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Sequential Induction of Phenylalanine Ammonia-lyase and a Lyase-inactivating System in Potato Tuber Disks

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Abstract. The light induced synthesis of phenylalanine ammonia-lyase in disks cut from potato tubers is very sensitive to cycloheximide. Synthesis is inhibited 50 % in disks cultured on 5 μM cycloheximide instead of water and almost completely in disks aged in the presence of 10 μM inhibitor. Inhibition is irreversible. Fresh disks exposed only 1 hour to 10 μM cycloheximide do not synthesize enzyme during the subsequent 24 hours.

Normally a maximal enzyme activity develops in disks about 24 hours after being cut from the tuber. Thereafter enzyme activity declines. The disappearance of enzyme is not affected by concentrations of cycloheximide sufficient to inhibit the synthesis of enzyme initially. No disappearance of enzyme is noted during the initial phase of induction if enzyme synthesis is inhibited by cycloheximide. However, enzyme does disappear from the tissue if more than half the maximal enzyme content is allowed to form before synthesis is inhibited. If cycloheximide at a concentration 10-fold that needed to inhibit synthesis completely is added to disks after they have attained a maximal enzyme level, then subsequent loss of enzyme activity from the tissue is prevented. The initial stability of the enzyme in the absence of further synthesis and the inhibition of enzyme disappearance by high concentrations of cycloheximide suggest A) that early phases of induction involve synthesis of enzyme protein in the absence of turnover, B) that a system capable of degrading or inactivating the lyase subsequently forms in the tissue, and C) that the formation of the degrading or inactivating system requires protein synthesis.

The effect of cycloheximide on uptake and incorporation of L-isoleucine-U-^{14}C into soluble and insoluble proteins of tuber disks was also examined. During induction the rate of uptake increased 3 to 4-fold, and the rate of incorporation into protein, corrected for change in uptake, increased 25-fold. Cycloheximide inhibited incorporation of isoleucine-^{14}C into proteins of fresh disks more than 80 %. It did not prevent activation of general protein synthesis during induction and inhibited incorporation in induced disks only 20 %. At all times incorporation of amino acid into the soluble, lyase-rich, protein fraction was more sensitive to cycloheximide than the insoluble fraction.

Studies of enzyme induction in plant (11, 14) and animal (10, 26, 27) tissues have shown that protein turnover may be intimately associated with the induction process. Changes in the rate of degradation independent of changes in rate of synthesis can occur during induction of specific enzymes (15, 26). These studies suggest that specific turnover systems exist and that their existence in the tissue is regulated by mechanisms similar to those controlling the synthesis of inducible enzymes. Evidence of protein turnover in plants concerns the overall synthesis and degradation of the total bulk of protein in tissues under examination (5, 6, 13, 29). Little information exists on the formation or specificity of individual turnover systems. The induction of phenylalanine ammonia-lyase in disks of tissue cut from potato tubers appears to involve both the synthesis and turnover or inactivation of the enzyme (33). Cycloheximide under suitable conditions will inhibit either the appearance or the disappearance of the enzyme in the tissue (34). Engelsma (9) has recently described a similar effect of cycloheximide on synthesis and disappearance of a phenylalanine ammonia-lyase in gherkin seedlings. This paper describes the conditions under which the selective effects of cycloheximide on potato disks are obtained. The results presented suggest that the induction process involves the synthesis of both lyase protein and the protein components of a system degrading or inactivating the enzyme (inactivating system).

Materials and Methods

Kennebec potato tubers stored less than 6 months at 4° were used for studies of the cycloheximide inhibition of enzyme synthesis. Incorporation stud-

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ies were performed on tissue from tubers stored 6 to 8 months. Disks 2 mm thick and 1.5 cm in diameter were cut from the inner tissue of the tuber. The original fresh weight of a single disk was approximately 0.4 g. Treatments for induction studies consisted of 25 disks moistened with 3.5 ml of solution and incubated at 21° under 400 ft-c of light. Disks increased approximately 10 % in fresh weight in 24 hours. All data are expressed per g original fresh weight.

Enzyme Assay. Phenylalanine ammonia-lyase was extracted from acetone powders of potato disks and assayed by following changes in absorbance at 290 mμ as previously described (33). One unit of activity is that amount of enzyme producing an increase in A_{290} mμ of 0.01 per hour (3.3 mμmoles of cinnamic acid).

Direct aqueous extraction of the enzyme did not previously yield consistently active extracts. Inclusion of mercaptoethanol in the extracting medium corrected the situation and made it possible to obtain reproducible results without preparing acetone powders. Samples of 5 disks were homogenized with a high speed grinder in 25 ml of cold 0.025 M borate buffer (pH 8.8) containing 0.4 ml of mercaptoethanol per liter (approx. 5 mM). Extracts were clarified by filtration under suction through Whatman No. 1 filter paper. Direct aqueous extraction yielded about twice as much activity per disk as corresponding acetone powder extracts. Since the protein concentration of aqueous extracts was also higher, the specific activity of both types of extract was about the same. The activity of aqueous extracts, although higher, was always proportional to that of corresponding acetone powder extracts under every experimental treatment examined. Aqueous extraction is preferred to preparation of acetone powders because it is rapid and decreases the variation between samples introduced by powdering the tissue.

