NITRATE ASSIMILATION IN SEEDLINGS OF *ZEA MAYS* L.

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by

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SCOPE AND CONTENTS: The experiments described in this thesis were performed to investigate the:

1. Role of nitrate in the growth of maize seedling and
2. Role of amino acids in the assimilation of nitrate by the growing embryo.

Nitrate stimulated the germination of Zea mays L. seeds by 10 to 15 percent. Further growth of the embryo axis, up to 6 days, however, was not affected by nitrate. During the early growth of the seedling, endosperm nitrogen was able to support the requirements of the embryo for 6 to 8 days. After a lag of 2 days, the protein content of the embryo increased linearly up to 6 days at a rate of 597 μ g a day. Some increase was observed between 6 and 8 days also. After 8 days, the protein level of embryo plateaued. Addition of 10 mM nitrate caused an increase in the protein and total nitrogen of the

embryo only after 6 days. The endosperm mutant of maize, opaque-2, also responded to the nitrate in the same way.

The protein content of the primary leaves increased linearly between 5 and 7 days. After 7 days, there is no increase in the leaf protein. Nitrate increased the protein level of primary leaves by 25 percent after 7 days. It protected against the further loss of protein in mature leaf. These results suggest that the primary role of nitrate in the growth of maize seedlings is to prevent protein loss.

Out of 8 amino acids tested individually, only lysine and to a lesser extent arginine, inhibited the induction of nitrate reductase in the maize root tips. Different ammonium salts had no effect on the induction of nitrate reductase. The initial rate of induction in opaque-2 mutant (high lysine) was lower than the wild type, W64A (low lysine). From a comparison of the rate of induction of nitrate reductase between young and mature leaf, it was suggested that the amino acid supply from the endosperm may inhibit the induction of nitrate reductase. In the young maize seedling, this effect of amino acids may be more effective in vivo and in this way the assimilation of exogenous nitrate could be restricted, when the endosperm amino acids are supporting the growth of the embryo.

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INTRODUCTION

Plants are able to reduce nitrate to ammonia via nitrite. The ammonia thus formed is converted mainly to glutamic acid (Steward and Pollard, 1957). Protein is the ultimate product of nitrate assimilation. When applied to various cereal crops, nitrate increases the dry matter production (Gasser and Iordanou, 1967) and the total grain yield (Widdowson, et al., 1971) in long term experiments. It also increases the protein levels of relatively mature maize seedlings (Hageman and Flesher, 1960). For example with 15 mM nitrate, the protein contents of shoots from 15, 21 and 27 day old plants were respectively 2.2, 7.4 and 8.5 times higher than the nitrate free control in HY2 x OH7. The hybrid WF9 x C103 also showed a similar response to nitrate. In a mature shoot, however, some of the older leaves start senescing and supply organic nitrogen to the younger leaves. The addition of exogenous nitrate may induce protein synthesis in younger leaves and/or prevent the degradation of protein in mature leaves.

In microorganisms, ammonia and amino acids are a better source of nitrogen than nitrate and the suppression of nitrate assimilation by

these potential end products is common. In higher plants growing in soil, on the other hand, the main source of nitrogen is nitrate. In many cases nitrate supports better growth than ammonia. For example, Joy (1969) found that nitrate is a good source of nitrogen for the growth of Lemna minor. Ammonia even in combination with nitrate gave poor results. In many systems, ammonium inhibits the absorption of nitrate directly or indirectly. In wheat seedlings, for example, the absorption of ammonium preceeds the absorption of nitrate, when both are present in the medium (Minnoti et al., 1969). Since the inhibition of nitrate uptake was related to the extent of ammonium uptake, a product of ammonium assimilation, perhaps was regulating nitrate uptake. In some other cases, however, ammonium has been found to cooperate with the nitrate. Weissman (1959) found that wheat seedlings grown in the dark for four days and supplied with both ammonium and nitrate nitrogen in the culture solution produced higher protein than that achieved by seedlings with either ammonium or nitrate alone. This result has also been confirmed for the shoots of sunflower (Weissman, 1964). Effect of amino acids on growth has been studied less in higher plants. When added together with nitrate in tobacco cell suspension cultures (Filner, 1966) or in Lemna minor (Sims et al., 1968), amino acids cause a real but limited sparing

effect of nitrate assimilation. In most cases where amino acids have been used singly as a nitrogen source, they have caused severe growth inhibitions (Filner, 1966; Harris, 1956; Joy, 1969 and Miflin, 1969). Miflin (1969) using barley embryo and Dunham and Bryan (1969) Marchantia polymorpha gemmalings provided evidence for the interaction of amino acids. For example, L-forms of lysine, arginine, tyrosine, proline, threonine, methionine, valine and leucine at concentrations of 1 or 2 mM inhibit fresh weight accumulation of the barley embryo. The inhibition by valine is relieved by the further addition of isoleucine and that of leucine by the addition of both isoleucine and valine. These interrelations suggested that leucine and valine could inhibit acetolactate synthesis. This has since been confirmed by Miflin, who demonstrated that acetohydroxy acid synthetase, the first enzyme in the biosynthetic sequence leading to valine and leucine was inhibited by valine and leucine (Miflin, 1969b, 1971). The terminal amino acids of aspartate pathway also show the same kind of relationships in effecting the growth and development of gemmalings of Marchantia polymorpha (Dunham and Bryan, 1969). Lysine and threonine synergistically inhibit the growth of these plants and cause a loss of normal pigmentation at concentrations as low as 1 mM. These effects

are highly specific for this pair of amino acids and can be prevented by low concentrations of methionine or its metabolic precursor, homoserine.

