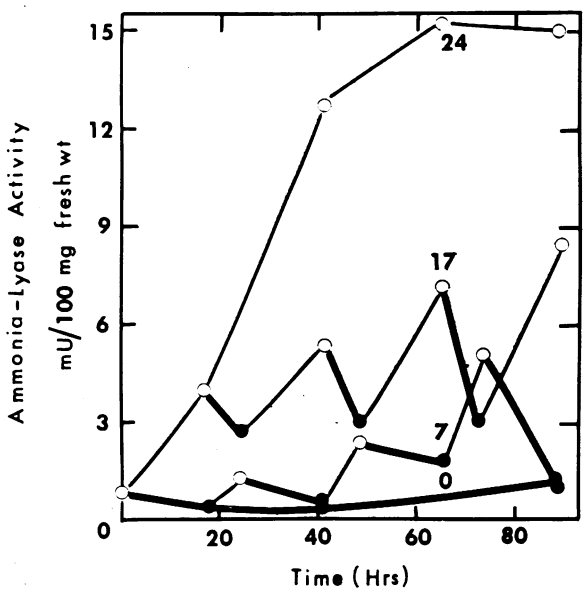

FIG. 7. Photoperiodic control of phenylalanine ammonia-lyase activity in *Xanthium* leaf disks. Disks cut from a large number of leaves were pooled and moistened with 0.1 M sucrose. Randomly chosen sets of disks were exposed to 24 hr cycles of light and darkness ranging from constant illumination (24 hr) to complete darkness (0 hr of light). The numbers above the curves indicate the length of the light period during each 24 hr cycle. Conditions of culture are those described in table I. Each point represents the mean value of enzyme assays from duplicate samples of disks extracted. Periods of illumination are indicated by thin lines followed by open circles; periods of darkness are thick lines followed by closed circles.

The total level of enzyme maintained in disks (as determined by the areas under the respective curves in Fig. 7) was directly related to the length of the light cycle to which the disks were exposed. The concentration of phenolic components accumulating in the disks [chiefly chlorogenic and 3,5-di-*O*-caffeoylquinic acid (32)] could be estimated from the maximum absorption at 330 mμ of alcoholic extracts. At the end of the experiment the amount of phenolic material in the disks was also related directly to the length of the light cycle and thus to the average level of ammonia-lyase maintained in the tissue.

The apparent increase in rate of lyase synthesis observed in each succeeding light cycle of Fig. 7 was not a constant feature of this type of experiment. But a correlation between the number of light cycles to which the disks had been exposed and the subsequent rate of enzyme synthesis in light was observed occasionally.

Discussion

The activity of phenylalanine ammonia-lyase increases dramatically in *Xanthium* leaf disks floated on sucrose in the light. An equally striking loss of enzyme activity occurs in disks transferred from light to darkness (Fig. 2). Data presented above suggest that these changes involve synthesis and degradation of lyase protein. The isolation of radioactive enzyme molecules from leaf disks fed labeled amino acids is indicative of *de novo* lyase synthesis. Cycloheximide is a very potent inhibitor of the change in lyase activity. A concentration, 50-fold greater than that needed to eliminate completely the

change in activity, inhibits general protein synthesis in *Xanthium* leaf disks only 50% (23). The incorporation studies together with the inhibitory effects of cycloheximide strongly suggests that the observed increase in enzyme activity results from induction of phenylalanine ammonia-lyase synthesis in the leaf disks.

The apparent loss of radioactive lyase protein from disks transferred to darkness is consistent with the view that the decay of enzyme activity results from proteolytic degradation of the enzyme. However, the absence of a radioactive peak in the lyase region of sucrose gradients containing material from dark-treated disks cannot be considered conclusive evidence for degradation. For instance, combination of lyase protein with an inhibitor molecule could inactivate the enzyme. If inactivation greatly altered enzyme solubility, then the enzyme-inhibitor complex might be lost during preliminary purification prior to density gradient centrifugation.

The requirement for external carbohydrate in lyase induction is by no means understood. Creasey had reported a similar effect of exogenous sugars on lyase synthesis in strawberry leaf disks (4, 5). The complete solubility of the *Xanthium* lyase and the potency of cycloheximide as an inhibitor of its synthesis suggests that the enzyme is formed in the cytoplasm. Synthesis of proteins in chloroplasts and mitochondria is reported to be much less sensitive to cycloheximide than that in the cytoplasm (19, 29). Metabolism of exogenous carbohydrate could provide a convenient source of ATP for the synthesis of cytoplasmic enzymes. A more interesting hypothesis is that exogenous sugar is metabolized in the light to an inducer of lyase synthesis.

Light induction of ammonia-lyase can occur in freshly cut disks in the absence of external carbohydrate. Since sucrose is a major product of photosynthesis in *Xanthium* leaves (33), the tissue may contain enough endogenous carbohydrate to support an initial period of enzyme synthesis. But eventually the endogenous factor is completely utilized and further lyase synthesis requires exogenous carbohydrate.

