The Role of Light and Nitrate in the Induction of Nitrate Reductase in Radish Cotyledons and Maize Seedlings[1, 2]

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Although the induction of enzyme synthesis has been extensively studied in microbial systems in recent years (21, 31), comparable studies with higher plants have received less attention. Substrate induction (or enhanced activity) of enzymes in higher plants has been reported for nitrate reductase, glycolic acid oxidase, alcohol dehydrogenase, and IAA oxidase (1, 8, 12, 14, 15, 18, 22, 36, 37). However, these studies have not achieved the level of sophistication of the microbial systems in which induction has been shown to involve de novo protein synthesis requiring messenger and transfer RNA, ribosomes, energy source, and amino acids.

In initial studies on nitrate reductase in higher plants, attempts by Evans and Nason (10) to show the adaptive (inductive) nature of the enzyme were inconclusive. Later, Candela et al. (7) found that nitrate fertilization increased the level of nitrate reductase in enzyme extracts from cauliflowers. Furthermore, molybdenum deficiency caused a decrease in nitrate reductase level and addition of molybdenum restored nitrate reducing capacity. Recently, Afridi and Hewitt (2, 18) confirmed the requirement for both nitrate and molybdenum for enzyme induction in cauliflower leaves. Comparable studies have been made by Champigny (8) with maize tissue.

Morris and Syrett (27) recently showed that nitrate was necessary for the induction of nitrate reductase in *Chlorella* and that ammonia prevented this induction. Thus, this system has some similarity to other microbial systems in which the end product prevents induction. Morton (28) and Kinsky (23) demonstrated the requirement for nitrate in the induction of the enzyme nitrate reductase in a variety of microorganisms, and in these instances, induction was prevented by the presence of ammonium ions.

As well as being induced by substrate, it has frequently been indicated that light may also control nitrate reductase activity. Evans and Nason (10) recorded that the level of nitrate reductase was low in extracts from plants immediately following a dark period and suggested that a limited availability of photosynthetically reduced pyridine nucleotide caused the decreased nitrate reduction. In contrast, other studies (7, 14, 16) indicated that enzyme inactivation or destruction occurred when the plants were subjected to dark or shade treatment. Hageman and Flesher (14) demonstrated that extracts prepared from green plants that had been exposed to increasing periods of darkness showed a progressive decrease in nitrate reducing ability. Nitrate reductase activity was high in extracts prepared from plants which had been returned to the light. Since an unlimited amount of reduced pyridine nucleotide was always present in the assay medium, it was suggested that light had a role in the induction of nitrate reductase (14) rather than in the generation of reduced pyridine nucleotides.

This present work attempts to : A) elucidate the effect of substrate, light, and temperature on the induction of nitrate reductase in radish cotyledons and corn seedlings, and B) demonstrate, by the use of appropriate inhibitors, whether induction of the enzyme was dependent on de novo protein synthesis.

Materials and Methods

Radish Seedlings. Cotyledons or intact seedlings were obtained by sowing seeds (*Raphanus sativus* L. var. Cherry Belle) on 4 layers of cheesecloth in 3-quart Pyrex utility dishes. After addition of 150 ml of dilute salt solution containing K_2SO_4, 1.25 mM; $Ca(H_2PO_4)_2$, 0.25 mM; and $MgSO_4$, 1.0 mM (adjusted to pH 7.4 with KOH), the dishes were sealed with Saran Wrap (Dow Chemical Company, Midland, Michigan). Twenty small aeration holes were punched in the Saran Wrap prior to placing the dish in the controlled environment room (16-hr photoperiod, 2000 ft-c at 20° and 8-hour dark period at 15°). Etiolated seedlings were produced by placing the dishes in a dark germinator at 27°. Most of the work was done with 5-day-old material; exceptions will be detailed later.

With the intact seedlings, induction was initiated by the addition of appropriate amounts of substrate solution (KNO_3) to the Pyrex dish; nonsubstrate solutions for controls were added in the same manner. Cotyledons were removed from this tissue at various time intervals for assay. In the excised tissue study, cotyledons were removed and immersed in 25 ml of appropriate medium (substrate, inhibitors, or control) contained in Erlenmeyer flasks. Samples for assay were taken as described.

