CHAPTER IV

CHAPTER IV

INDUCTION OF NITRATE REDUCTASE IN THE LEAVES OF RICE

INTRODUCTION

The biosynthesis of nitrate reductase (NADH) (NADH : nitrate oxidoreductase, E.C. 1.6.6.1) is subject to repression, induction and derepression in an independent manner. Induction by nitrate is almost universal (Hewitt et al., 1976). Light is another factor whose mode of operation on the induction of nitrate reductase by nitrate is complex and multiple (Beevers and Hageman, 1969 and Hewitt, 1975). Whether the role of light in the nitrate metabolism resides in its effect on nitrate reductase level per se, availability of nitrate, the generation of reductant, or a combination of all is still not well understood. However, it was recognised that the stimulating effect of light on induction of nitrate reductase may be associated with the increased protein synthesis occurring after illumination. Travis et al. (1970) reported that nitrate reductase induction was positively correlated with the level of the cytoplasmic polyribosomes. Travis and Key (1971) suggested that light per se is not required for nitrate reductase induction in leaves of dark grown seedlings but is needed to reactivate the protein synthetic apparatus of older leaves. The observations of

Travis and Key (1971), Aslam et al. (1973), Sluiters-Scholten (1975), Aslam and Oaks (1975), and Sasakawa and Yammamoto (1977) that exogenously supplied sucrose or glucose stimulated induction of nitrate reductase in darkness suggests that light per se is not an absolute requirement and one of the primary functions of light may be to furnish photosynthathes. Aslam et al. (1976) suggested that nitrate accumulates in a storage pool when grown in darkness and that the transfer of this nitrate to the metabolic pool is mediated by light but not by glucose. Gandhi and Naik (1974) have shown that synthesis of nitrate reductase is mediated via photosynthetic reactions. Kannangara and Woolhouse (1967) found that in addition to light and nitrate the presence of carbon dioxide was also necessary for the formation of nitrate reductase. Canvin and Atkin (1974) showed that nitrate assimilation was greatly reduced when carbon dioxide was removed from the air. Sawhney et al. (1972) investigated the synthesis of nitrate to nitrite in ethyl methane sulphonate (EMS) induced albino mutants of barley and found the synthesis of nitrate reductase to be intimately linked with photosynthesis involving chlorophyll. Jones and Sheard (1975) showed that nitrate reductase induction in etiolated pea terminal buds was increased by 5-min exposure to red light than by similar exposure to blue or far red light. Sawhney et al. (1978) assumed that a regulatory mechanism must exist in leaves which shuts of nitrate reduction immediately when light is

extinguished, so that the accumulation of toxic amounts of nitrite which can only be reduced by photosynthetic reactions is avoided. They proposed that this regulation functions through mitochrondrial respiration which operates in the dark, but is inhibited in light because of increased cytoplasmic adenylate change due to photosynthesis. According to these authors light, A.T.P. and mitochondrial respiration together play a very elegant mechanism in the reduction of nitrate in green leaves. Experiments conducted by Duke and Duke (1979) in Zea mays seedlings lacking chlorophyll due to chemical treatment (fluridone) or genetic lesions indicate that photosynthetic pigments are only secondarily involved in nitrate reductase induction.

Borriss (1967) discovered the effect of cytokinins on nitrate reductase. Roth-Bejerano, and Lips (1970) have reported that in the case of tobacco leaves light requirement for induction of nitrate reductase could be replaced by appropriate concentrations of gibberellic acid and kinetin in the dark. Chantarotwong and Huffaker (1971) observed a greater polyribosomal content in kinetin treated tissue. Rijven and Parkash (1971) and Kendre et al. (1971) working on cotyledons of fenugreek and Agrostemma githago reported enhancement of nitrate reductase with kinetin.

Repression of amino acids has been reported in yeasts (Sims et al., 1968), Fungi (Cove, 1966), green algae (Losada et al., 1970) and in several higher plants (Hewitt et al.,

1976). Some amino acids appear to derepress nitrate reductase (Rigano, 1971). Choudary and Rao (1976) reported that a wide range of D-amino acids induced the synthesis of nitrate reductase in yeast. Radin (1975) observed induction with certain amino acids and suggested that the effect of amino acids on induction appeared to be independent of nitrate uptake.

The effect of inhibitors of RNA and protein synthesis has been studied by several workers (Schrader et al., 1967, Stewart, 1968; Sawhney and Naik, 1972; Jackson et al., 1973; Sorger and Davies, 1973; Choudary and Rao, 1976 and Sihag et al., 1979). Beevers et al. (1965) earlier reported chloramphenicol to be relatively inaffective in inhibiting induction in radish cotyledons and corn seedlings unless used at higher concentrations. Rao and Rains (1976) have shown that cycloheximide inhibited nitrate absorption and Stewart (1968) has reported inhibition of nitrate reductase synthesis by cycloheximide in Lemna minor. Much of the work using inhibitors has shown that it interferes with protein synthesis after the production of mRNA. Actinomycin D an inhibitor of protein synthesis if added after the inducer prevents the synthesis of only a part of the inducible enzyme. Rijven and Parkash (1971) reported that inhibitors of RNA and protein synthesis suppressed the induction of nitrate reductase in cotyledons of fenugreek.

The object of the present study was to elucidate the

role of light in the induction of nitrate reductase by the inducer nitrate i.e. to see whether light affects the uptake of nitrate and/or induction. Investigations were also conducted to study the effect of kinetin on the activity of nitrate reductase. To examine whether the induction of nitrate reductase in *in vitro* conditions is mediated through the transcription and translation processes the effect of inhibitors of protein synthesis such as actinomycin D and cycloheximide on the induction of the enzyme was studied. Attempts were also made to find out whether selected amino acids could induce nitrate reductase in rice leaves and the results are presented below.

MATERIALS AND METHODS

Seeds were germinated as described in Chapter I, but in complete darkness for 9 days. They were supplied with 25 mM KNO_3 or distilled water. About fifty 1st leaves (cut into 3 cm pieces) excised from these etiolated seedlings raised in the presence of 25 mM KNO_3 were used in experiments I and II. Similarly about 50 first leaves (cut into 3 cms) excised from etiolated seedlings grown in water were used for all the other experiments. All manipulations and operations were conducted under green safe light.

I. Effect of light on the induction of nitrate reductase in etiolated rice leaves.

1. Leaf tissues were incubated at 28±1 °C on 50 ml of

water and kept in darkness for 12 and 24 hours respectively.

2. Leaf tissues were floated on 50 ml of water and exposed to light at an intensity of 3000 lux for a duration of 12 and 24 hours.

3. Leaf tissues were transferred to a solution containing 50 ml of 100 mM KNO_3 and exposed to light at the same intensity and duration as in the above experiment.

II. Effect of glucose on the induction of nitrate reductase

1. Leaf tissues were floated on 50 ml of 100 mM glucose and incubated in the dark for 12 and 24 hours.

2. Leaf tissues were floated on 50 ml of 100 mM glucose in 100 mM KNO_3 and kept in darkness of 12 and 24 hours respectively.

III. The influence of varying concentrations of exogenous nitrate on the induction of nitrate reductase in the presence of light.

1. Leaf tissues were floated on 50 ml of KNO_3 solution, concentrations of which ranged from 20-100 mM in the presence of light at an intensity of 3000 lux for a duration of 3, 6, 12 and 24 hours.

IV. Effect of varying concentrations of KNO_3 and glucose on the induction of nitrate reductase.

Leaf tissues were incubated at 28±1°C in 50 ml of

1. 20 mM KNO_3 in 50 mM glucose.
2. 25 mM KNO_3 in 50 mM glucose.
3. 25 mM KNO_3 in 100 mM glucose.
4. 50 mM KNO_3 in 100 mM glucose.
5. 100 mM KNO_3 in 100 mM glucose.
6. 100 mM KNO_3 in 150 mM glucose.
7. 100 mM KNO_3 in 200 mM glucose.

The operations were carried out under green safe light. The sets were kept in darkness for a duration of 3, 6, 12 and 24 hours.

V. Effect of KNO_3 and sucrose on the induction of nitrate reductase.

Leaf tissues were treated with 50 ml of 100 mM KNO_3 and 150 mM sucrose and incubated in the dark for a duration of 3, 6, 12 and 24 hours.

VI. Influence of ATP on the induction of nitrate reductase.

Leaf tissues were floated on 50 ml of 5 mM ATP and kept in darkness for 3, 6, 12 and 24 hours.

VII. Effect of kinetin on the induction of nitrate reductase.

Leaf tissues were floated on 50 ml of kinetin at the

following concentrations 5, 10 and 20 mg/l and kept in darkness for 12 hours.

1) In the absence of the inducer KNO_3.

2) In the presence of the inducer KNO_3 at a concentration of 100 mM.