The period of assay was shortened and the sensitivity increased by running the reaction at 40° instead of at room temperature. The complete reaction mixture was incubated 5 minutes at 40° before measurements of A_{290} mμ were begun. Readings were taken at 5 or 10 minute intervals, and the reaction was returned to the 40° bath between readings. The elevated temperature had no apparent effect on the enzyme and increased the rate of reaction 2 to 3-fold over that at room temperature.

Protein was determined with the biuret reagent or with Nessler's reagent after Kjeldahl digestion of alcohol insoluble precipitates.

Uptake and Incorporation Studies. L-Isoleucine-U-^{14}C (234 mc/mmole) was obtained from New England Nuclear Corporation. The radioactivity of an infinitely thin layer of each sample dried on planchets was measured in a Nuclear-Chicago Corporation gas-flow counter with about 25 % efficiency.

A single treatment consisted of 5 tuber disks (original fr wt of 2.0 g) moistened with 0.5 ml of a solution containing 3.0 mμmoles (3.8×10^5 cpm) of isoleucine-U-^{14}C. Disks were allowed to absorb the amino acid from solution for 2 hours while exposed to 400 ft-c of white light at 21°. The disks were then washed thoroughly with tap water and distilled water to remove the unabsorbed isoleucine-^{14}C. Inclusion of unlabeled isoleucine in the washing medium did not increase the amount of radioactivity washed from the disks.

Where only incorporation into total protein was measured, the washed disks were ground with 10 ml of 95 % ethanol in a Ten Broeck glass homogenizer and 0.1 ml aliquots of the finely suspended homogenate were counted to determine the total uptake. An aliquot of the homogenate was centrifuged 15 minutes at 20,000 × *g* to collect the protein precipitate. The precipitate was washed 3 times with ethanol, the first wash containing 50 μmoles of unlabeled isoleucine. The washed precipitate was then suspended and homogenized in ethanol, and aliquots were counted to determine incorporation into total protein. Aliquots of the alcoholic supernatant from which the precipitate had first been removed were also counted as a measure of incorporation into the soluble free amino acid pools. When paper chromatograms of samples of the alcoholic supernatant were run in 1-butanol-acetic acid-water (25:4:10) and scanned in a Packard radiochromatogram scanner, at least 95 % of the radioactivity was located in the region of the chromatogram containing isoleucine.

When incorporation into the soluble and insoluble protein fractions was studied, disks were homogenized in 10 ml of cold 0.025 M borate buffer, (pH 8.8) containing 5 mM mercaptoethanol. One ml of the aqueous homogenate was suspended in 4 ml of ethanol and ground in a glass homogenizer to produce a suspension uniform enough for counting. The radioactivity of aliquots of this fine suspension served as a measure of total uptake. Five ml of the remaining aqueous extract were centrifuged for 30 minutes at 20,000 × *g* to precipitate the insoluble protein not extracted from the disks by borate buffer. The precipitate was washed 2 times with more buffer and then suspended in ethanol and homogenized. Aliquots were counted to determine incorporation into insoluble protein. The first wash containing 50 μmoles of unlabeled isoleucine was added to the original aqueous supernatant. The soluble protein in the combined supernatant and wash was precipitated by adding 1 ml of extract to 4 ml of ethanol. After 15 minutes in the cold, the suspension was centrifuged and the precipitate washed several times with ethanol. The washed precipitate was then suspended by grinding it in ethanol, and aliquots were counted to measure incorporation into soluble protein. Aliquots of the 80 % (v/v) ethanol supernatant were also counted to determine radioactivity in the soluble pools. The

sum of activity in the free amino acid pools, soluble protein, and insoluble protein fractions accounted for very close to 100 % of the total activity measured directly in the original homogenate.

During the course of experimentation, it was discovered that exposure to cycloheximide decreased the natural resistance of tuber disks to infection by a fluorescent *Pseudomonas* contaminating the deionized water used in the laboratory (35). Usually 48 to 72 hours elapsed before fluorescence under ultraviolet light (a sensitive test for infection) could be detected on the surface of the disks. Comparison of lyase activity extracted from disks not visibly contaminated with that from obviously contaminated disks showed little difference in enzyme level. Nevertheless, care was taken in cycloheximide experiments of long duration to assay disks from samples that showed no visible signs of infection.

Incorporation of isoleucine-^{14}C was apparently not affected by contamination in experiments where exposure to cycloheximide was only of 2 hours duration. Comparison of incorporation under aseptic and nonsterile conditions yielded identical results within experimental error. Fresh disks removed from tubers under aseptic conditions (35) did not contain any viable organisms when tested on nutrient agar. Normally resistant disks maintained on contaminated water under nonsterile conditions supported less than 1000 organisms per disk after 48 hours of culture. Use of aseptic conditions was therefore not deemed necessary for experiments described below.

Results

Effect of Cycloheximide on Lyase Synthesis. Relatively low concentrations of cycloheximide inhibited the appearance of phenylalanine ammonia-lyase activity in tuber disks. When disks were incubated for 24 hours in the presence of 5 μM cycloheximide instead of water, only 50 % as much enzyme activity could be extracted from them. Extracts of disks treated with 10 μM cycloheximide showed almost no enzyme activity. Higher concentrations of cycloheximide completely inhibited the appearance of activity during culture. No effect of cycloheximide was noted on the activity of the enzyme *in vitro*. The potency of cycloheximide as an inhibitor of enzyme formation in tuber disks is shown quantitatively in figure 1. Under the experimental conditions used, an inhibition of 50 % was achieved with less than 1 μg of cycloheximide per g initial fresh weight of tissue.