Corn Seedlings. One hundred corn seeds (*Zea*

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mays L. var., Hy2 × Oh7) were planted embryo down on a layer (2 liters) of Krum, a commercial soil conditioner (Ryolex Corporation, Champaign, Illinois), and covered with a second layer (0.8 liter). The containers were 10-quart plastic pans with perforated bottoms. The Krum was saturated by subirrigation with a solution of $CaCl_2$ 10^{-4} M. Containers were covered with perforated Saran Wrap and placed in a dark germinator at 27° and 90% relative humidity. To obtain the light grown material, the pans were transferred when the emergence of the coleoptiles was complete (3 days) to growth chambers (3000–4000 ft-c, 15 hr light at 27° and 9 hr dark at 23°, with 65% relative humidity at all times). From the fourth day on, the seedlings were subirrigated, as needed, with nutrient media of the following composition: K_2SO_4, 0.25 mM; KH_2PO_4, 0.5 mM; $MgSO_4$, 0.75 mM; $CaSO_4$, 1.0 mM; $(NH_4)_2SO_4$, 2.5 mM; NH_4Cl, 5.0 mM; Fe (as Sequestrene 330, Geigy Agricultural Chemical Company, Yonkers, New York), 0.3 mM and micronutrients (19) and the pH adjusted to 4.0 with H_2SO_4. Since 7 to 11-day-old material was used for the induction studies, it was found necessary to add ammonium salts to obtain material of reasonable induction potential.

For the induction studies, the seedlings were excised at ground level and placed in 430 ml of medium containing the desired level of substrate, inhibitor, or nonsubstrate salts (control). (Inhibitors were always added 30 min prior to substrate.) The medium was held in 3-quart Pyrex dishes. A uniformily perforated plate (200 holes, 5 mm diam) of Plexiglas (2 mm inch thick), cut to fit the inside dimensions of the Pyrex dish, was used to submerge the excised seedlings in the medium. Thin Plexiglas strips (2 mm × 10 mm) glued to 1 side of the plate provided 6 compartments to keep the seedling material evenly distributed over the bottom of the Pyrex dish. Normally, 3 seedlings were placed in each compartment. The dishes were placed on a shaker (100 cycles/min) in a controlled environment chamber for the induction of the enzyme.

Extraction and Assay. The tissue, radish cotyledons or expanded corn leaves (the nongreen tissue was discarded prior to extraction), was removed at desired intervals from the incubation medium, rinsed twice with deionized water, and blotted dry. The tissue was minced with a razor blade and transferred to the blendor cup (MSE microhomogenizer, Instrumentation Associates Inc., 17 West 60th Street, New York). Normally, 10 radish seedlings or 20 radish cotyledons and leaves from 6 corn seedlings were used for a sample and duplicate or triplicate samples were taken from each treatment. The tissue was homogenized in an extraction medium in a 4:1 or 6:1 ratio (v/w) for 2 minutes at maximum speed. The extraction medium was a mixture of 0.01 M potassium phosphate, 0.01 M EDTA and 5×10^{-3} M cysteine adjusted to pH 8.2 with HCl for radish tissue and 0.025 M potassium phosphate, 0.005 M EDTA and 10^{-2} M cysteine adjusted to pH 8.8 with HCl for corn tissue. The homogenate was strained through 4 layers of cheesecloth into plastic centrifuge tubes and centrifuged at 15,000 × *g* for 15 minutes. The supernatant was saved and used for assay of enzyme (14), protein (24), and nitrate (40).

Inhibitor Studies. In order to determine if the induction involved or was dependent upon protein synthesis, use was made of inhibitors known to function specifically in preventing polypeptide formation. The inhibitors used were as follows: Actinomycin D, which associates with the guanine residues of DNA and thus prevents DNA-dependent synthesis of RNA by RNA polymerase (13) and, in turn, protein synthesis. Puromycin has been shown to compete with the transfer-RNA-amino acid complex and thus prevents polypeptide formation (30). Chloramphenicol has been shown to prevent protein synthesis and at the same time stimulate RNA synthesis in bacterial systems (6, 35, 39). While chloramphenicol has been shown to inhibit protein synthesis in plant systems (26, 34), the concentrations were considerably higher than those used in microbial systems. Since it has been demonstrated recently that chloramphenicol prevents mitochondrial oxidative phosphorylation (17), its mode of action in preventing protein synthesis in plants is somewhat dubious.