VIII. Induction of nitrate reductase by selected D- and DL-amino acids.

Leaf tissues were supplied with 50 ml of selected D- and DL-amino acids (1. D-alanine, 2. D-aspartic acid, 3. D-serine, 4. D-phenylalanine, 5. D-tryptophan, 6. DL-Leucine, and 7. DL-threonine) at the following concentrations (2.5, 5, 10 mM) and exposed to light at an intensity of 3000 lux for a duration of 6 and 12 hours.

Appropriate controls were maintained for all the experiments.

Experiments were replicated thrice.

Dark experiments were conducted under green safe light.

The tissues (in all cases) on removal from the incubation medium were rinsed with distilled water, blotted and then used for the extraction of the enzyme.

The enzyme was extracted and assayed as mentioned in Chapter II.

Protein and nitrite were estimated as mentioned in Chapter II.

IX. Effect of RNA and protein synthesis inhibitors on the induction of nitrate reductase.

Leaf tissues were floated on 50 ml of

1. Actinomycin-D at 40, 80 and 160 µg/ml concentrations for a duration of 6 and 12 hours in the presence of 100 mM KNO_3 and light at an intensity of 3000 lux.

2. Cycloheximide at 5, 10 and 20 ug/ml in 100 mM KNO_3. The duration of the incubation was 6 and 12 hours. One set was exposed to a light intensity of 3000 lux and the other was kept in darkness with ·1 M glucose.

3. Chloramphenicol at 0.5, 1 and 2 mg/ml concentrations in the presence of the inducer KNO_3 at 100 mM or in its absence and exposed to light intensity of 3000 lux for a duration of 6, 12 hours respectively.

RESULTS

The activity of nitrate reductase was not detectable in the leaves of 9-day-old etiolated seedlings of rice supplied with 25 mM KNO_3 (Table 4.1). When the leaf tissues of etiolated seedlings raised in 25 mM KNO_3 were exposed to light, activity of nitrate reductase was observed both at the end of 12 and 24 hours of incubation (Table 4.1). Induction of nitrate reductase in light did not require the presence of extra nitrate in the induction medium. When the

tissues were given an exogenous supply of nitrate (100 mM), and exposed to light, the uptake of nitrate was higher as evidenced by the higher activity of nitrate reductase both at the end of 12 and 24 hours of incubation (Table 4.1). When the leaf tissues of etiolated seedlings raised with 25 mM KNO_3 were floated on 100 mM glucose for a duration of 12 and 24 hours and incubated in complete darkness, activity of nitrate reductase was not detected (Table 4.2). Nevertheless, when the leaf tissues of 9-day-old etiolated seedlings were incubated in the dark for a duration of 12 and 24 hours in a medium containing glucose (100 mM) and KNO_3 (100 mM), activity of nitrate reductase was observed (Table 4.2). Induction of nitrate reductase by glucose required the presence of nitrate in the induction medium.

When the leaves of etiolated seedlings grown in water were excised and floated on varying concentrations of KNO_3 and exposed to light at an intensity of 3000 lux for a duration of 3, 6, 12 and 24 hours, activity of nitrate reductase was found to increase with increase in the concentration of KNO_3 and the duration of the exposure to light (Fig. 4.1). When the excised leaves were incubated with KNO_3 at a concentration of 20 mM for various time periods (3, 6, 12 and 24 hours) activity of nitrate reductase was low during the initial periods of incubation i.e. at the end of the 3rd and 6th hour and as the period of incubation was extended the

Table 4.1. Effect of light on the induction of nitrate reductase in etiolated rice leaves.

Medium of incubation	Period of incubation (hours)	Activity of nitrate reductase
Water	12	-
Water	24	-
Water	12 *	32
Water	24 *	35
KNO_3 **	12 *	70
KNO_3 **	24 *	80

The seedlings were grown in 25 mM KNO_3 in darkness for 9 days.

Manipulations and incubations were carried out in darkness or under green safe light.

Activity of nitrate reductase expressed as nmoles no_2^-/mg protein/15 min.

* Tissues were exposed to light

** 100 mM

Table 4.2. Effect of glucose*** on the induction of nitrate reductase in etiolated rice leaves.

Medium of incubation	Period of incubation (hours)	Activity of nitrate reductase
Glucose	12	-
Glucose	24	-
Glucose	12 *	20
Glucose	24 *	7
Glucose + KNO_3**	12 *	24
Glucose + KNO_3**	24 *	23

The seedlings were grown in 25 mM KNO_3 in darkness for 9 days.

Manipulations and incubations were carried out in darkness or under green safe light.

Activity of nitrate reductase expressed as nmoles no_2^-/mg protein/15 min.

* Tissues were exposed to light

** 100 mM.

*** 100 mM.

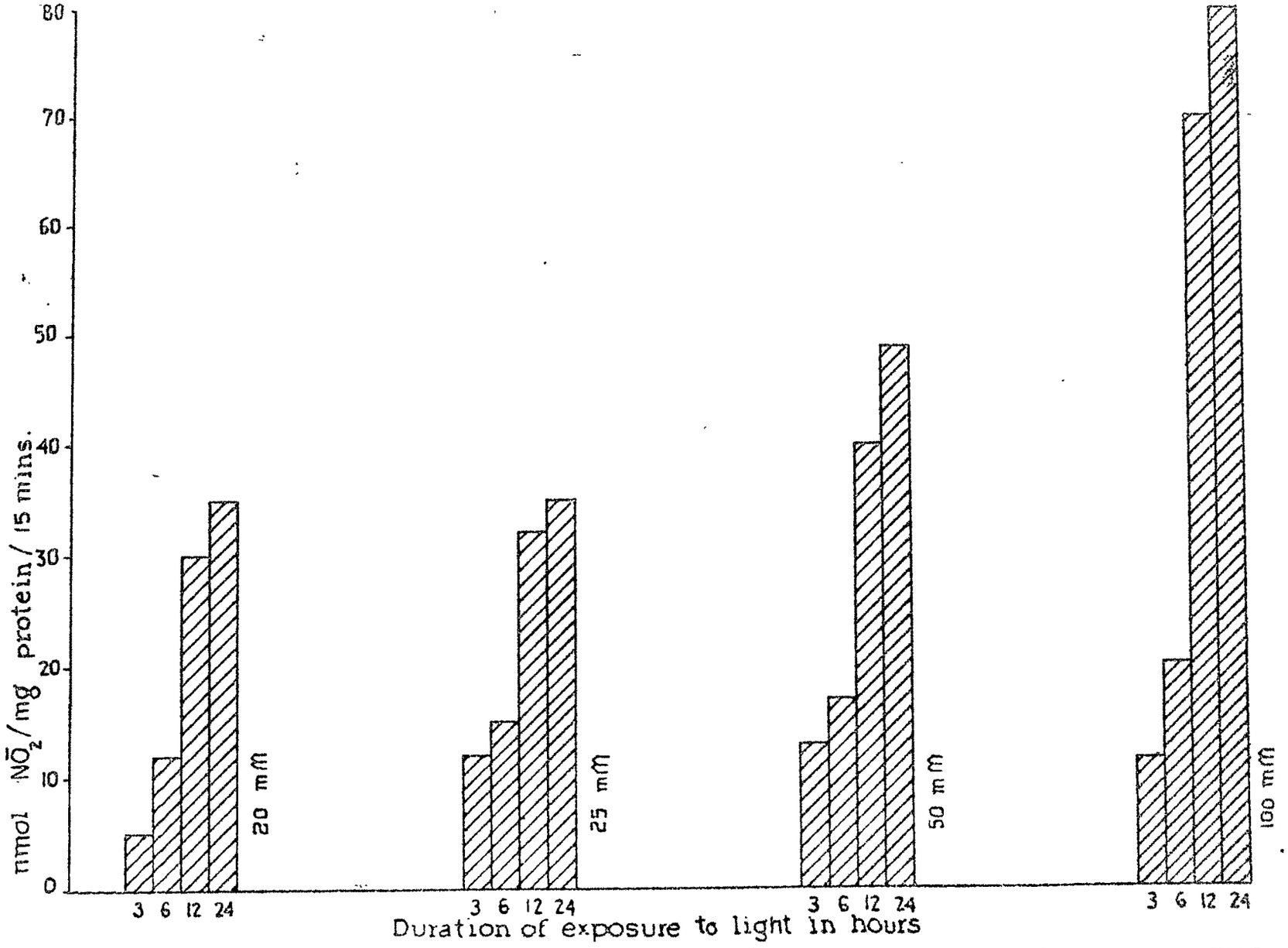

FIG NO 4.1 EFFECT OF VARYING CONCENTRATIONS OF EXOGENOUS NITRATE ON THE INDUCTION OF NITRATE REDUCTASE IN THE PRESENCE OF LIGHT

activity of nitrate reductase showed a marked increase (Fig. 4.1). An increase in the substrate concentration from 20 to 25 mM brought about an increase in the activity of nitrate reductase at the end of the 3rd and 6th hour of incubation. Activity thereafter doubled as can be seen in Fig. 4.1. When the excised leaves were subjected to a substrate concentration of 50 mM and exposed to light, the activity of nitrate reductase showed a rise especially at the end of 12 and 24 hours of incubation (Fig. 4.1). Doubling the substrate concentration (100 mM) resulted in almost a two-fold increase in the activity of nitrate reductase. Higher activities of nitrate reductase was obtained when the tissues were incubated in light for a duration of 12 and 24 hours (Fig. 4.1).