Cycloheximide had no visible effect on the disks other than to prevent the surface from turning light brown. The inhibition of surface browning was directly correlated with the inhibition of chlorogenic acid biosynthesis in the disks (35). Usually the amount of soluble protein extracted from disks

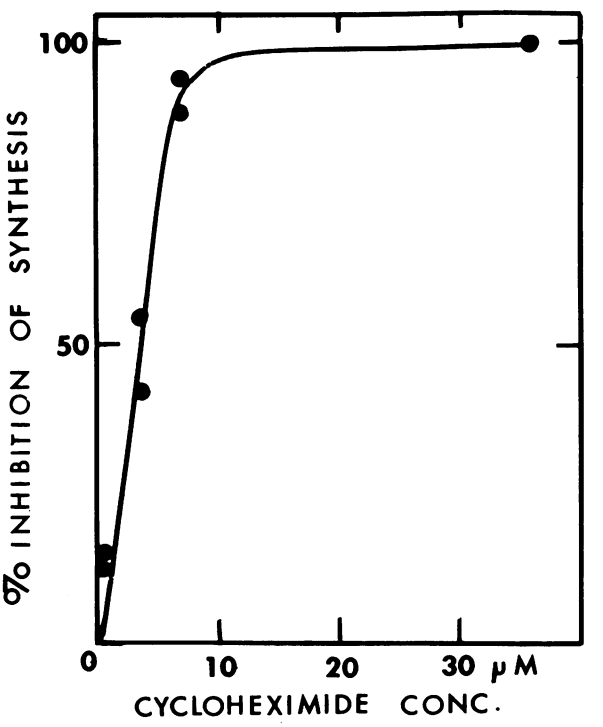

FIG. 1. Effect of cycloheximide concentration on synthesis of phenylalanine ammonia-lyase in disks of potato tuber. Disks were aged 24 hours in light in the presence of water or cycloheximide before enzyme was extracted and assayed. Activity of water control, 145 units/g original fresh weight.

maintained 24 hours in the light was slightly more than that obtained from fresh disks, the increase being 10 to 15 % at most. The small extent of net change and the variation between tissue samples made it difficult to observe any significant effect of cycloheximide on the amount of soluble protein extracted.

Cycloheximide inhibition of protein synthesis can be reversed in many systems by removing the inhibitor (12). However, inhibition of lyase appearance in potato disks was irreversible. If freshly cut disks were moistened with a 10 μM solution of cycloheximide for just a few minutes and then washed free of inhibitor and maintained subsequently on water alone for 24 hours, significantly less activity could be extracted from treated disks compared to tissue not exposed to cycloheximide at all. Quantitative effects of short initial exposures to cycloheximide are shown in figure 2. An exposure of 1 hour to cycloheximide was sufficient to inhibit enzyme formation 90 % during the remaining 23 hours of incubation. Normally no enzyme activity can be detected in disks after an incubation of only 1 hour on water alone. Possibly enough inhibitor was absorbed by disks during the short exposure to maintain an internal inhibitory concentration during the subsequent period of formation of enzyme activity. However the results

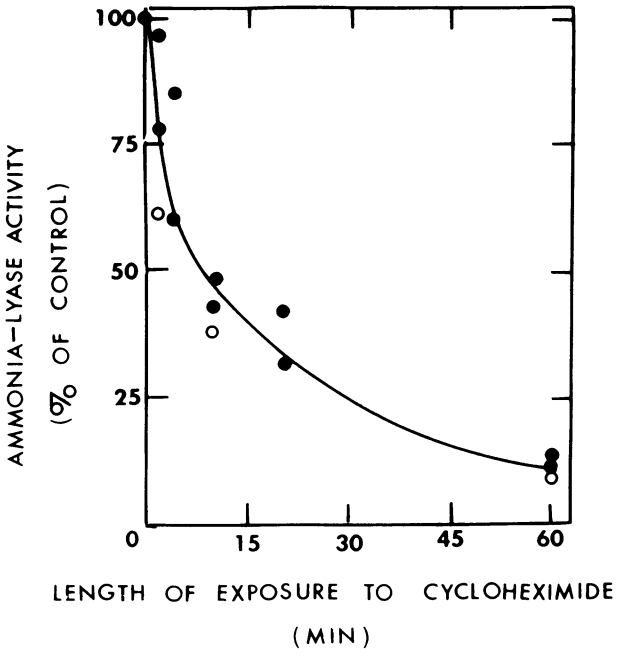

FIG. 2. Effect of length of exposure to cycloheximide on subsequent appearance of ammonia-lyase activity in the tissue. Freshly cut disks were moistened with 36 μM cycloheximide for lengths of time indicated, washed thoroughly with water and maintained for the remaining period of aging on water alone. After a total of 24 hours of treatment and aging, disks were extracted directly with buffer, and enzyme was assayed at 40° (see Methods). Disks maintained on water alone during the experiment served as controls. Different symbols represent different experiments. Activity of water controls, 800 units/g original fresh weight.

of table I make this explanation unlikely. When disks were treated with a low concentration of cycloheximide (3.6 μM) that produced only partial inhibition of synthesis, an initial exposure of 30 minutes was as effective as continuous exposure to inhibitor during the entire incubation of 24 hours. The fact that inhibition did not disappear once cycloheximide was removed even when sub-maximal levels of inhibitor were used suggests that the inhibition is irreversible once it occurs.