In growing seedlings, the hydrolysis of storage proteins provides amino acids and amides for the growth of embryonic axis. As the endosperm nitrogen supply is exhausted, most seedlings switch to inorganic nitrogen assimilation. The embryos from the seed can be grown on nitrate. Isolated oat embryos are able to meet their amino acid requirement from nitrate (Harris, 1956). Oaks and Beevers (1964) also found that when the excised embryos of maize were grown on nitrate, there was a lag of 72 hours before there was an increase in the organic nitrogen of embryo. After this time, the organic nitrogen of the excised embryo increased at a rate equal to the control. They suggested that the normal supply of amino acids from the endosperm repressed the amino acid biosynthetic enzymes and that during the 72 hours of limited amino acid supply, the amounts of these enzymes would have been derepressed. After that the seedlings could exist with nitrate as a sole source of nitrogen.

The assimilation of nitrate by plants is initiated by the activity of nitrate reductase. Hageman and Flesher (1960) describe a positive correlation between nitrate reductase activity and growth and protein contents of two varieties of

young maize plants. Shoots and leaves of the hybrid HY2 x OH7 had higher levels of nitrate reductase activities than similar tissues from WF9 x C103 plants. Correspondingly, protein levels of HY2 x OH7 tissues were higher than WF9 x C103. Thus the activity of nitrate reductase could determine the capacity of the seedling to assimilate exogenous nitrate into protein. Nitrate reductase is a substrate inducible enzyme (Beevers et al., 1965; Beevers and Hageman, 1969). Thus, when nitrate is supplied to the maize seedling an increase in nitrate reductase is to be expected. However, if the endosperm amino acids supplied the nitrogen requirements, the induction of nitrate reductase would not be necessary. Thus in the presence of an efficient regulatory mechanism, this enzyme may not be induced or may not be maximally induced. A study of the development of nitrate reductase in the embryonic axis of the young maize seedling was undertaken to define the degree of control.

Inhibition of nitrate reductase by the potential end products of nitrate reduction has been shown in algae and fungi (Morris and Syrett, 1963; Losada et al., 1970 and Kinsky, 1961). Working with the enzyme from Chlorella fusca, Losada et al., (1970) found that ammonium does not inhibit the induced synthesis of the enzyme but rather inhibits the activity of enzyme both in vivo and in vitro.

When ammonia was dialyzed away from cell free extracts, the reactivation of nitrate reductase occurred. This reactivation also occurred in intact cells. In the fungus Ustilago maydis ammonium triggered the disappearance of nitrate reductase activity (Lewis and Fincham, 1970). Cycloheximide and actinomycin prevented the ammonium mediated loss of nitrate reductase. They claim that the loss of nitrate reductase by ammonium is actually due to its degradation. The findings of Subramaniam and Sorger (1972) in Neurospora crassa also indicate an almost similar role of ammonia. They observed that unlike Chlorella fusca, inactivation by ammonium is not reversible when ammonium is withdrawn. Hence inactivation is not merely due to a change in the activity of the enzyme. Cycloheximide protected the enzyme from this inhibition. Therefore, it appears that ammonium mediated inactivation of nitrate reductase in Neurospora crassa in vivo is due to an active degradation of the enzyme.

End product inhibition of nitrate reductase has been reported in higher plants also, although at the present time these cases appear to be exceptional (Beevers et al., 1965; Ingle et al., 1966 and Schrader and Hageman, 1967). Ferguson (1969) found that ammonium inhibits the nitrate reductase activity in the leaves of Spirodella oligorhiza. This effect was observed only on in vivo activity of the enzyme.

The inhibition of nitrate reductase by ammonium has also been observed in barley roots (Smith and Thompson, 1971). In excised barley roots, however, ammonium did not inhibit the uptake or assimilation of nitrate but rather the development of nitrate reductase itself. Amino acids, as end product inhibitors of nitrate reductase, have also been tested. Filner (1966) observed that nitrate reductase in cultured tobacco pith cells is repressible by casein hydrolysate and eleven amino acids, when supplied one at a time. Arginine and lysine could counteract the 'repressive' action of any one of the eleven amino acids. Cysteine and isoleucine were also able to 'derepress' but not in the presence of either methionine or alanine. Wallace and Oaks (ms in preparation) also found that arginine and lysine enhance the induction of nitrate reductase in maize root tips, whereas proline, leucine and glutamine inhibit the induction. In tobacco suspension cells, it was found that amino acids inhibit growth when nitrate is the nitrogen source, but not when urea or α - amino butyric acid was the nitrogen source (Heimer and Filner, 1970). Amino acids inhibited nitrate uptake and the development of nitrate reductase in a cell line, which was susceptible to inhibition by threonine. In a mutant cell line (XDR^{thr}), which was resistant to threonine, amino acids inhibited the development of nitrate reductase only. From their findings, they deduced

that amino acids inhibited growth by specifically inhibiting the nitrate assimilation.