When leaf tissues of etiolated seedlings grown in water were supplied with KNO_3 at a 20 mM concentration and glucose at a concentration of 50 mM and incubated in the dark for a duration of 3, 6, 12 and 24 hours, activity of nitrate reductase was found to increase with increase in the period of incubation (Fig. 4.2). When the substrate concentration was increased to 25 mM and the glucose concentration was kept constant i.e. at 50 mM, increase in the activity of NR was not observed (Fig. 4.3). However, when the glucose concentration in the incubation medium was doubled (100 mM) and the substrate concentration was kept constant, there was a considerable increase in the activity of nitrate reductase

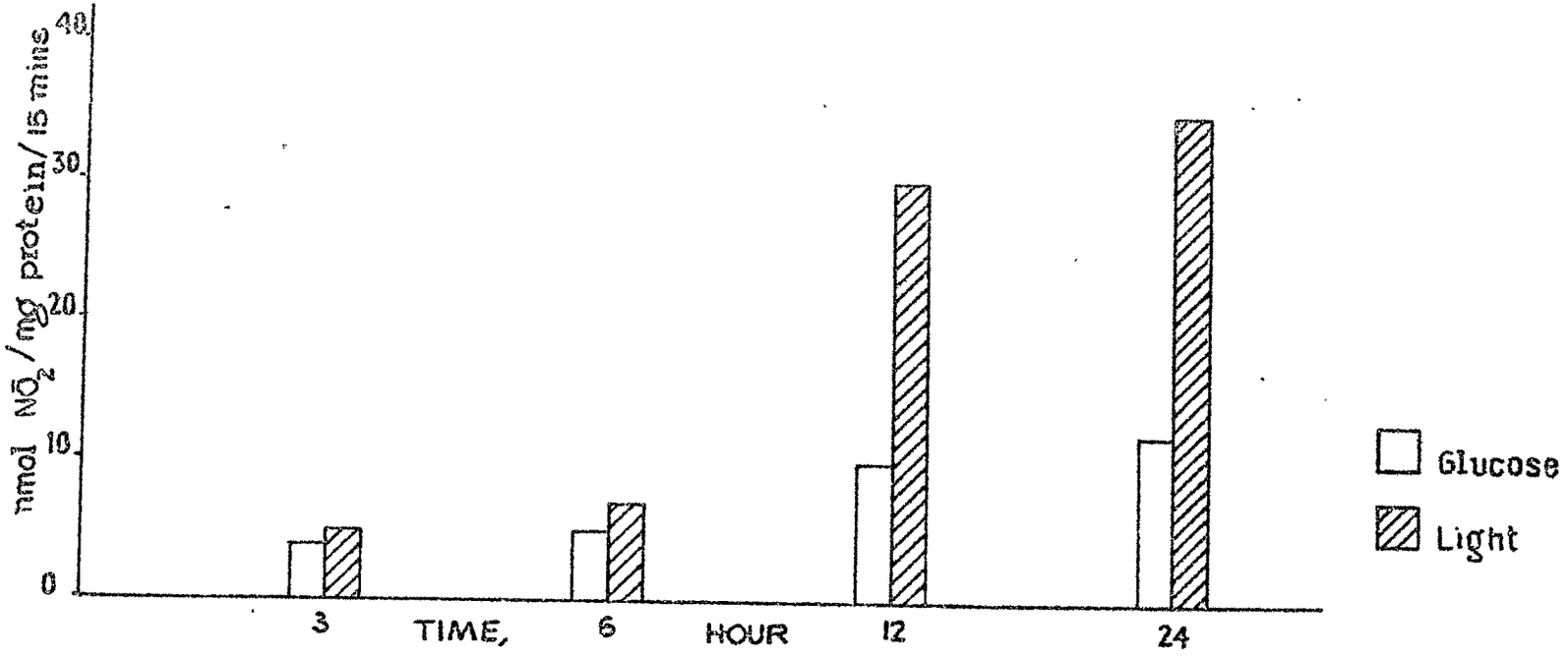

FIG NO 4 2 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (20 mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (50 mM) ON THE INDUCTION OF NITRATE REDUCTASE

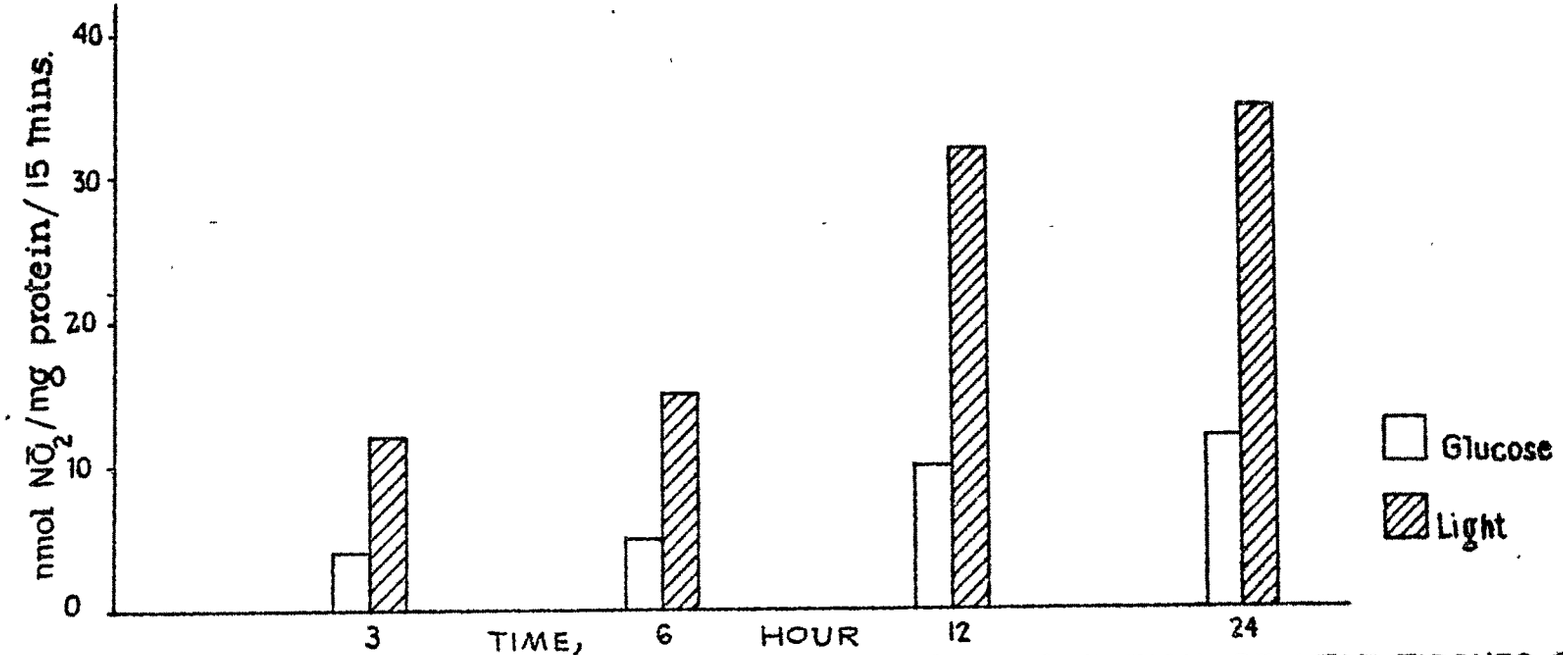

FIG NO 4.3 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (25mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (50mM) ON THE INDUCTION OF NITRATE REDUCTASE.

(Fig. 4.4). When the tissues were supplied with 50 mM of the substrate and 100 mM of glucose, the activity of NR showed a remarkable increase (Fig. 4.5) when compared to the results of experiment conducted earlier, i.e. with 25 mM KNO_3 and 100 mM glucose. When the glucose concentration in the incubation medium was kept constant (100 mM) and the substrate concentration was doubled (100 mM) the activity of nitrate reductase nearly doubled when compared to the previous experiment (Fig. 4.6). When the exogenous concentration of glucose was increased to 150 mM and substrate concentration was 100 mM, the activity of nitrate reductase increased considerably (Fig. 4.7) Activity of NR doubled when the substrate concentration was at 100 mM and the glucose concentration was increased to 200 mM (Fig. 4.8). The activity obtained at this concentration almost equalled the activity obtained under the influence of light at an intensity of 3000 lux and a substrate concentration of 100 mM.