Effect of Cycloheximide on Lyase Disappearance. The potency and irreversibility of cycloheximide as an inhibitor of lyase appearance made it a useful tool for investigation of events occurring during the induction. The kinetics of induction originally described (33) and illustrated again as a control in figure 3A (closed circles) indicates that both appearance (synthesis or activation) and disappearance (degradation or inactivation) of enzyme occur. If active enzyme were allowed to form in the disks before treating them with an inhibitory concentration of cycloheximide, it should be possible to study the behavior of the enzyme in the absence of further synthesis or activation.

Table I. *Effect of Cycloheximide Concentration During Short Exposure on the Inhibition of Phenylalanine Ammonia-lyase Synthesis*

Each treatment consisted of 25 disks cultured on 3.5 ml of solution at 21° under 400 ft-c of light for 24 hours. Disks treated with cycloheximide for only 0.5 hour were washed and transferred to water for the remaining 23.5 hours of culture. The enzyme was extracted with buffer from fresh disks after 24 hours of culture and assayed at 40° as described under Methods. The ammonia-lyase activity of water controls was 795 units/g original fresh weight.

Cycloheximide conc	Length of exposure	Lyase activity
μM	*hr*	*% of water control*
3.6	0.5	54
	24	47
36	0.5	11
	24	10

Data thus far have referred to treatment with cycloheximide begun immediately after induction was initiated by cutting disks from the tuber and exposing them to light. The curves in figures 3A and 3B describe some typical effects of delayed additions of the inhibitor. In experiments shown in figure 3A disks were allowed to form active enzyme for an initial period of 12 hours before some of them were transferred from water to 7 μM cycloheximide, a concentration capable of producing

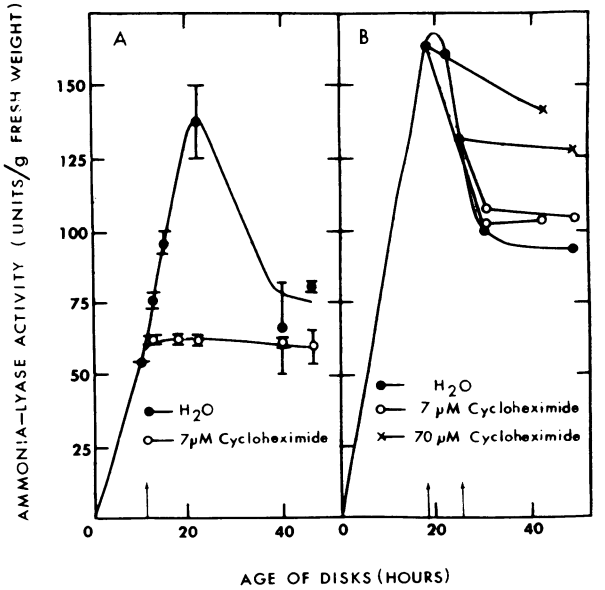

FIG. 3. Effect of delayed addition of cycloheximide on level of enzyme activity in disks. Samples of disks initially moistened with water were then transferred to cycloheximide solutions at times indicated by arrows along absicca. Acetone powders of disks were prepared at appropriate times, and enzyme activity was assayed at room temperature. The extent of variation between duplicate samples is shown in figure 3A.

an inhibition of 85 to 90 %. At the time of transfer only 40 % of the maximal enzyme level had been attained. Within an hour after transfer to cycloheximide further increase in enzyme level halted (open circles, fig 3A), and the enzyme activity remained unchanged thereafter. The failure of the enzyme to disappear from the treated tissue in which formation of active enzyme was inhibited, suggested either that turnover or inactivation was also inhibited or that the system responsible for disappearance of enzyme activity was not present during the initial stage of lyase induction. If the addition of 7 μM cycloheximide were delayed until the enzyme normally began to disappear from the tissue after 24 hours of induction, then the low concentration of inhibitor did not affect the rate or extent of disappearance (open circles, fig 3B) from the tissue. Consequently the failure of enzyme to disappear from disks treated during an early stage of induction (fig 3A) cannot readily be explained on the basis of inhibition of degradation or inactivation by 7 μM cycloheximide. Rather, the initial phases of induction seem to involve only the formation of active enzyme in the absence of any turnover or inactivation.

Cycloheximide added to the disks at any time during the initial 24 hour period of increase in enzyme activity quickly halted further increase in level of activity. However if the disks had already attained 60 % or more of the maximal enzyme level before addition, then not only was further increase stopped but, unlike the results pictured in figure 3A, enzyme began to disappear from the tissue. In these instances the rate of disappearance was less than that normally observed in untreated disks between 24 and 48 hours of induction, but approached the normal rate in those disks allowed to form close to maximal amounts of enzyme before being exposed to cycloheximide.