Although amino acids and ammonia can be seen as end product inhibitors of nitrate reductase, a minimum level of amino acids is always required for the induced synthesis of any enzyme. In the pyrimidine or amino acid requiring mutants of Neurospora crassa, corresponding nucleotides or amino acids are required for the induction of nitrate reductase by nitrate (Sorger, 1965). In maize seedlings, Schrader and Hageman (1967) reported about 31 percent enhanced induction of nitrate reductase, when nitrate was supplemented with ammonium salts. In young maize seedlings, amino acids coming from the reserve proteins are able to maintain the growth of the embryo axis. In a mature seedling, there is no such supply of amino acids. The amino acids from the degraded proteins in the senescent tissues, however, may maintain a minimum level for the induction of the enzyme. If the amino acids coming from the endosperm inhibit the induction of nitrate reductase, we might expect a lower induction in young seedlings than in mature seedlings. A comparison of the induction of nitrate reductase between the leaves of two ages, may show this type of effect. For this reason, the induction of nitrate reductase in the leaves from five day and nine day old seedlings has been compared.

Folkes and Yemm (1958) observed that, during early growth of the seedling, barley embryo obtains many of its amino acids from the hydrolysis of endosperm proteins. From the wide differences in composition between the storage proteins of the endosperm and proteins of the embryo, it was concluded that an extensive conversion of amino acids occurs during the early growth of barley seedling. During the growth period of ten days, aspartate, alanine, glycine, lysine and arginine increased considerably. There was a significant decrease in amides, glutamate and proline. Changes in the amount of other amino acids were less significant. Increases in the amounts of arginine, lysine, aspartate and glycine and decreases in leucine, glutamate, proline and amides during the early growth of seedling have also been observed in wheat (Pion et al., 1968). Normal maize endosperm is rich in proline, leucine, glutamate and amides. Opaque-2, a mutant maize has relatively less zein and relatively more glutelin. Due to the redistribution, the mutant has a higher amount of lysine and tryptophan and a lower amount of alanine, proline and leucine (Sodek and Wilson, 1971). The importance of proline for the growth of maize embryo has been observed by Oaks and Beevers (1964). Maize embryos have a large pool of free proline. When the embryo is excised from the endosperm and allowed to grow, this pool is largely depleted after 72 hours

of excision. A mixture of amino acids, similar to zein in composition is able to support protein synthesis in embryo. When proline is omitted from the amino acid mixture, normal increases in protein nitrogen of the embryo are restricted by 85 percent. Omission of other amino acids has a less severe effect. If proline or other zein amino acids are important in the growth of maize embryos, the single gene mutant opaque-2 could have a poorer embryo growth. In order to make some of the deficient amino acids, there may be a requirement for an external source of nitrogen, in addition to the nitrogen of the endosperm. To test this possibility, the effect of nitrate on the protein and total nitrogen contents of the growing embryo of opaque-2 and on nitrate reductase have been compared to that of the wild type.

The current investigation was undertaken to determine: (i) The role of nitrate in the germination of maize seed. (ii) The role of endosperm amino acids in the assimilation of nitrate by the embryo and (iii) The role of amino acids (exogenous and endosperm) in the induction of nitrate reductase.

CHAPTER I

MATERIALS AND METHODS

Seed Material

Zea mays L. seeds, used in the present investigation, were supplied by Warwick Seed Company, Blenheim, Ontario. Routinely the hybrid variety W64A x W182E was used. It was selected because it had a good percentage of germination, good early growth and high levels of nitrate reductase activity (Steven's personal communication). The inbred line W64A and its isogenic mutant W64A opaque-2 were used for comparing the role of endosperm amino acids on the assimilation of nitrate by the embryo and the induction of nitrate reductase in the root tips.

Growth Conditions

Seedlings were grown either in a growth room or in a Controlled Environment growth chamber, model E7H. Both were regulated under the same environmental regime. A 16 hour photoperiod of 1,000 lux light intensity and temperature at 26°C was maintained during the growth of the seedlings. Relative humidity ranged between 60 to 80 percent. Before planting into the trays, containing vermiculite and sand mixture, the seeds were sterilized with a commercial bleach (Javex) for a brief period, and then washed thoroughly with sterile distilled water. They were watered daily with 1/10 strength Hoagland's solution, which included nitrate as required.

Germination Experiments

Maize seeds were planted in soluble nitrogen free vermiculite and sand mixture in small trays. Alternatively, they were planted in large petri plates (14 cm. diameter) on 0.9 percent agar which contained 1/10 strength Hoagland's solution plus nitrate when required. The seeds planted in vermiculite were watered twice a day with 1/10 strength of Hoagland's solution, which contained nitrate when required. The seeds were then grown either in complete darkness or in the light. The photoperiod was 16 hours light and 8 hours darkness. The light intensity was 1,000 lux. Seeds were considered germinated, when the radicle was visible, through a scar in the seed coat over embryo part. For comparing the effect of nitrate on the germination of different varieties of maize seeds, 7 varieties and 1 mutant were grown on agar in the dark.