The activity of nitrate reductase obtained with 150 mM sucrose and 100 mM of the substrate (Fig. 4.9) was similar to the activity obtained under the influence of 200 mM glucose and 100 mM of the substrate as can be observed in Fig. 4.10.

The activity of NR obtained in the case of tissues incubated in complete darkness with ATP at a concentration of 5 mM and 100 mM of the substrate was comparable to the activity obtained under the influence of light (at the end

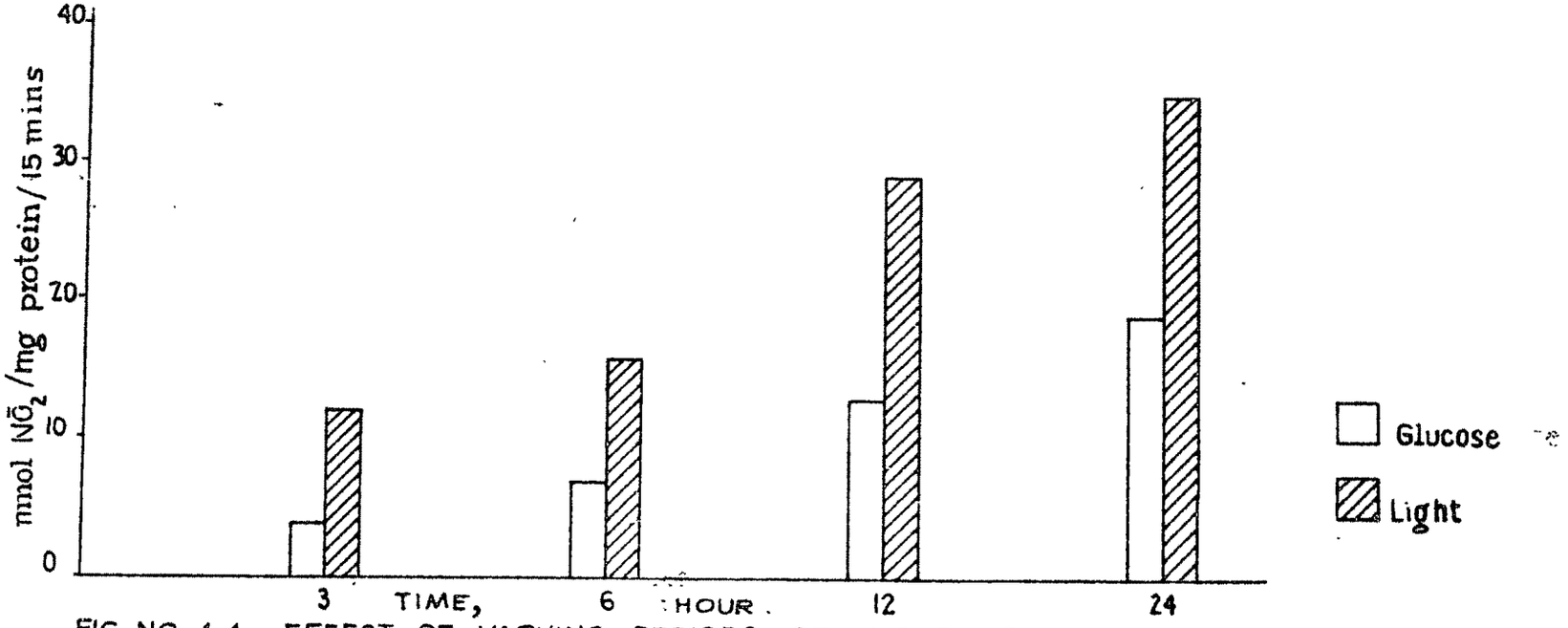

FIG NO 4.4 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (25 mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (100mM) ON THE INDUCTION OF NITRATE REDUCTASE.

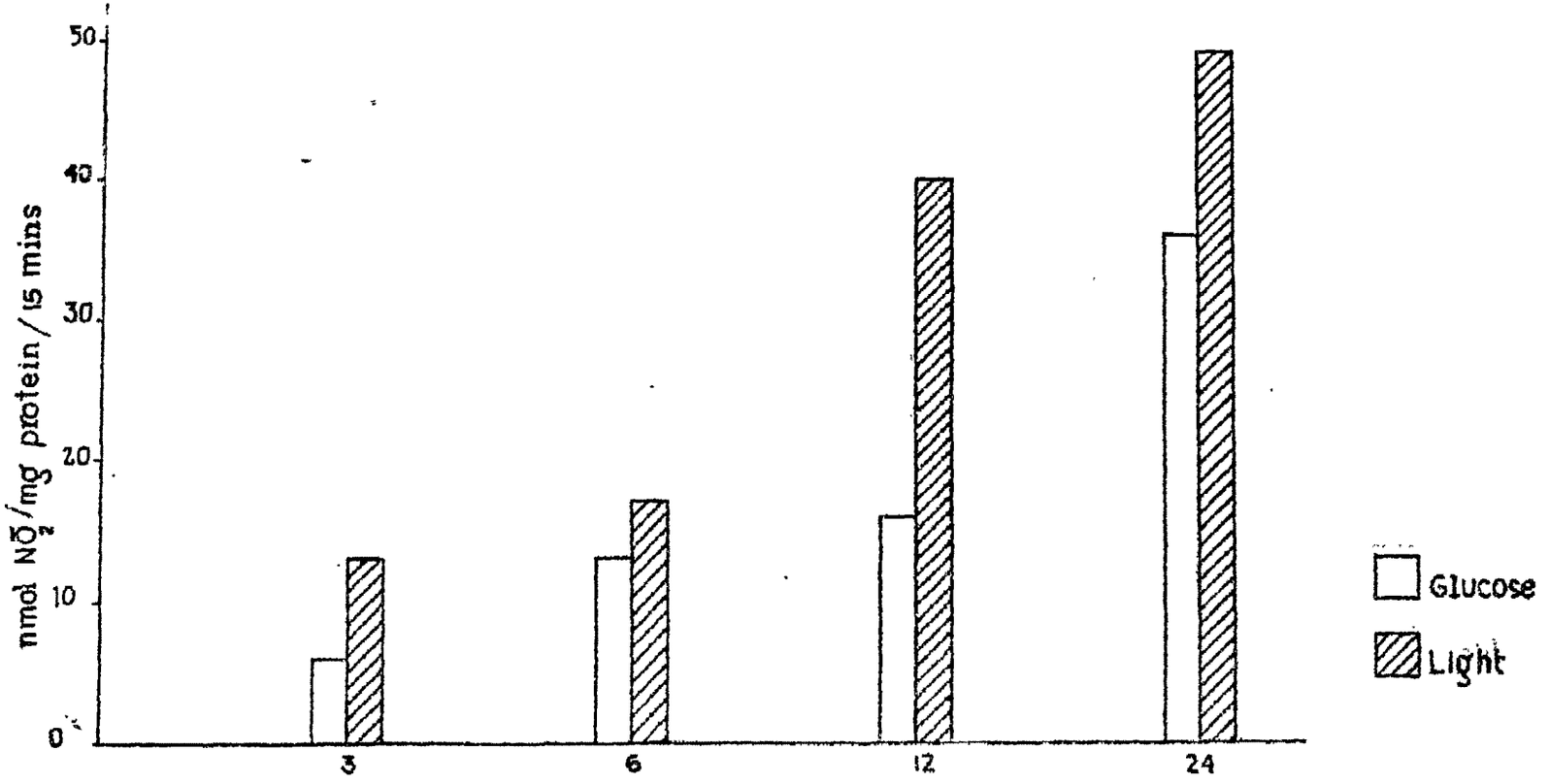

FIG NO 4.5 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (50 mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (100 mM) ON THE INDUCTION OF NITRATE REDUCTASE