Although 7 μM cycloheximide inhibited lyase formation (fig 1, 3A, open circles), I have already pointed out that this low concentration of cycloheximide did not affect its disappearance during later stages of induction. Data presented below indicate that cycloheximide is not as effective an inhibitor of protein synthesis in light-induced disks as in fresh tissue. Consequently higher concentrations of cycloheximide were tested in delayed addition experiments to determine whether the inhibitor had any effects on the latter stages of induction. When the concentration of cycloheximide added after 18 to 24 hours of culture was increased to 10 times that needed to prevent lyase appearance initially, the disappearance of enzyme from the tissue did not occur (fig 3B, Xs). The quantitative effects of increasing concentrations of cycloheximide on prevention of the loss of enzyme activity are shown in figure 4. In these experiments, disks were maintained on water for 24 hours to allow maximal formation of active enzyme. At that time, enzyme activity was assayed, and samples of disks

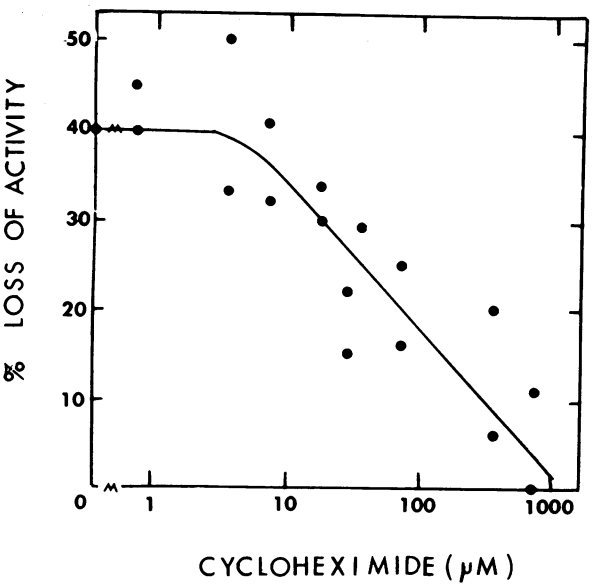

FIG. 4. Effect of cycloheximide concentration on loss of enzyme activity between 24 and 48 hours of aging. Disks were moistened 24 hours with water and assayed for lyase activity. Samples of disks were then transferred to solutions of cycloheximide at concentrations indicated. After an additional 24 hours enzyme activity of all samples was determined. Activity of disks aged 24 hours, 154 units/g original fresh weight (acetone powder extracts).

were transferred to solutions of cycloheximide at concentrations indicated. After an additional 24 hours of culture during which enzyme activity began to disappear from water controls, all samples were extracted and assayed for activity. The percent loss of enzyme between 24 and 48 hours of culture is plotted in figure 4 against the concentration of cycloheximide to which the disks were exposed during this time. Although 40 % of the enzyme activity disappeared from disks maintained on water alone, almost no loss of activity occurred in disks transferred to 1 mM cycloheximide. Disks transferred to 50 μM cycloheximide lost only half as much enzyme activity as those cultured continuously on water alone. Only one-tenth the concentration of cycloheximide *i.e.* 5 μM, produced a comparable 50 % inhibition of the initial appearance of lyase activity. Addition of cycloheximide at concentrations as high as 700 μM after the enzyme level had declined to a steady plateau failed to produce any further alteration of activity in the disks.

Incorporation Studies. The effect of cycloheximide on incorporation of isoleucine-U-^{14}C into protein of tuber disks was investigated to determine whether cycloheximide inhibited protein synthesis under the same conditions that it inhibited the induction of the lyase. Both the rate of uptake and the rate of incorporation of the amino acid into protein increased during light induction (fig 5).

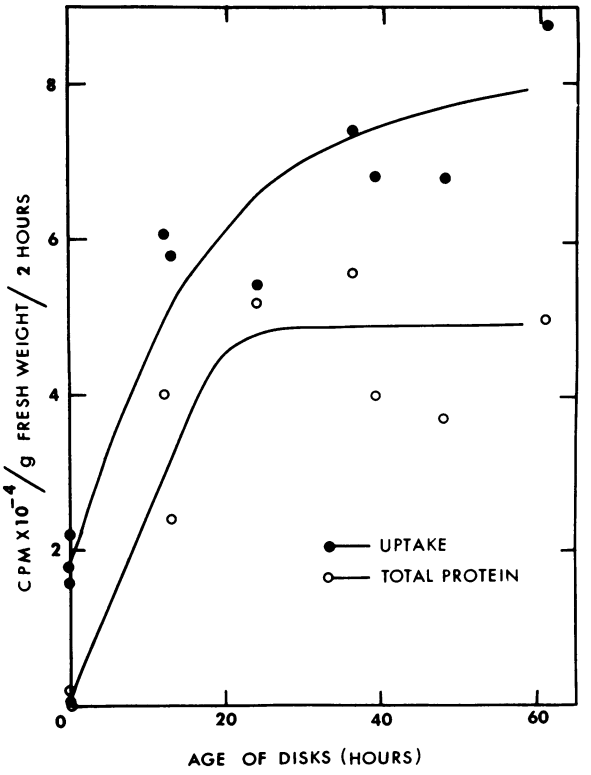

FIG. 5. Change in rate of uptake and incorporation of isoleucine-U-^{14}C into protein of tuber disks during aging. Disks were aged on water at 21° under 400 ft-c of light for the periods of time indicated. Appropriately aged samples of 5 disks (2 g original fr wt) were fed 3 mμmoles of isoleucine-^{14}C (3.8×10^5 cpm) for 2 hours before being washed and extracted as described under Methods. Kinetic studies indicated that steady-state rates of uptake and incorporation were achieved within 20 minutes after labelled amino acid was supplied to the tissue.