It has been shown that 8-azaguanine and 5-fluorouracil inhibit protein synthesis in bacterial and plant systems (4, 9, 33), presumably due to the formation of nonfunctional or spurious RNA.

Ethionine and *p*-fluorophenylalanine, as well as other amino acid analogues, prevent protein synthesis in many systems; however, the extent of their action is variable (32).

In order to study the effect of these inhibitors on the induction of nitrate reductase by nitrate, cotyledons or leaves were detached from nitrate deficient radish or corn seedlings and floated for 30 minutes in solutions of the various inhibitors adjusted to pH 7.4. After this 30-minute preincubation period, nitrate was added to the bathing solution to a final concentration of 10^{-2} M for radish cotyledon and 10^{-1} M for corn leaves. Incubation in the presence of inhibitor and nitrate was continued for 3 hours, after which time the materials were removed and extracts prepared for assay.

Experimental Results

Nitrate and Light. Initial experiments with intact radish seedlings demonstrated (table 1A) that of the 3 salt solutions [KCl, $(NH_4)_2SO_4$, and KNO_3] used, only the KNO_3 treatment resulted in enzyme induction. There was a progressive increase in nitrate reductase activity with time; however, a plot of this data suggests an initial lag in induction. Subsequent experiments (table 1B) confirmed the occurrence of a 30-minute lag period.

Table I. *Requirement for Nitrate for the Induction of Nitrate Reductase*

Induction media Induction time (hr)	A. Intact 5-day-old radish seedlings (22°–2000 ft-c) KCl(10^{-2} M)	 $(NH_4)_2SO_4$(10^{-2} M) Activity (NR*)	 KNO_3(10^{-3} M)
0	0.017	0.017	0.017
1	0.011	0.010	0.041
2	0.015	0.020	0.092
4	0.010	0.021	0.174

Induction media (KNO_3) Induction time (min)	B. Excised radish cotyledons (30°–200 ft-c) 10^{-2} M NR	 NO_3-N**	 5×10^{-2} M NR	 NO_3-N**	 10^{-1} M NR	 NO_3-N**
0	0.11	0.0	0.11	0.0	0.11	0.0
15	0.15	8.3	0.10	75	0.11	140
30	0.15	25.0	0.12	166	0.12	315
45	0.21	33.3	0.19	233	0.15	458
60	0.26	50.0	0.27	291	0.19	550
120	0.32	100	0.28	432	0.23	990

Induction media	C. Excised 11-day-old corn seedlings (35°–3500 ft-c) KNO_2(10^{-1} M) NR	 NO_3-N**	 NH_4NO_3(10^{-1} M) NR	 NO_3-N**	 KNO_3(10^{-1} M) NR	 NO_3-N**
Induction time (4 hr)	0.0	...	0.84	296	0.77	377

* Nitrate reductase activity as μmoles No^-_2/mg protein per hr.
** NO_3N as μg N/g fresh weight.

The nitrate content of the excised radish cotyledons was roughly proportional to the concentration of nitrate of the induction media and the time of induction (table IB). The optimum nitrate concentration of the induction media used for the excised radish cotyledons was 10^{-2} M. Higher concentrations of inducer (KNO_3 at 5×10^{-2} and 10^{-1} M) suppressed the induction, as indicated by the relatively small increase in nitrate reductase between 60 and 120 minutes.

Initial (0 time) values and rate of induction varied from experiment to experiment. These variations are attributed to the metabolic status of the plant at time of experimentation, even though the seedlings were grown under standardized conditions. This is confirmed not only with variable induction but by variable nitrate uptake by the tissue. The third set of data (table IC) shows that nitrite did not induce nitrate reductase in the corn seedlings, which is in agreement with the findings of Afridi and Hewitt (2) with cauliflower leaves. There is no indication that the presence of ammonium ions in the induction medium suppressed induction of nitrate reductase, which is in contrast to the findings of Morris and Syrett (27) with *Chlorella*. Furthermore, the corn seedlings had been grown on a nutrient solution containing ammonium ions (10 mM, methods), prior to induction. A higher nitrate content in the induction medium was required for optimum induction in corn than in radish cotyledons (table IB, C). This is attributable to the differential in rates of nitrate uptake by radish cotyledons and corn seedlings.