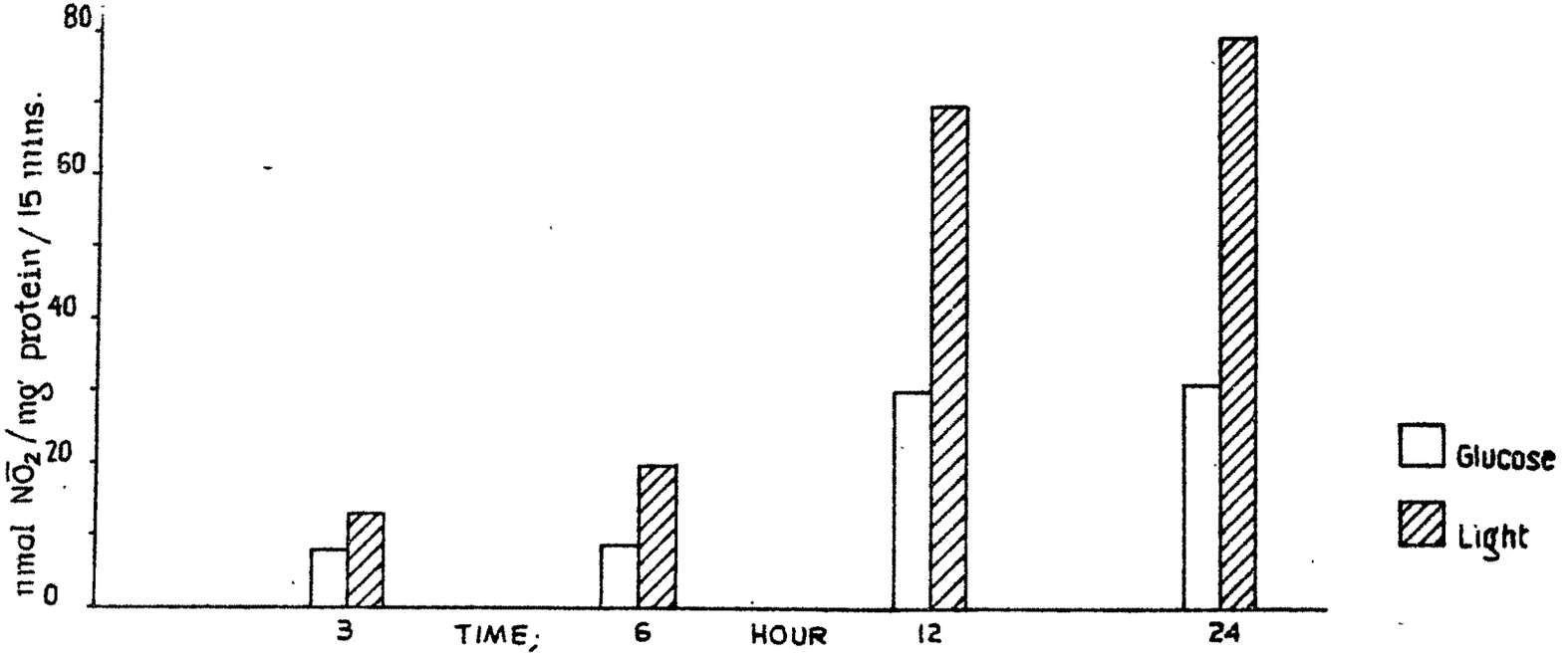

FIG NO 4.6 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (100 mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (100 mM) ON THE INDUCTION OF NITRATE REDUCTASE.

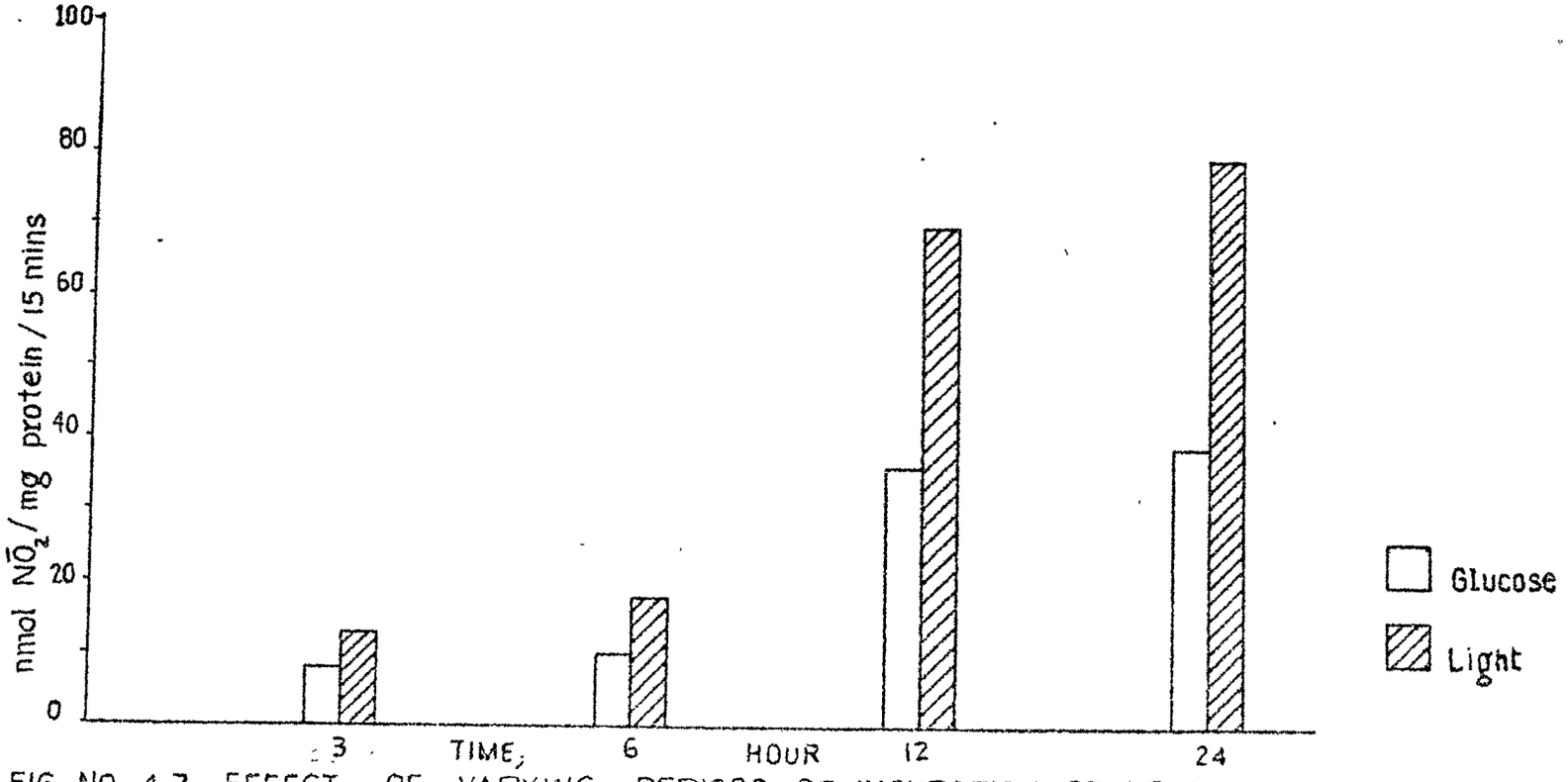

FIG NO 4.7 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (100 mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (150 mM) ON THE INDUCTION OF NITRATE REDUCTASE