At about the time lyase activity reached a maximal level in the disks, the rate of protein synthesis as measured by incorporation attained a steady level which was more than an order of magnitude greater than that observed in fresh disks. Less than 10 % of the isoleucine-^{14}C taken up by fresh disks during a 2-hour period of feeding was incorporated into protein, while almost 70 % of the amino acid taken up by disks maintained in the light for 24 hours was incorporated into protein during a similar 2-hour period.

About two-thirds of the radioactivity incorporated into proteins of fresh disks was found in the soluble fraction. Aging produced a large increase in rate of incorporation into both soluble and insoluble proteins. The change in rate of incorporation into the insoluble fraction was much greater than that of the soluble fraction. Consequently more radioactivity was found in the insoluble proteins than in the buffer extracted proteins of disks cultured in light.

Cycloheximide inhibited the incorporation of labeled amino acid into both fractions. However, the extent of inhibition declined appreciably with age of disk (fig 6). Incorporation by fresh disks was inhibited more than 90 % by 36 μM cycloheximide, an effect comparable to that on formation of lyase activity. After 24 hours of aging, less than half of the incorporation could be eliminated by administering isoleucine-^{14}C in cycloheximide instead of in water. Increasing the concentration of inhibitor from 36 to 360 μM (Xs in fig 6) did not increase the inhibition very much.

Although cycloheximide inhibited protein synthesis in fresh disks, it did not prevent the development of a very rapid rate of incorporation during aging in the light. Disks cultured 12 to 16 hours on cycloheximide instead of water did not differ in rate of incorporation from disks cultured on water during this period and exposed to cycloheximide only during the 2-hour period of isoleucine-^{14}C feeding. Results of a typical experiment are shown in table II. An effect not mentioned previously but illustrated in the table is the inhibition of uptake by cycloheximide. Inhibition of uptake was never more than 50 % and did not occur in disks aged 24 hours or more. If a correction is made for the

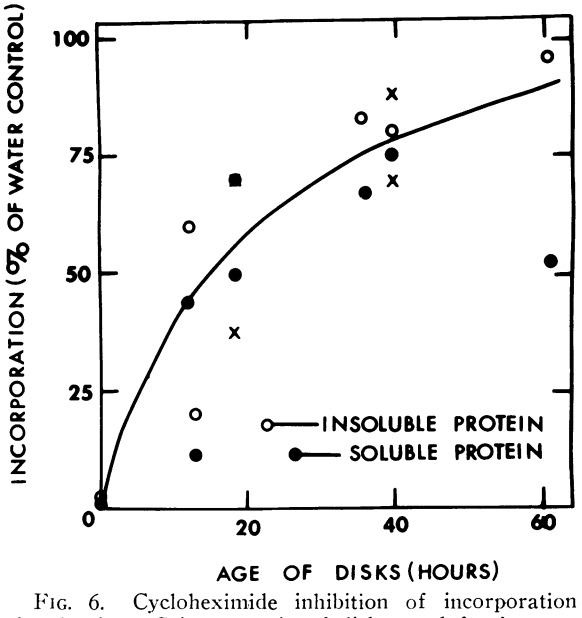

FIG. 6. Cycloheximide inhibition of incorporation of isoleucine-^{14}C into protein of disks aged for increasing periods of time. Conditions of incorporation were the same as those in legend to figure 5 except that in addition to supplying isoleucine-^{14}C in 0.5 ml of water, duplicate samples of disks of each age examined were also fed isoleucine-^{14}C in 0.5 ml of cycloheximide. Soluble and insoluble protein fractions were prepared as described under Methods. Circles are results obtained with 36 μM cycloheximide. Xs with 360 μM inhibitor. Xs above the line, insoluble protein; below the line, soluble protein. Correction for cycloheximide inhibition of uptake in fresh disks does not change the shape of the curve substantially.

Table II. *Comparison of Uptake and Incorporation of Isoleucine-^{14}C into Proteins of Disks Cultured on Water or on Cycloheximide*

Disks were cultured 12 hours on water or on 36 μM cycloheximide (10 μg/ml). Samples of 5 disks weighing 2 g original fresh weight were then exposed for 2 hours to 3.8 $\times$ 10^5 cpm of isoleucine-U-^{14}C (3 mμmoles) in the absence or presence of 36 μM cycloheximide. Soluble and insoluble protein fractions were prepared from the labeled disks as described in the text. Phenylalanine ammonia-lyase activity was measured in extracts of duplicate sets of disks taken at the end of the feeding period. Values in the table are reported per g original fresh weight of tissue.