Radish seedlings were grown on the normal light regime (16 hr light–8 hr dark) for 4 days and nitrate supplied at the beginning of the last dark period. Cotyledons removed at the end of this dark period contained essentially no nitrate reductase activity. Within 1 hour after the initiation of the light phase of the fifth day, appreciable levels of activity were obtained. The activity continued to increase with continued illumination over a 4-hour period; however, when the lights were turned off, the activity decreased to the original level (fig 1). This cycling

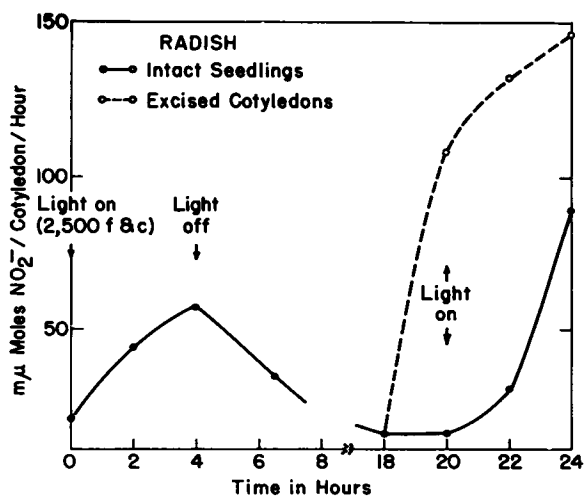

FIG. 1. The effect of light and dark environment on induction of nitrate reductase in intact radish seedlings and on excised radish cotyledons.

Table II. *Nitrate Reductase Levels in Relation to Nitrate Present in Intact Radish Seedlings and Excised Cotyledons under Varying Light and Dark Treatment*

Seedlings were irrigated with, and cotyledons floated in 5×10^{-2} M KNO_3.

Cotyledons from intact seedlings after:	μg NO_3/N Cotyledon	Nitrate reductase mμmoles KNO_2/per mg prot per hr
10 hr dark	1.65	22
12 hr dark	1.50	13
12 hr dark + 3 hr light	4.65	98
Excised cotyledons* after		
0 hr	1.65	22
2 hr dark	9.50	96
2 hr dark + 3 hr light	8.60	195

* From seedlings supplied with nitrate but previously exposed to 10 hours dark.

of activity with fluctuating illumination was consistent with previous findings (14) that suggested the requirement of both light and nitrate for induction. That light per se was not essential for induction was established when cotyledons were removed (fig 1, 18 hr) and placed in KNO_3 (0.01 M) induction media. A high level of enzyme was extractable from these cotyledons after 2 hours of dark incubation.

These findings suggest that the light effect is indirect and that the light is involved in the movement of the substrate into the cotyledon of these seedlings. Subsequent experiments confirmed this suggestion (table II) and demonstrated that the induction was dependent upon an adequate level of nitrate in the tissue.