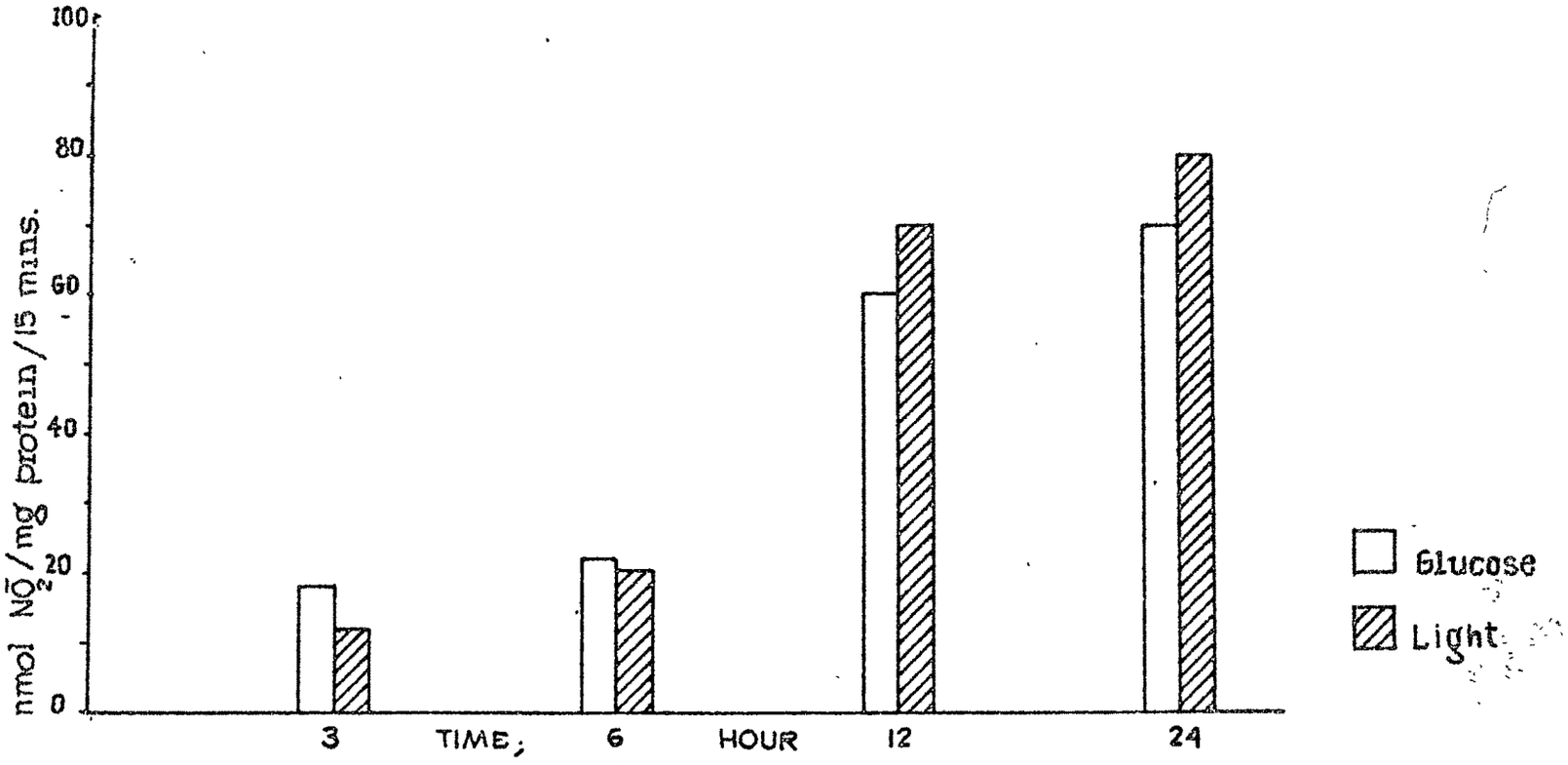

FIG NO 4.8 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUES OF RICE IN NITRATE MEDIUM (100 mM) IN THE PRESENCE OF LIGHT OR GLUCOSE (200 mM) ON THE INDUCTION OF NITRATE REDUCTASE.

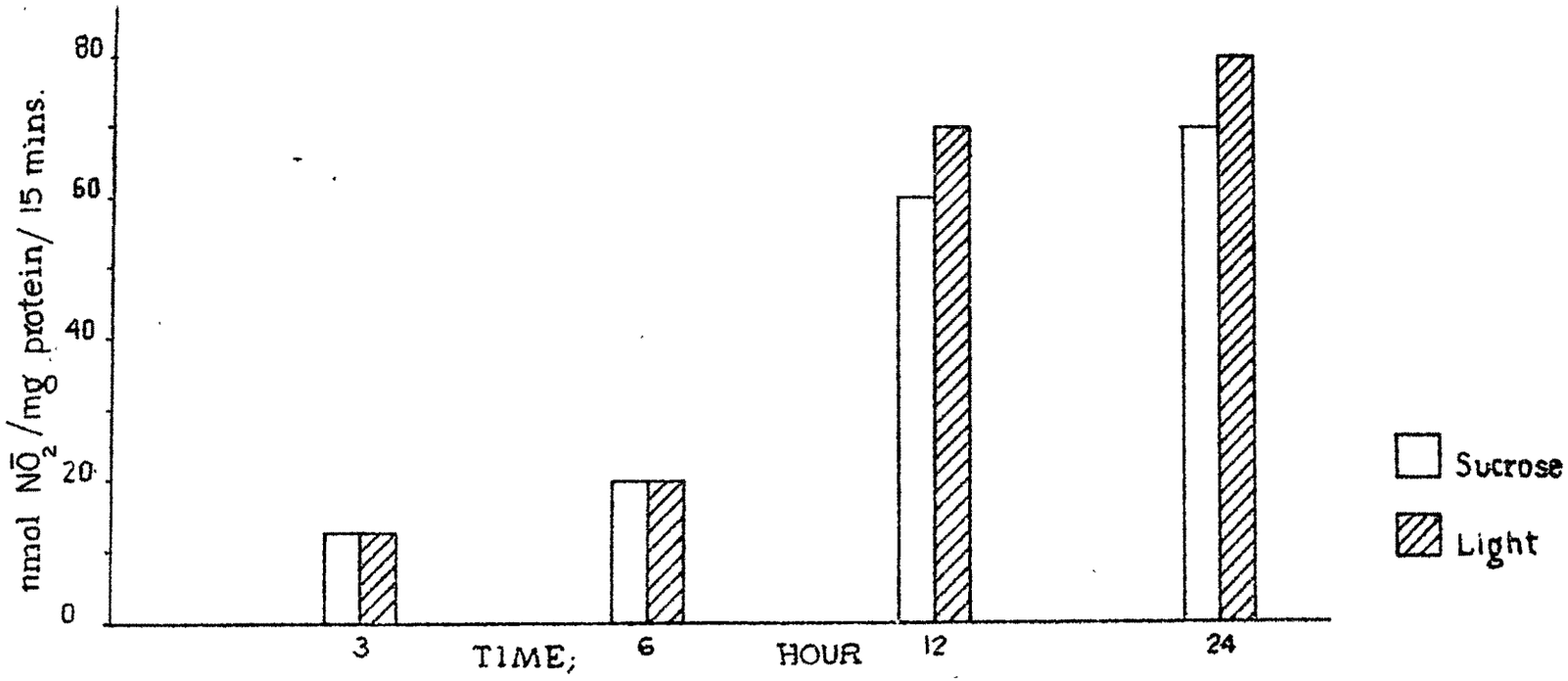

FIG NO 4.9 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUE OF RICE IN NITRATE MEDIUM (100 mM) IN THE PRESENCE OF LIGHT AND SUCROSE (150 mM) ON THE INDUCTION OF NITRATE REDUTASE.

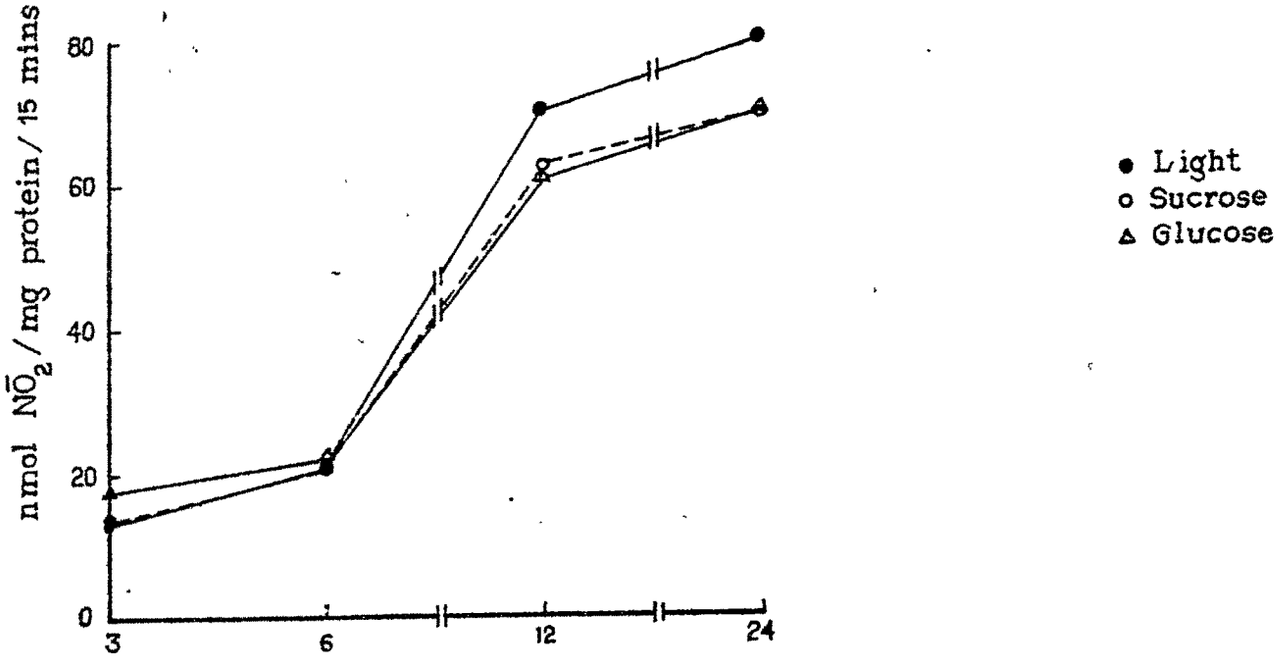

FIG: 4.10 EFFECT OF VARYING PERIODS OF INCUBATION OF LEAF TISSUE OF RICE IN NITRATE MEDIUM (100 mM) IN THE PRESENCE OF LIGHT, GLUCOSE (200 mM) AND SUCROSE (150 mM) ON THE INDUCTION OF NITRATE REDUCTASE.

of the 3rd and 6th hour of incubation) at an intensity of 3000 lux and 100 mM of the substrate. However, at the end of 12 hours there was a 37% decrease in the activity of nitrate reductase (Fig. 4.11).

When the tissues of etiolated leaves were treated with kinetin at a concentration of 5, 10 and 20 mg/l in the absence of light and nitrate, activity of nitrate reductase could be detected. Among the different concentrations of kinetin tried, kinetin at a concentration of 10 mg/l gave the maximum activity (Table 4.3). The activity of nitrate reductase almost doubled when the tissues were incubated in darkness with kinetin along with the inducer KNO_3 (100 mM).