Culture solution	Solution during isoleucine-^{14}C feeding	Uptake	Incorporation as % of uptake		Ammonia-lyase activity
			Sol. protein	Insol. protein	
		cpm $\times$ 10^{-3}			*units*
Water	Water	60	17	50	750
Water	Cycloheximide	48	9	38	650
Cycloheximide	Cycloheximide	44	8	49	75

effect on uptake by calculating incorporation as percent of uptake, then little inhibition of incorporation of the amino acid into insoluble protein was evident in disks cultured on cycloheximide or exposed to inhibitor during feeding. These data indicate that cycloheximide does not prevent activation of synthesis of insoluble protein during incubation of tissue in light. The increased incorporation probably reflects the formation and development of mitochondria (18) and plastids (32) in the aging tissue. Incorporation into soluble protein was inhibited about 50 % by cycloheximide. Culturing disks on cycloheximide for 12 hours previous to administering isoleucine-^{14}C had little further effect on the extent of inhibition. In contrast, disks cultured on inhibitor had very little enzyme compared with those cultured on water (last column, table II). Consequently most radioactivity must have been incorporated into proteins other than the lyase. This conclusion was verified by measuring the distribution of radioactivity upon ammonium sulfate fractionation of soluble proteins and upon sucrose density centrifugation of the lyase-rich fraction. Examination of the lyase fractions in the sucrose gradient indicated that they contained less than 10 % of the total radioactivity incorporated into the soluble proteins.

Discussion

The appearance of phenylalanine ammonia-lyase activity in disks of tissue cut from potato tubers has been interpreted as an induction of enzyme synthesis (33). The present study has shown that low concentrations of cycloheximide inhibit both the appearance of enzyme activity and the synthesis of proteins. These results lend further support to the hypothesis that increase in activity involves the synthesis of enzyme protein. A similar inhibition of lyase formation by cycloheximide and other inhibitors of protein and nucleic acid synthesis have been reported in a number of tissues (8, 9, 20, 30). Nitsch and Nitsch (21) described the action spectrum of the light stimulated induction.

The ability of cycloheximide to inhibit the disappearance of enzyme activity which normally occurs about 24 hours after lyase induction is initiated in the disks suggests that synthesis of proteins is also required for the degradation or inactivation of the lyase. Engelsma (9) has recently described the induction of phenylalanine ammonia-lyase synthesis in gherkin seedlings transferred from darkness to light. He observed that enzyme accumulating in the light would disappear from the seedlings if they were transferred back to darkness, but that cycloheximide could prevent the loss of activity. He concluded that *de novo* protein synthesis was required for the loss of enzyme in darkness. Hotta and Stern (14) observed that disappearance of the induced thymidine kinase in lily anthers required conditions which favored protein synthesis.

If loss of activity resulted from actual degradation of enzyme protein as in the case of classical protein turnover, then cycloheximide might act to prevent synthesis of proteolytic enzymes in the disks. Kenny (16) has demonstrated that cycloheximide inhibits the basal turnover of an inducible tyrosine transaminase in rat liver. He concluded that continuous synthesis of protein, presumably of proteolytic enzymes, was required to maintain the turnover of the transaminase.

Disappearance of lyase activity could involve inactivation rather than degradation. Specific proteins that combine stoichiometrically with enzymes to inhibit their activity have been demonstrated in other induction systems (2, 3, 25). Potato tubers seem to be a particularly rich source of inhibitor proteins. Crystalline protein inhibitors of proteolytic enzymes have been isolated from tubers (1), and their induction in the potato plant has been studied (24). Pressey (22) has isolated a protein inhibitor of invertase from potatoes. The regulation of invertase activity in tubers by the protein inhibitor (23) provides an interesting model for the

lyase system. If this type of stoichiometric inactivation were involved, then cycloheximide could maintain the enzyme level by preventing the synthesis of a protein inhibitor. Enzymatic modification of the active site of the lyase would also provide an inactivation dependent on other protein molecules.

Whatever the nature of the system responsible for disappearance of activity, be it turnover, inactivation, etc., protein synthesis appears to be required for the decline in lyase activity.

The delayed addition experiments in which low concentrations of cycloheximide were employed to inhibit enzyme synthesis without affecting loss of activity provide additional information concerning the formation of a degrading or inactivating system. If formation of lyase was stopped during the initial period of induction before the lyase concentration had reached 50 % of the maximal level, no subsequent loss of enzyme occurred. A similar stability of phenylalanine ammonia-lyase was observed when early phases of induction in sweet potato roots were inhibited with puromycin (20). If more lyase was allowed to form in the potato disks by delaying the addition of cycloheximide, then a subsequent loss of activity was observed. The more enzyme allowed to accumulate before inhibition of synthesis, the greater was the rate of disappearance. These results suggest that no inactivation of the lyase occurred during the initial phase of induction. Subsequently though, an inactivating system capable of degrading or inactivating the lyase appeared in the tissue and reached a maximal activity at about the time or shortly after a maximal lyase concentration was attained. The fact that the eventual loss of enzyme could be inhibited by high concentrations of cycloheximide suggests that the appearance of the inactivating system in the tissue involved the induction of protein synthesis. That is, cutting disks of tissue from the potato tuber induced the synthesis of the ammonia lyase. Subsequently the synthesis of an inactivating system was induced, perhaps by the ammonia-lyase itself or by a product derived from its catalytic activity.

This pattern of sequential induction is illustrated diagramatically in figure 7. The solid bar extending from 0 to 24 hours after cutting represents the period during which addition of low concentrations of cycloheximide stop further increase in enzyme activity and is considered to be the period of lyase synthesis. The bar extending from approximately 12 to 36 hours after placing the disks in the light covers the period during which disappearance of enzyme from the tissue occurs in the presence of low concentrations of cycloheximide but not in the presence of high concentrations of inhibitor. This phase is equivalent to the period of synthesis of the inactivating system. The sequential induction is similar to that described by Sussman for the induction of a galacturonic acid transferase and its inactivating system in slime molds (28). It contrasts with the inductions of invertase in sugar cane (11) and of transaminase (10) in liver where continuous turnover exists throughout the process.