Sampling of Material for Nitrogen Determination

A. Embryo Growth - The seedlings were taken out daily for the first 4 days and then on alternate days for the next 6 or 8 days. For each treatment, about 25 to 30 uniformly grown plants were selected from two trays. Care was taken to cause as little damage as possible to the roots, while taking the seedlings out. The vermiculite was flooded with water before uprooting the seedlings. Endosperm and embryo were separated with the help of sterile forceps and frozen in the liquid nitrogen. Scutellum was dissected from the

embryo when required. The seed coats were not included in any of the portions.

B. Growth of the Primary Leaves - The seedlings were watered with 1/10 strength Hoagland's solution without nitrate for the first 3 days. The irrigation water was supplemented with different concentrations of nitrate on fourth and subsequent days. The samples (about 30 primary leaves) were taken from 5, 7, 8 and 12 day old seedlings. Further treatment of these samples was similar to that of endosperm and embryo.

Measurement of Growth

Fresh weight of embryo and of primary leaves was taken as an index of growth. The embryo axis or leaves were blotted dry before freezing in liquid nitrogen and then weighed. Freezing in liquid nitrogen does not change their weight. The fresh weight of one part is the average of 40 to 50 plant parts.

For comparing the growth of W64A and it's opaque-2 mutant, the dry weight of the embryo was measured. Embryos were frozen in liquid nitrogen and dried in lyophilizer for 48 hours. They were weighed immediately after removal from the lyophilizer.

Analytical Tests

A. Determination of Total and Protein Nitrogen - For each treatment, aliquots of 200 milligrams each were taken from the powdered dried tissue. The samples, were then extracted with excess of 80 percent ethanol.

Nitrogen contents of the soluble and insoluble portions were determined by microkjeldahl method. The extract and the residue were dried and then digested in 2.0 ml. of 6N sulfuric acid overnight in a fuming chamber. Next morning, about 10 drops of hydrogen peroxide were added to make this digested material clear. After one hour of further heating, it was cooled and made up to 50 or 100 ml. with double distilled water. To 0.5 ml. of this, 3.0 ml. of water and 2.0 ml. of Nessler's reagent was added. After 20 minutes, the intensity of colour was read at 440 nm. Readings in duplicate were taken for each sample. The range of the readings was kept between 0.15 to 0.65 on the absorbance scale.

B. Determination of Chlorophyll - About 30 primary leaves from uniformly grown seedlings, were selected. They were blotted dry, frozen and then powdered in liquid nitrogen. An aliquot of 1.0 gm was extracted several times with 80 percent acetone, until the residue was almost colourless or light brown. The supernatant solutions from each extraction were cleared by centrifugation and filtering. Finally, all fractions were combined and made up to a known volume. The absorbance of each extract was read at 665 nm and 449 nm. Pure solvent was used as blank. Total chlorophyll was calculated according to the equation of Strain and Svec (1965), which is as follows:

Total chlorophyll μgm/ml = $6.45(A_{665}) + 17.72(A_{649})$

Induction of Nitrate Reductase

A. In Leaves - For studying the induction of nitrate reductase in intact leaves, seedlings grown in minus nitrate medium were watered with the desired concentrations of nitrate in 1/10 strength Hoagland's solution. The solution was also supplemented with additional molybdenum at a concentration of 0.05 ppm. The samples of 30 to 40 primary leaves were collected after a 3, 6, 12 or 24 hour induction period. The leaves were frozen in liquid nitrogen, ground to a fine powder and stored overnight at -20°C before the enzyme was extracted.

The induction procedure for excised leaves was similar to that described by Beevers et al. (1965). Thirty excised corn leaves were immersed in about 400 ml. of induction medium in a flask. Composition of the induction medium was same as for intact leaves. The flasks were placed on a shaker at low speed. At the required intervals of time, the leaves were removed, washed in cold distilled water and then blotted dry. Further treatment was similar to the intact leaves.

B. In Root Tips - Maize seedlings were grown on 0.9 percent agar, which contained 1/10 strength Hoagland's solution without nitrate. When the primary roots were 4-5 cm. long, about 65 hours at 26°C, suitable seedlings were selected for the induction studies. Induction medium was contained in a 250 ml. beaker with a plastic mat on the top. The holes in the mat were approximately 0.6 cm^2 in area. About 50 seedlings

were placed on the mat in such a way that their roots were completely immersed in the induction medium. Composition of the induction medium was similar to that described for the leaves. The medium was maintained fully aerated during the induction period. After the required period of induction, the seedlings were washed with cold distilled water and then blotted dry. From these seedlings, 0-10 mm. root tips were excised. Routinely, the samples were quick frozen in liquid nitrogen, weighed and stored overnight at -20°C before extracting the enzyme.