When etiolated leaves of rice were incubated with some selected D- and DL-amino acids, induction of NR was observed. D-aspartic acid at a concentration of 5 mM showed a 43% activity of NR when compared to the control (Table 4.4). It was found to be the most effective. DL-Leucine was also effective in inducing NR. The other amino acids D-serine and DL-threonine induced marginal levels of NR. All amino acids tried at concentrations exceeding 5 mM were found to inhibit the activity of the enzyme (Table 4.4).

When the tissues of etiolated leaves were incubated in a medium containing nitrate (100 mM) and actinomycin D at 40, 80 and 160 ug/ml concentrations, in the presence of light for a duration of 6 hours, ~~activity~~ the level of nitrate

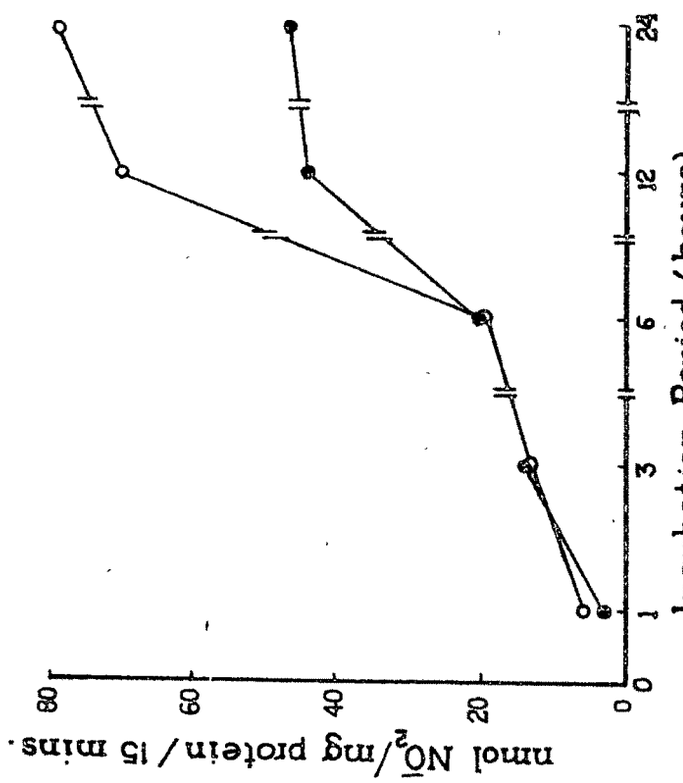

FIG: 4.11 ROLE OF ATP ON THE INDUCTION OF NITRATE REDUCTASE IN ETIOLATED LEAF TISSUES OF RICE BY NITRATE IN THE ABSENCE OF LIGHT.

Table 4.3. Effect of kinetin on the induction of nitrate reductase in paddy.

Kinetin concentration mg/l	Incubation period (hours)	Activity of nitrate reductase
Nil (Control)	-	-
5 *	12	13
10 *	12	20
20 *	12	14
5 **	12	30
10 **	12	52
20 **	12	40

* Incubated without KNO_3.

** Incubated with 100 mM KNO_3.

All operations and incubation were carried out in darkness.

Prior to assaying the samples were dialyzed.

Activity of nitrate reductase expressed as nmoles no_2^-/mg protein/15 min.

Table 4.4. Induction of nitrate reductase in paddy by D- and DL-amino acids.

Amino acids	Concentration mM	Nitrate reductase relative activity %
KNO_3 (Control)	100	100
Alanine *	2.5	-
	5	-
	10	-
Aspartic acid *	2.5	20
	5	43
	10	19.5
Serine *	2.5	7
	5	26
	10	13.8
Phenylalanine *	2.5	-
	5	-
	10	-
Tryptophan *	2.5	-
	5	-
	10	-
Leucine **	2.5	7.3
	5	30
	10	6
Threonine **	2.5	7.3
	5	23
	10	7.1

* D- amino acid

** DL- amino acid

reductase was inhibited. The inhibition was partial at 40 and 80 ug/ml concentrations of actinomycin D and about 33% inhibition was observed at a concentration of 160 ug/ml (Table 4.5). However, when the incubation period was prolonged to 12 hours, the inhibition was partial at 40 ug/ml concentration of actinomycin D and nearly 50% inhibition was obtained with actinomycin D at a concentration of 80 and 160 ug/ml (Table 4.5).

When the etiolated leaf tissues were incubated in a medium containing cycloheximide at 5, 10 and 20 ug/ml concentrations in the presence of the substrate at a concentration of 100 mM, total inhibition of the induction of NR was observed with light at an intensity of 3000 lux and also in darkness (Table 4.5).

When leaf tissues were incubated with chloramphenicol at 0.5, 1 and 2 mg/ml concentrations for a duration of 6 and 12 hours in light, induction of NR was marginal (Table 4.6). When the tissues were incubated with chloramphenicol at 1 mg/ml concentration along with the substrate KNO_3 in the presence of light for a duration of 6 and 12 hours, there was an increase in the activity of NR by about 13%. When the tissues were incubated with lower concentrations of chloramphenicol (0.5 mg/ml), the activity was similar to the control at a 12 hour incubation period. When tissues were incubated with chloramphenicol at a concentration of

Table 4.5. Effect of actinomycin D and cycloheximide on the induction of nitrate reductase in paddy.

Antibiotic supplied exogenously	Final concentration µg/ml	Addition of inducer	Incubation period (hours)	Nitrate reductase specific activity
A. Nil(Control)	-	+	6	30
	-	+	12	77
B.Actinomycin D	40	+	6	15
	80	+	6	22
	160	+	6	10
	40	+	12	56
	80	+	12	42
	160	+	12	42
C.Cycloheximide	5	+	6	-
	10	+	6	-
	20	+	6	-
	5	+	12	-
	10	+	12	-
	20	+	12	-
	5	+	6 *	-
	10	+	6 *	-
	20	+	6 *	-
	5	+	12 *	-
	10	+	12 *	-
	20	+	12 *	-

The first leaves of 9-day-old etiolated seedlings were incubated in an exogenous supply of the antibiotic and the inducer KNO_3 (100 mM) at 30°C at a light intensity of 3000 lux.

* Incubation in darkness with 100 mM glucose.

Activity expressed as nmoles NO_2^-/mg protein/15 min.

Table 4.6. Effect of chloramphenicol on nitrate reductase induction in paddy.

Antibiotic supplied exogenously	Final Concentration mg/ml	Addition of inducer	Incubation period (hours)	Nitrate reductase specific activity
Chloramphenicol	0.5	-	6	3
	1	-	6	12
	2	-	6	6
	0.5	-	12	5
	1	-	12	14
	2	-	12	8
	0.5	+	6	10
	1	+	6	39
	2	+	6	20
	0.5	+	12	80
	1	+	12	96
	2	+	12	20

The tissues were exposed to light at an intensity of 3000 lux.

Activity expressed as nmoles NO_2^-/mg protein/15 min.

2 mg/ml there was a marked inhibition of NR both at end of 6 and 12 hours of incubation (Table 4.6).

DISCUSSION

The failure of the leaves of rice seedlings grown in the dark with 25 mM KNO_3 to synthesize NR is in conformity with the results of Aslam et al. (1976). As suggested by Beevers and Hageman (1969), nitrate which accumulates in the dark is inaccessible to the enzyme and does not function as an inducer. Aslam et al. (1976), reported that nitrate passes from the external medium to the storage pool, through the metabolic pool in the cytoplasm. The level of the nitrate in the metabolic pool is promoted by either glucose or light. Once in the storage pool, which could represent the vacuole, it is relatively inaccessible. Since neither light nor glucose was available in the present investigation nitrate reductase could not be induced. The nitrate taken up by the etiolated seedlings must have entered the storage pool. Ferrari et al. (1973) working with tobacco cells reported the existence of two nitrate pools, a metabolic and a storage pool. Heimer and Filner (1971) reported that nitrate in the storage pool does not induce the enzyme and the work of Aslam and Oaks (1975) suggested that nitrate in the storage pool does not stabilize the enzyme.

The detection of activity of NR in the case of etiolated

seedlings of rice raised with KNO_3 and exposed to light is probably because of the availability of the inducer no_3^- in the metabolic pool. Light mediates the transfer of the nitrate from the storage to the metabolic pool (Aslam et al., 1976). The induction of NR was greater when nitrate was present in the incubation medium. This is presumably because of the availability of more nitrate in the induction medium.

The absence of induction of NR in etiolated leaves transferred to a 100 mM glucose solution but kept in darkness is also similar to the results of Aslam et al. (1976). It is interesting to note that these authors noticed small traces of nitrate in the "metabolic pool" and reported that addition of glucose did not alter the distribution of this nitrate. On the other hand, they found that exposure of etiolated seedlings grown with nitrate to light resulted in an appreciable accumulation of nitrate in the metabolic pool. They therefore, suggested that nitrate accumulates in the storage pool when seedlings were grown in continuous darkness and that the transfer of this nitrate to the active pool is mediated by light, but not by glucose. They were convinced that the transfer of no_3^- to the active pool leads to induction. These statements further support the results obtained in the present experiments. The failure of dark-grown seedlings supplied with nitrate to produce NR might possibly be due to the accumulation of the no_3^- in the storage pool.