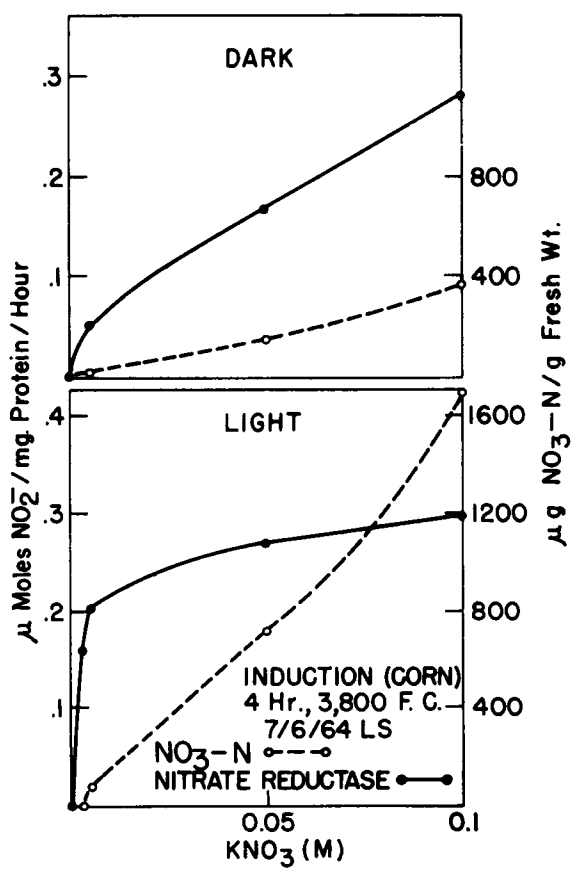

FIG. 2. The effect of light and dark environment and increasing levels of nitrate on uptake of nitrate and induction of nitrate reductase in excised corn seedlings.

Other experiments with corn seedlings clarified the interaction of light, temperature, and nitrate concentration of the induction media on the induction of nitrate reductase. Initially, all attempts to induce nitrate reductase in the dark with corn seedlings failed; however, in these instances, the concentration of nitrate in the induction media was 0.01 M. Adequate induction was obtained in the dark when the nitrate concentration of the induction media was increased.

Figure 2 illustrates the effect of light and dark environment and increasing levels of nitrate on the uptake of nitrate by the tissue as well as enzyme induction. In the dark, there was essentially a linear increase in nitrate content and enzyme induction in the tissue, with increased nitrate content of the induction media. In the light, the uptake of nitrate was greater (4-fold) than in the dark and proportional to the amount of nitrate supplied. Enzyme induction was linear only up to 10^{-3} M KNO_3 supplied. At higher concentrations of substrate, only a slight increase was noted (fig 2). These data suggest that excess nitrate may limit the induction process.

The view that light exerts an indirect effect on the induction of nitrate reduction by increasing the uptake of nitrate from the induction media is further supported by the data presented in figure 3. There is no known reason for the low uptake of nitrate and lower induction at 2800 ft-c (28°). Since other experiments at 28° showed a progressive increase in both nitrate and enzyme with increasing light intensity, this set of data was selected because it emphasizes the dependence of induction upon uptake of nitrate.

The fact that nitrate reductase was induced in the green cotyledons or corn seedlings supplied (induced) with nitrate in the dark did not completely eliminate the possibility that light was necessary for induction. Thus, it is conceivable that the re-

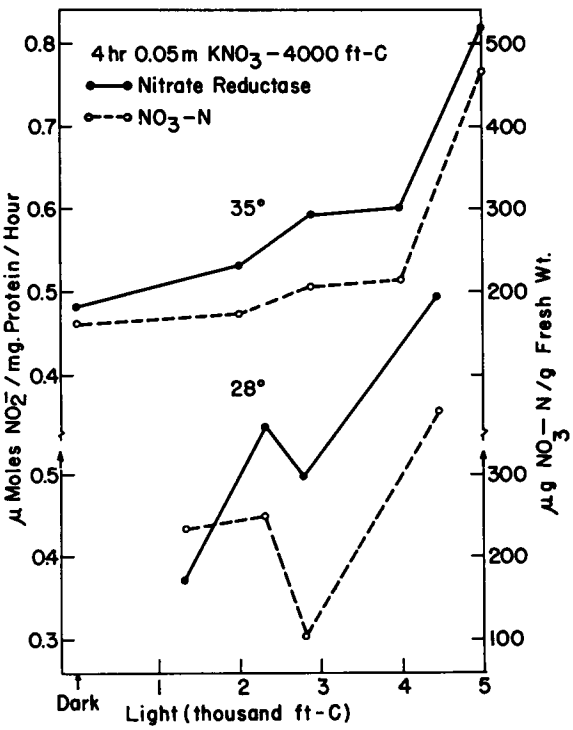

FIG. 3. The influence of light intensity and temperature on nitrate uptake and induction of nitrate reductase in excised corn seedlings.

quired photo-response had already occurred in the green tissue prior to dark induction. This postulate was tested by using radish cotyledons from seedlings grown in complete darkness. Experimental details and results for these experiments are given in table III. Although the amount of enzyme induced was small, these results show that induction is not directly dependent upon light. The low level of induction achieved in these experiments may be attributed either to a deficiency of substrates required for enzyme synthesis (amino acids, nucleotides, ATP, etc.) or to a slow or retarded transport of synthetic or inducer (NO^-_3) substrates to the site required for synthesis. Although the nitrate present in the tissue should be adequate for a high level of induction, nothing is known of its localization within the tissue. Not even a trace of activity was detectable in the control (KCl or water as inducer media) material (etiolated cotyledons).