The inhibition of phenylalanine ammonia-lyase synthesis by CMU indicates that the light requirement in *Xanthium* leaf disks is one for photosynthesis. Ammonia-lyase synthesis in strawberry leaves is also stimulated by photosynthesis (4, 5). However, the reported photosynthetic requirements in these 2 tissues appear to be quite different. Low light intensities produce a maximal effect in *Xanthium* whereas high intensities are needed to obtain maximal rates of lyase synthesis in the strawberry system. Photosynthesis functions in part by supplying substrate amounts of carbohydrate in strawberry leaf disks (5). The low light saturation curves observed in the present study indicate that only a very minimal rate of photosynthesis is needed to sustain maximal rates of lyase synthesis in *Xanthium* leaf disks.

Downs *et al.* (6) have demonstrated that photosynthesis is required for anthocyanin pigmentation in apple skins and that sucrose also stimulates pigmentation. High light intensities were required for maximal effects. However, the rate of photosynthesis in apple skin tissue was so low that net CO_2 fixation was not always observed. In this regard, the photosynthetic requirement of the apple skin is similar to that of the *Xanthium* system. Both tissues appear to require only a trace quantity of some photosynthetic product, if a source of exogenous carbohydrate is available. The stimulatory effect of sucrose and light on lyase synthesis in strawberry leaf disks exposed to CO_2-free air (5) could reflect a similar trace requirement as well. The basipetal translocation of photosynthate from sugarcane leaves is another phenomenon reported to involve a low light intensity, CMU-sensitive photosystem (13, 14).

The low intensity photosynthetic requirement of *Xanthium* leaf disks appears to represent a type of photocontrol of phenylalanine ammonia-lyase synthesis different from those previously reported. The phytochrome system has been implicated in the induction of lyase synthesis in several tissues (1, 7). A high energy blue light reaction appears to control induction in Jerusalem artichoke (22) gherkin seedlings (8) and buckwheat (27). CMU does not inhibit the high energy light stimulation of lyase synthesis in potatoes (unpublished observations).

The half-life of the *Xanthium* ammonia-lyase, calculated from rates of enzyme decay in darkness, ranged from 6 to 15 hr in the leaf material examined. The apparent constituitive nature of the *Xanthium* degrading system contrasts with the inducible lyase-inactivating systems of potato tuber disks (37) and bean seedlings (34). In all of these tissues though, as in gherkin seedlings (9), the decay of enzyme activity can be prevented by cycloheximide. The cycloheximide inhibition suggests that *de novo* synthesis of protein is required to maintain turnover. Within 6 to 12 hr after addition of cycloheximide to leaf disks in the dark, the decay of lyase activity stops. Thus the degrading system itself must disappear rapidly from the tissue once its synthesis is inhibited by cycloheximide. Recently, the disappearance of a light inducible nitrate reductase in barley leaves was shown to be inhibited by cycloheximide (17).

The photoperiodic regulation of ammonia-lyase activity in *Xanthium* leaf disks is atypical. It apparently involves the chlorophylls rather than phytochrome as the photoreceptors. This diurnal fluctuation in enzyme activity depends on 2 opposing responses of the *Xanthium* system. One is the repeated induction of lyase synthesis that occurs during each light cycle. The other is the rapid turnover of the enzyme.

Xanthium leaf disks have the ability to synthesize ammonia-lyase continuously during an extended period of illumination or to start and stop synthesis repeatedly during alternating cycles of light and

darkness. This pattern of induction is fundamentally different from that in potatoes (37) and gherkins (8). Light stimulated lyase synthesis in these tissues becomes irreversibly repressed within a relatively short time after induction has begun. Once repression is complete, exposure to light can no longer induce further synthesis of the lyase.

The continuing synthesis of ammonia-lyase in *Xanthium* leaf disks suggests that repression, if it occurs at all, is reversible. Cessation of lyase synthesis in darkened *Xanthium* leaf disks may not require the formation of effector molecules. For instance, polyribosomes needed for lyase synthesis may be unstable in darkness. Clark, *et al.* (3) have demonstrated a diurnal fluctuation in the concentration of polysomes in chinese cabbage leaves.

The large changes in rate of lyase synthesis in leaf disks compared with those in whole plants suggests that excision is required for rapid induction of lyase synthesis in *Xanthium* leaf tissue. Ryan (25, 26) has shown that the light-induced synthesis of a trypsin inhibitor protein occurs only in leaves that have been excised from potato or tomato plants.

Under normal growing conditions, daylength has a small effect on the level of ammonia-lyase in attached *Xanthium* leaves. Nevertheless, the effect would be sufficient to account for the relation Taylor (31) observed between the steady-state concentration of phenolic components in *Xanthium* leaves and the length of the photoperiod under which his plants were grown.

The physiological or ecological importance of the regulatory systems controlling phenylalanine ammonia-lyase synthesis in *Xanthium* leaf disks is difficult to assess at present. However, the light-sensitive synthesis of certain leaf proteins such as the ammonia-lyase presents an intriguing phenomenon. The study of such systems may provide further clues to the biochemical mechanisms whereby light controls the growth and development of plants.

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