The activity of NR observed in the case of etiolated leaves grown with nitrate and given an exogenous supply of nitrate and glucose is presumably because of the increase in the incorporation of NO_3^- into the metabolic pool. Aslam *et al.* (1976) have suggested two mechanisms for regulating the metabolic nitrate pool a) a transfer from the storage pool which requires light, b) a transfer from the external medium which requires either glucose or light. Aslam and Oaks (1975) reported that since the induction of NR by NO_3^- involves the de novo synthesis of protein, there must be a requirement for energy and amino acids. These requirements in the case of light grown leaves are met by photosynthesis. In etiolated or carbohydrate deficient green leaves, an exogenous supply of energy generating compounds such as glucose or sucrose was required to obtain maximum induction in darkness. Travis and his coworkers (1970) have shown that light or glucose is responsible for maintaining polyribosomes. Their results suggest that by increasing the protein synthesis machinery, light or glucose have enhanced the synthesis of NR presumably of other proteins. Energy, therefore, seems to be playing an important role in the induction process.

Light was found to increase the movement of the nitrate already in the cells into the metabolic pool. Glucose was unable to influence this movement. Jones and Sheard (1975) have suggested that a phytochrome system might regulate the activity of NR in etiolated pea buds by inducing NO_3^- movement

across membranes by enhancement of protein synthesis. In view of these results, it seemed possible that glucose or light could regulate the availability of no_3^- within the cells of leaves.

Induction of NR was obtained when 9-day-old rice seedlings raised in water in complete darkness were provided with an exogenous supply of nitrate and exposed to light. A steady increase in the activity of NR was observed both with increase in the concentration of exogenous nitrate and the duration of incubation. Dusky and Galitz (1977) reported concentrations ranging from 1-10 mM for maximum activity of NR in different grass species, Aslam et al. (1976) used 100 mM KNO_3 in the induction studies conducted by them with etiolated leaves of barley. However, induction was obtained after a lag period of 3 hours. Sawhney and Naik (1972) working with etiolated rice seedlings reported a lag period of 3 hours before the commencement of enzyme synthesis. In the present investigation activity of NR increased with time. Increased activity was observed at the end of 12 and 24 hours of incubation. Jordan and Huffaker (1972) observed increased activity of nitrate reductase after continuous illumination of leaves of dark-grown barley seedlings for 24 hours.

The activity of NR was found to increase not only with increase in the substrate concentration but also with increase in the glucose concentration. It is interesting to

note that with exogenous glucose at a concentration of 200 mM, the activity of NR almost equalled the activity obtained with leaves exposed to light. Glucose substituted for light in these dark-grown carbohydrate depleted leaves. The data presented by Jackson et al. (1973) on nitrate uptake by dark-grown corn seedlings suggest that the development of the maximal accelerated rate of no_3^- uptake depended upon continuous protein synthesis. Glucose presumably supplies the energy for maintaining the level of polyribosomes and thereby causes the induction of NR. Aslam and Oaks (1975) reported that an exogenous supply of glucose or sucrose is required to obtain maximum induction in darkness. It is presumed that glucose allowed the synthetic or activation phase of the induction to proceed more rapidly. The results obtained with sugars (glucose and sucrose) supports the hypothesis that one of the main effects of light may be supply photosynthates to support respiration, which in turn drives the induction process.

The marked increase obtained with sucrose along with the inducer KNO_3 is in conformity with the results reported by Sluiters-Scholten (1975). The increase in the activity of NR obtained with increases in the substrate KNO_3 and sugars or with a supply of ATP shows that NR is induced by no_3^- and that there is a clear relationship between the exogenous supply of nitrate and the activity of NR as shown in Fig. 4.1. Increase in the exogenous supply of energy generating compounds such

as glucose or sucrose or a supply of ATP resulted in maximum induction of NR. Sihag et al. (1979) with the help of tracer incorporation studies conducted in pea seedlings were able to show that both sucrose and light acted by enhancing the rate of protein synthesis. Sluiters-Scholten (1975) reported that sugars controlled NR level in various ways. The author suggested that NADH generated by photosynthesis (sugars) protected the NR against the 'break down' enzyme. The investigation undertaken with sugars indicates that glucose or sucrose enhanced the synthesis of NR& thereby almost equalled the activity of NR obtained in the presence of light.

Activity of nitrate reductase could be detected with kinetin in the absence of light. Among the different concentrations of kinetin tried, kinetin at a concentration of 10 mg/l gave the maximum activity. Enhancement of the activity of nitrate reductase in embryonic tissues of Agrostemma githago by kinetin in the absence of exogenously supplied nitrate has been reported by Kendre et al. (1971). However, the activity of NR doubled when the etiolated tissues were treated with kinetin along with the inducer nitrate. Similar enhancement of nitrate reductase by kinetin along with nitrate has been reported by Kendre et al. (1971) in Agrostemma githago and Chantarotwong and Huffaker (1971) in detached corn and barley leaves. The enhancement of activity of NR by kinetin and nitrate suggest that kinetin

may be partially replacing the role of light in the induction of nitrate reductase. Induction of nitrate reductase in tobacco leaves deprived of nitrate occurred in darkness when they were placed in a nitrate containing solution and sprayed with cytokinins (Roth-Bejerano and Lips, 1970). Rijven and Parkash (1971) obtained induction of nitrate reductase in cotyledons of fenugreek by nitrate in the presence of light or kinetin. They suggested that kinetin may be removing a limitation that prevents the synthesis of RNA and genome expression.

Nitrate reductase was induced in etiolated leaves by certain D- amino like aspartic acid and serine and DL-amino acid leucine and threonine. Similar induction of nitrate reductase by amino acids has been reported in yeast (Choudary and Rao, 1976) and in cotton (Radin, 1975). Radin (1975) has also shown that the induction of nitrate reductase by amino acids is independent of nitrate uptake. The induction of nitrate reductase by amino acids may be suggestive of the adaptive ability of the organisms to induce the enzyme in absence of nitrate.

When etiolated leaves were incubated in a medium containing NO_3^- (100 mM) and actinomycin D at 40, 80 and 160 µg/ml concentrations, in the presence of light of a duration of 6 hours, activity of NR was inhibited. The inhibition was partial at 40 and 80 µg/ml concentrations of actinomycin D and about 33% inhibition was observed at a concentration of 160 µg/ml.

When the incubation with actinomycin D was prolonged to 12 hours about 50% inhibition of nitrate reductase was observed with 80 and 160 µg/ml concentrations of actinomycin D. The activity of nitrate reductase seen (even in the presence under the influence) of actinomycin D may be due to the presence of preformed mRNA in the tissues, as actinomycin D is known to block the production of mRNA, Radin (1974) observed an early peak activity of NR in soybean and sunflower seedlings treated with actinomycin D. Actinomycin D inhibits NR only after 3 hours of its application.

The total inhibition of induction of NR observed when tissues were treated with cycloheximide may be due to its interference in the synthesis of protein at the translation level as suggested by Stewart (1968). As reported by Jackson et al. (1973), this inhibitor of protein synthesis may perhaps be drastically inhibiting nitrate uptake. Rao and Rains (1976) investigated the influence of protein synthesis and nitrate reductase activity on nitrate absorption of barley. Pretreatment studies conducted by them showed that cycloheximide affects either energy transfer or nitrate reductase activity or both.

The induction of nitrate reductase obtained with 0.5 and 1.0 mg/ml concentrations of chloramphenicol is relatively inaffective in inhibiting the induction of nitrate reductase e/ by the substrate. Schrader et al. (1967) reported that

synthesis of chloroplast proteins is inhibited by chloramphenicol in excised and intact plants. In contrast, protein synthesis in the cytoplasm of photosynthetic cells appears to be comparatively insensitive to chloramphenicol. Sawhney and Naik, (1972) observed 40% inhibition of nitrate reductase in green seedlings of rice. However, Beevers et al. (1965), have reported that chloramphenicol was ineffective in inhibiting the nitrate reductase in radish and corn leaves at levels not exceeding 1 and 5 mg/ml respectively. Perhaps the stimulation of nitrate reductase with chloramphenicol is due to the nitro group in which the oxidative level of the nitrogen atom is the same as in nitrates as speculated by Shen (1972). This postulation is supported by the fact that other nitro compounds also induced nitrate reductase in rice seedlings. The stimulation of nitrate reductase observed in the present studies under low concentrations of chloramphenicol may be due to the availability of extra energy (Shuiters-Scholten, 1973) and amino acids because of the inhibition of protein synthesis in the chloroplast.

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