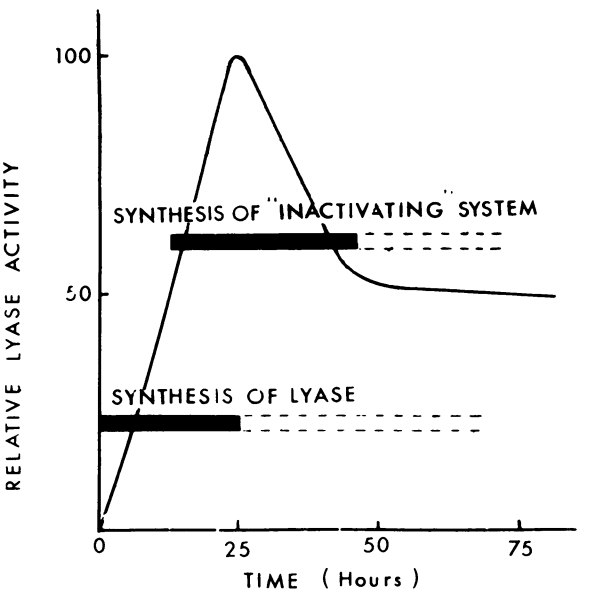

FIG. 7. Diagrammatic scheme of the sequential induction of phenylalanine ammonia-lyase and a system degrading or inactivating the lyase. Bars represent proposed periods of synthesis of the 2 components in the induction system and are superimposed on the normal pattern of change in lyase activity (solid line) which occurs during aging. Absicca represents age of disks.

Lyase synthesis is pictured as stopping at 24 hours in figure 7 because low concentrations of the inhibitor no longer influence the level of enzyme after this time. However, the increasing insensitivity to cycloheximide of general protein synthesis in aging disks complicates interpretations. If synthesis actually stops at 24 hours, then the inactivating system must eventually disappear from the tissue. Otherwise the decline in lyase activity would continue until complete loss occurred. On the other hand, a continued synthesis of lyase throughout the induction (indicated by dotted lines in fig 7) would only require that the rate of breakdown or inactivation become greater than the rate of synthesis during the period of enzyme disappearance. Oscillation in the rate of breakdown or synthesis such as that observed in other induction systems (4) could account for the peak of enzyme activity followed by a decline to a lower steady-state level where synthesis balanced degradation or inactivation. The fact that concentrations of cycloheximide up to 700 μM do not alter enzyme level once the final plateau concentration is reached, argues against this final phase of induction as a steady-state. However, an equal inhibition of synthesis and breakdown such as that observed by Kenny (16) or a complete insensitivity of aged disks to cycloheximide could account for the lack

of effect. The nature of the plateau region will only become clear when it is possible to measure synthesis or breakdown of enzyme directly by incorporation studies with radioactive amino acids.

The irreversible inhibition of lyase synthesis contrasts with the usual reversible effects of cycloheximide in other tissues (12) and with the reversible inhibition of induction of fatty acid synthetase in the potato tuber itself (31). Above 10 μM concentration, cycloheximide could produce a complete inhibition of lyase synthesis whereas cycloheximide inhibitions of 80 to 90 % are maximal in reversible systems. Grollman (12) has suggested that the irreversibility of certain cycloheximide derivatives and glutarimide analogues is conferred by secondary binding sites not associated with those involved in inhibition of protein synthesis. Cycloheximide bound irreversibly to ribosomal structures could prevent lyase synthesis even though the external source of inhibitor had long been washed away. In this instance, the irreversibility of binding would involve secondary sites specific to the ribosomes rather than to the inhibitor.

The large increase in rate of incorporation of amino acids into protein which occurs during aging of potato disks parallels changes in lyase activity. Although the apparent increase in rate could reflect a corresponding shrinkage of metabolic pools (13), the change has been considered to result from the activation of protein synthesis in the disks (7, 31). The activation of protein synthesis in aging carrot disks initially involves the aggregation of inactive ribosomes to form active polysomes, the aggregation being completed within a few hours after the disks are prepared (17). An analogous formation of active polysomes also occurs during the germination of seeds (19). Marcus and Feeley (19) have shown that cycloheximide will inhibit the *in vitro* aggregation of inactive monosomes to active polysomes in extracts of wheat embryoes. If cycloheximide were to inhibit formation of polysomes in freshly cut tuber tissue, then the inhibitory effect could occur before a net synthesis of lyase was detected. The presence of cycloheximide would not be necessary during the subsequent period of lyase synthesis.

Cycloheximide inhibition in potato disks showed a considerable degree of specificity. The 10-fold difference in concentration between that needed to stop lyase synthesis and lyase disappearance has already been mentioned. Under conditions where lyase synthesis was inhibited at least 90 % by culturing disks on 10 μM cycloheximide, little apparent inhibition of the activation of general protein synthesis was observed. In contrast, puromycin was shown to prevent any increase in rate of incorporation of leucine into protein during aging of potato disks (7). The selectivity of cycloheximide inhibition makes it a promising tool for further investigation of ammonia-lyase induction in tuber disks.

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