Assay of the Nitrate Reductase

A. Extraction Procedure - An aliquot of 0.5 gm of leaf tissue was extracted in a cold mortar and pestle with the extraction medium. The extraction medium consisted of either 0.05 M phosphate buffer of pH 7.5 (Method I) or 0.1 M Hepe's buffer of pH 7.4 (Method II), plus 5×10^{-4} M EDTA and 10^{-3} M cysteine. In case of root tips, the whole sample of 50 root tips was extracted. Four ml. of cold extraction medium was added for each gram of tissue. The homogenate was then centrifuged for 30 minutes at 30,000 x g. The clear supernatant was used as enzyme preparation. The homogenates and extracts were kept cold (3-5°C) throughout.

B. Assay - The activity of nitrate reductase was determined by the method described by Wallace and Oaks (ms in preparation) as modified by Stevens (Ph.D. transfer report). In principle it involves the

measurement of the production of nitrite during the reaction. Two types of assay mixtures were used. We shall refer to them as Method I and Method II.

Method I. Phosphate buffer (0.05 M) of pH 7.8, 20 µmoles of KNO_3, 1.12 µmoles of NADH and the enzyme all in a total volume of 1.5 ml.

Method II. Hepes buffer (0.1 M) of pH 7.8, 20 µmoles of KNO_3, 5 µmoles of phosphate buffer, 0.56 µmoles of NADH and the enzyme all in a total of 1.5 ml.

The reaction was started by the addition of enzyme preparation. The assay was carried out for 30 minutes at 28°C. The reaction was stopped by adding 10 µmoles of oxalacetic acid and enough malic dehydrogenase to completely oxidize all the NADH in a 2 to 3 minute period. After that 1.0 ml of 1.0 percent sulphanilimide in 1.0 N HCl was added followed by the addition of 1.0 ml of 0.02 percent N-1-naphthyl ethylene diamine hydrochloride (NED) solution. After 30 minutes the absorbance was read at 540 nm. Readings were kept in the range of 0.15 to 0.60.

Protein concentrations in crude extracts were determined by Folin's method as described by Lowry et al., (1951). Bovine serum albumin was used as the standard.

Malic dehydrogenase used in the assay of nitrate reductase was extracted and purified by the

method described by Stevens (Ph.D. transfer report). The enzyme was extracted with 0.1 M phosphate buffer from young maize leaves. The precipitate obtained by adding 66 percent ammonium sulphate was dissolved in phosphate buffer and purified through sepadex G-25 column. The same phosphate buffer was also used as eluent. The activity of malic dehydrogenase in the purified preparation was measured by the oxidation of NADH. Decrease in absorbance was read at 340 nm. Aliquots of malic dehydrogenase were placed in small tubes and frozen for further use.

CHAPTER II

RESULTS

I. Effect of Nitrate on Germination of Seeds

Nitrate is known to induce the germination in certain seeds (Koller et al., 1962). In an attempt to study the role of nitrate in the early growth of maize embryo, its effect on germination of the seed was studied.

The results in table 1 show that nitrate has a slight promoting effect on the germination of maize seeds. This effect is seen whether the seeds are grown in vermiculite or on agar and whether in light or dark. In these tests, seeds germinated in the dark for 30 hours showed a higher rate of germination than seeds grown on a 16 hour light and 8 hour dark regime. Because of this observation other tests were performed in the dark.

The results in table 2 show that the stimulation of 10 to 15 percent is specific for the nitrate ion. Sodium nitrate was as effective as potassium nitrate whereas potassium or sodium chloride were without effect.

The effect of various concentrations of nitrate were tested next and the results are presented in table 3. They show that 5, 10 or 20 mM nitrate stimulated germination. With 50 mM nitrate, however, germination was inhibited by 4 percent at

TABLE 1

Effect of Nitrate on the Germination of Maize Seeds

Growth Medium	Photoperiod	Percentage of seeds germinated Hr. after planting					
		30		48		72	
		$-NO_3$	$+NO_3$	$-NO_3$	$+NO_3$	$-NO_3$	$+NO_3$
Vermiculite	16 hrs light	37	48	89	94	95	96
	dark	65	75	100	97	98	99
Agar	16 hrs light	44	60	88	94	98	96
	dark	78	88	98	99	100	98

Nitrate was supplied at concentration of 10 mM with 1/10 strength Hoagland's solution. Seeds were grown at 26°C.

TABLE 2

Effect of Different Salts on the Germination of Maize (Var. W64A x W182E) Seeds

Salts	Percent germination. Hrs. after planting			
	30	40	50	60
None	4	45	70	90
Potassium chloride	0	34	69	92
Potassium nitrate	8	56	84	97
Sodium nitrate	7	56	81	95
Sodium chloride	6	44	71	94

Seeds were germinated at 20°C in the dark. Salts were provided at the concentration of 10 mM with 1/10 strength Hoagland's solution.

TABLE 3

Effect of the Concentration of Nitrate on the Germination of Maize (Var. W64A x W182E) Seeds

Concentration of nitrate mM	Percentage germination Hr. after planting			
	30	40	50	60
0	4	33	66	88
5	11	49	83	93
10	10	45	79	96
20	7	52	84	91
50	7	43	62	84

Seeds were germinated at 20°C in the dark. Desired concentrations of potassium nitrate were supplied with 1/10 strength Hoagland's solution.