Temperature Effects. Initial work showed that the induction of nitrate reductase in excised radish cotyledons was temperature dependent, with an optimum at 31° for a 2- to 4-hour induction period. Comparable results were obtained with excised corn seedlings (fig 4), except that the optimum temperature was 38°. The higher optimum temperature for corn is consistent with the normal growth response of these 2 species to temperature. Nitrate uptake was also temperature dependent over the entire range tested (fig 4), and closely paralleled enzyme induction until the temperature reached a lethal point for the induction process.

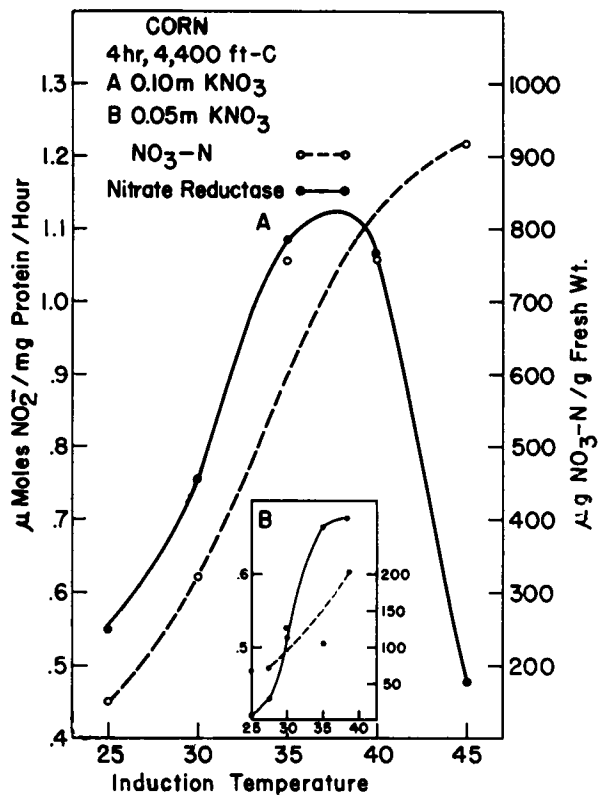

FIG. 4. The influence of increasing temperature on the uptake of nitrate and induction of nitrate reductase in excised corn seedlings (insert B provides evidence that 38° is the optimum temperature for induction of the enzyme).

Table III. *Induction of Nitrate Reductase and Nitrate Content of Excised Etiolated Radish Cotyledons Induced in the Dark (3 hr)*

		µg NO_3–N Cotyledon	mµmoles NO^-_2/per mg prot per hr
Control (KCl, 0.05 M or water)	(a)	0.0	0.0
	(b)	0.0	0.0
	(c)	0.0	0.0
Induced (KNO_3), 0.05 M	(a)	3.7	23.0
	(b)	2.5	16.3
	(c)	1.4	27.0

Cursory examination of the data presented leads to the conclusion that nitrate reductase induction is primarily controlled by the nitrate content of the tissue. This can be but partially true because no numerical correlation between nitrate content and induction is evident from experiment to experiment. Thus, while nitrate must be present for induction, other factors such as metabolic activity of the tissue are also important. The data of figure 4 support, but do not prove conclusively, that protein synthesis is involved in induction.

An Arrhenius constant, μ, for the induction process for corn was estimated at 14,700 calories (data of fig 4B) and 14,000 calories (fig 4A) for the 25 to 35° temperature range. Comparable calculations with the radish data gave a value of 19,000 calories for the 20 to 30° temperature range. These values are comparable with those obtained for similar complex physiological processes (11).