TABLE 4

Effect of Nitrate on the Germination of Different Varieties of Maize Seeds

Varieties	Percent germination. Hr. after planting					
	$-NO_3$			$+NO_3$		
	30	40	50	30	40	50
W64A x W182E	4	41	68	5	51	79
W64A x A635	4	40	80	9	50	93
W59M x W117	3	30	49	7	49	82
A495 x C06124	9	48	83	10	60	84
SL510	2	29	59	1	40	68
C0106 x C0303	0	27	56	1	41	67
W64A wild	2	31	66	0	38	76
W64A opaque-2	0	21	56	0	43	65

Seeds were grown at 20°C in the dark. Concentration of nitrate provided was 10 mM.

60 hours. In further experiments with germination, 10 mM nitrate was used because this concentration was sufficient to give a maximum effect but not high enough to cause an inhibition.

The effect of nitrate on the germination of different varieties of maize seeds was studied and the results are presented in table 4. There was little difference in the magnitude of the stimulation, with the exception of the hybrid line W59M x W117. In this case nitrate stimulated germination by 19 to 33 percent.

These observations show that nitrate has definite positive effect on the germination of different varieties of maize seed.

II. Effect of Nitrate on Seedling Growth

Application of nitrate as fertilizer is known to induce the growth and dry matter of plants (Gasser and Iordanou, 1967). Hageman and Flesher (1960) have shown that application of nitrate increases the protein levels of shoots from maize seedlings. Since nitrate promotes the germination of maize seed, its effect on the further growth of embryo axis was studied.

The results presented in the figures 1 and 2 show that nitrate has no effect on growth of the embryo up to 6 days. In hybrid maize, there is a 12 to 15 percent increase in the fresh weight of embryos from nitrate treated seedlings, after 6 days. The effect of nitrate

Figure 1. Effect of nitrate on the growth of embryonic axis and the primary leaf of maize.

The plant material was Zea mays L. var. W64A x W182E. With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required. Growth conditions are described in the text.

In the figure, 0 day for leaf age represents 4 day old seedling. The dotted lines represent minus nitrate condition and the solid lines plus nitrate condition. Fresh weight of the embryonic axis is shown by circles and that of primary leaves by squares. Each point represents the average from four experiments.