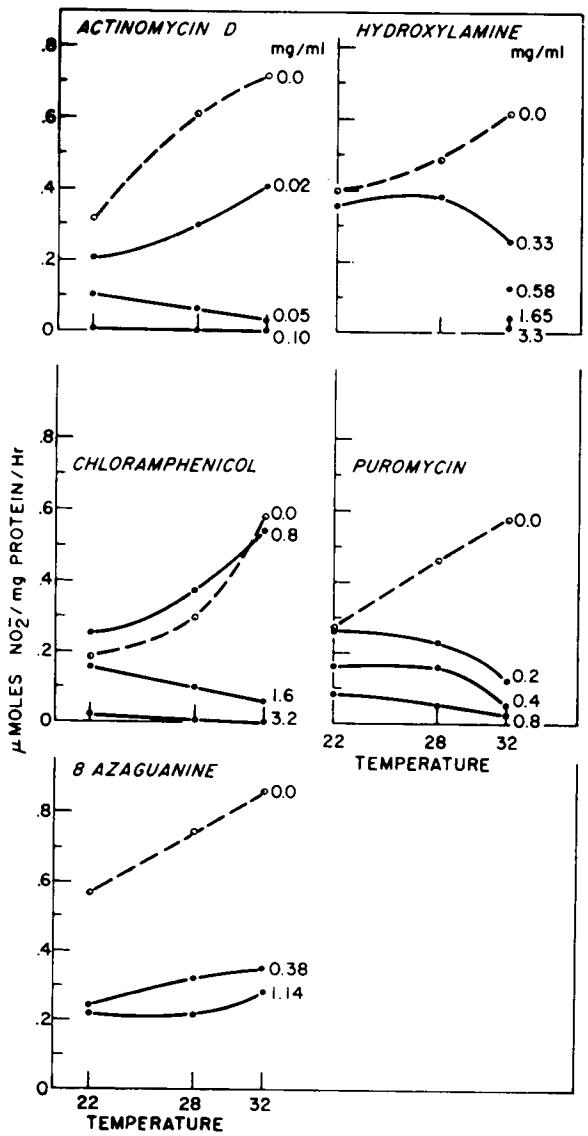

FIG. 5. The effect of various concentrations of 5 inhibitors at 3 temperatures on the induction of nitrate reductase in excised radish cotyledons.

Inhibitor Studies. The effect of various concentrations of 5 inhibitors at 3 temperatures on the induction of nitrate reductase in radish cotyledons is shown in figure 5. Comparable studies with 5-fluorouracil (0.06 mg/ml), *p*-fluorophenylalanine (9.0 mg/ml), and ethionine (1.6 mg/ml) at 32° for 3 hours gave no inhibitory effects.

Although it is possible that the inhibitors were indirectly inhibiting the induction by restricting nitrate uptake, this is most doubtful. Even chloramphenicol, which has been implicated in restricting ion uptake by plants (34), failed to influence P^{32} phosphate incorporation (unpublished data) in similar studies with radish cotyledons. Corn seedlings treated with chloramphenicol (6.4 mg/ml, solubilized with 95% ethyl alcohol, 35°, 4 hr) during induction showed a 66% inhibition of nitrate reductase, but only a 12% reduction in nitrate content. However, the location of the nitrate within the tissue was not determined.

Discussion

Substrate (nitrate) induction of nitrate reductase was demonstrated in radish cotyledons and leaves from corn seedlings. Induction was suppressed by Actinomycin D, 8-azaguanine, puromycin, and chloramphenicol. Although the specific action of these inhibitors vary, they all prevent protein synthesis. The fact that induction could be impeded by these inhibitors indicates that the production of nitrate reductase in response to substrate requires de novo synthesis rather than activation of some existing protein or proenzyme. The temperature dependence of the induction also supports the operation of a synthetic process rather than a simple activation.

The failure of 5-fluorouracil and the amino acid analogs to prevent induction is inconsistent with de novo synthesis during induction. These failures may be attributed to one of several factors; namely, penetration, enzyme specificity, time, and endogenous pool size. The amino acid analogs may have been incorporated at a nonactive site, thereby not influencing normal catalytic properties (32).

Ideally, the demonstration of incorporation of labeled amino acids throughout the newly synthesized protein would be the most conclusive evidence for de novo synthesis. However, all attempts to demonstrate such incorporation of labeled amino acids into the induced nitrate reductase were inconclusive. These inconsistent results were attributed to the small amount of enzyme present at the end of the induction period and the lack of a suitable method of purification of this very labile enzyme.