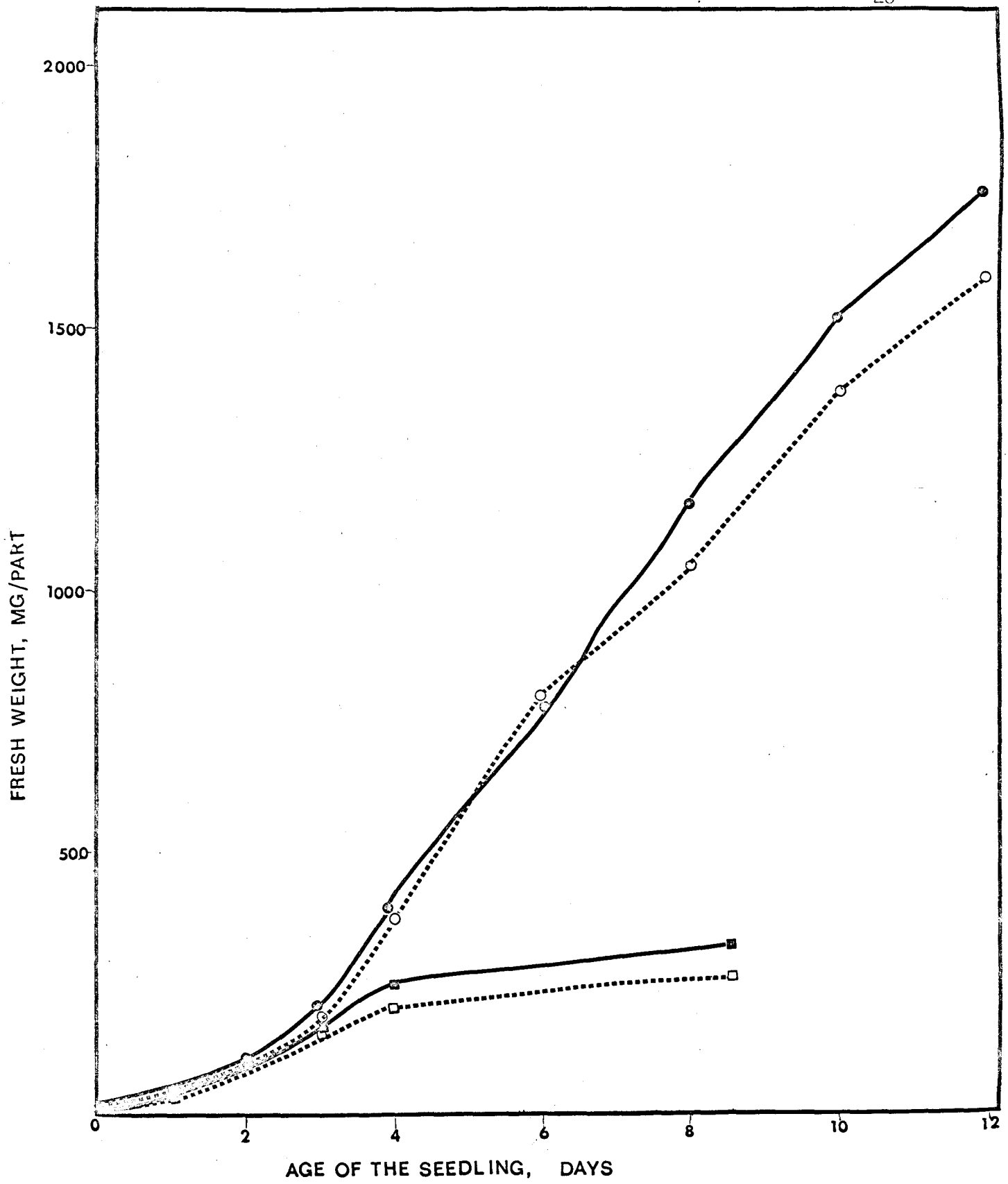
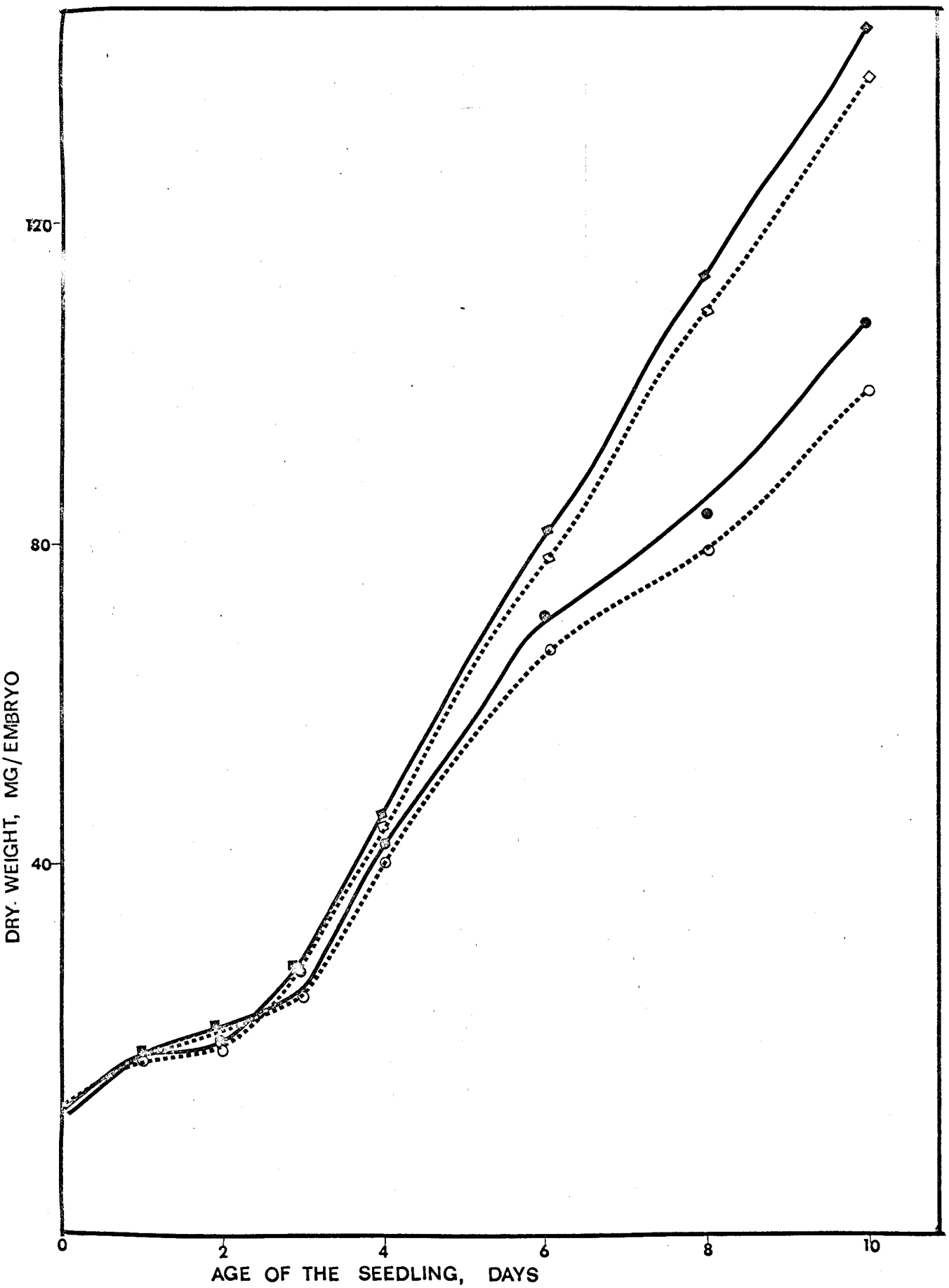

2000
1500
1000
500
FRESH WEIGHT, MG/PART
0
2
4
6
8
10
12
AGE OF THE SEEDLING, DAYS

Figure 2. Effect of nitrate on the growth of embryonic axis of two inbred lines of maize.