It is concluded that the role of light in the induction process is indirect in that it influences nitrate mobilization. Support for this statement is derived

from the observation that induction is related to the nitrate content of the tissue and that the amount of nitrate in the tissue is altered by the nitrate content of the induction media, light, and temperature (fig 2, 3, and 4). Furthermore, induction was demonstrated in etiolated radish cotyledons in the dark. Although light could have enhanced transpiration, and thereby nitrate content in the intact radish seedlings (fig 1), this cannot explain the results of later experiments (fig 3) where corn seedlings were submerged in the induction media during induction. Thus, it would appear that light has a more direct (metabolic) effect on the uptake of nitrate. In 1856, Bineau (5) suggested that light influenced nitrate uptake by alteration of membrane permeability. Later, Warburg and Negelein (38) also postulated that light functions in nitrate utilization in *Chlorella* by increasing permeability, and hence nitrate availability. Other workers (20, 25) have shown that light increases permeability of cell membranes to ions. Recently, Nagai and Tazawa (29) have shown that light alters the resting electro-potential of cell membranes and that this change was associated with ion uptake. They (29) divided the effect of light on ion uptake into 2 processes: A) movement across the plasmalemma, which is controlled by the potential gradient, and B) movement across the cytoplasmic layer and tonoplast, which is controlled by photosynthesis. Even though the rate of induction of nitrate reductase may be increased by light-enhanced metabolism, this does not provide evidence for an absolute and direct requirement for light for the induction process.

An unexplained anomaly of the relation of light and nitrate reductase is the observation that the level of nitrate reductase is low in plants grown under shaded conditions even though the nitrate content is high (16). It is possible that the nitrate which is accumulated under shaded conditions is retained in the vacuoles and in this location cannot function as an inducer. Alternatively, under shaded conditions, inorganic nitrogen metabolism may be impaired due to a limited availability of photosynthetically produced electron donors, and thus intermediate products may accumulate and repress further induction. This type of end-product repression has been shown in various enzyme systems in bacteria (21) and apparently functions in *Chlorella* (27) and *Neurospora* (23) where ammonia was found to prevent (repress) the induction of nitrate reductase by nitrate.

Attempts to demonstrate repression of substrate induction of nitrate reductase in radish cotyledons or corn seedlings with various end products (nitrite, ammonium ion, mixed amino acids, glutamine, and aspargine) were not successful. Although hydroxylamine will prevent induction of the enzyme (fig 5), it interferes with the reduction of nitrate, prevents incorporation of C^{14} leucine into protein in general, and interferes with normal RNA metabolism and thus cannot be considered a specific repressor.

Summary

Studies were made to determine the effect of substrate (nitrate), light, and inhibitors of protein synthesis on the induction of nitrate reductase in radish (*Raphanus sativus* L., var. Cherry Belle) cotyledons and corn (*Zea mays* L., var. Hy2 ×Oh7) seedlings.

Induction of nitrate reductase is dependent upon the presence of nitrate in the tissue; neither nitrite nor ammonia would induce the enzyme. The presence of ammonium ion in the media did not inhibit induction of nitrate reductase. Induction of nitrate reductase is roughly proportional to the amount of nitrate present in the tissue. A higher nitrate content in the induction medium is required for optimum induction of enzyme in corn than in radish cotyledon. This difference is attributable to differential rates of nitrate uptake.

Although the presence of light caused induction of nitrate reductase in radish cotyledons and increased light intensity resulted in increased induction in corn, the role of light was indirect. This was verified by the observation that induction did occur when both seedling materials were grown and induced in complete darkness. The effect of light on induction is attributed to enhanced nitrate uptake as a result of increased permeability of the tissue.

Induction of nitrate reductase is temperature dependent, and a part of this dependence is based on the increase in nitrate content of the tissue with increase in temperature during induction. Maximum induction temperatures were 31° and 38° for radish cotyledons and corn seedlings, respectively.

Inhibition of induction of nitrate reductase by actinomycin D, chloramphenicol and puromycin provide evidence that de novo synthesis is involved.

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