The plant materials were Zea mays L. var. W64A wild type and its isogenic mutant opaque-2. With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required. Growth conditions are described in the text.

In the figure, dotted lines show minus nitrate condition and solid lines plus nitrate condition. Dry weight of opaque-2 embryo is shown by circles and that of W64A wild type by squares. Each point is the average from two experiments.


120
80
40
DRY. WEIGHT, MG/EMBRYO
0
2
4
6
8
10
AGE OF THE SEEDLING, DAYS

on the dry weights of two inbred lines - W64A and its mutant opaque-2 is also similar. The general growth of the wild type is better than the opaque-2.

In the course of this experiment, it was observed that nitrate has no effect on the emergence and opening of the primary leaf. In both treatments, the primary leaf emerges above the ground on the fourth day, while it is still enclosed in the coleoptile sheath. It is fully expanded by the sixth day in the hybrid and W64A - wild type and by the seventh day in opaque-2.

The effect of nitrate on the growth of primary leaf was studied. The growth of primary leaf is not affected by nitrate up to six days (figure 1). The fresh weights of leaves from 7, 8 and 12 day old seedlings is stimulated by 18 to 27 percent, when nitrate is supplied. After 8 days, growth of the leaf is very slow in both treatments.

These experiments indicate that nitrate has no effect during the early rapid growth of the embryo or primary leaf. There is a clear cut effect only after the initial rapid growth phase.

III. Effect of Nitrate on the Nitrogen Balance of the Seedlings

1. Loss of Nitrogen from the Endosperm - In the hybrid maize, there is no transport of nitrogen from endosperm to the embryo during the first 2 days (table 5). On the other hand, there is significant redistribution

TABLE 5

Transport of Nitrogen from Endosperm to the Embryo of Growing Seedlings of maize var. W64A x W182E

Age of the Seedling Days	Milligram of nitrogen per part							
	Endosperm				Embryo			
	Soluble	Insoluble	Total	Total loss each day	Soluble	Insoluble	Total	Total Gain each day embryo + scutellum
0	1.04	3.67	4.71	-	0.01	0.17	0.18	-
1	1.36	3.28	4.63	0.08	0.02	0.16	0.18	0.02
2	1.69	2.98	4.67	0.04	0.10	0.09	0.19	0.031
3	1.87	2.31	4.17	0.50	0.22	0.45	0.67	0.52
4	1.56	1.61	3.16	1.01	0.63	0.96	1.59	0.98
6	0.91	0.70	1.61	1.55	1.05	2.48	3.53	1.37
8	0.66	0.47	1.12	0.49	1.18	2.60	3.78	0.63
10	0.30	0.31	0.61	0.51	1.29	3.45	4.74	0.47
12	0.21	0.19	0.40	0.21	1.18	3.62	4.74	0.018
			TOTAL	4.310				4.017

Seedlings were grown at 26°C with 1/10 strength Hoagland's solution without any nitrate.

of nitrogen between soluble and insoluble components of both parts. Insoluble nitrogen of the endosperm starts decreasing on the very first day and there is an equal increase in the soluble component. On the second day, an increase of 13 percent from zero time is observed in the soluble nitrogen. After 4 days, the rate of loss from the endosperm is equal to the rate of solubilization of nitrogen in the endosperm. Both soluble and insoluble components decrease at almost the same rate. Almost 65 percent of the total nitrogen is transported out of the endosperm by 6 days. By 8 days, only 23 percent of the original nitrogen remains in the endosperm. After this time, there is little further decrease. The residual nitrogen may be unavailable to the embryo, due to its complex form or to slower proteolytic activities in the endosperm. The supply of exogenous nitrate does not affect the loss or distribution of nitrogen in the endosperm.

2. Gain of Nitrogen in the Embryo - The increase in embryo nitrogen can be divided into 3 phases: (i) a phase of no change (ii) a phase of rapid increase and (iii) a phase of slow increase. The initial phase of no change is completed in 2 days of seedling growth. During this period, there is no gain in the total and protein nitrogen of the embryo. On the other hand, there is a significant redistribution between

soluble and insoluble components of the embryo. As seen in table 5, by the second day about 50 percent of the total embryo nitrogen is in the soluble form as compared to only about 6 percent at zero time. It appears that any increase in the fresh weight of embryo axis during this period (phase of no change) is supported by the nitrogen of the embryo itself.

During the phase of rapid increase from 2 to 6 days, there is a rapid increase in the total and protein nitrogen of the embryo. Table 5 shows that the total nitrogen of 6 day old embryo is 19 times higher than that of 2 days. This increase is correlated to a similar decrease in the total nitrogen of the endosperm. During this period, more than 60 percent of the total embryo nitrogen is protein. Thus it represents a phase of real growth.

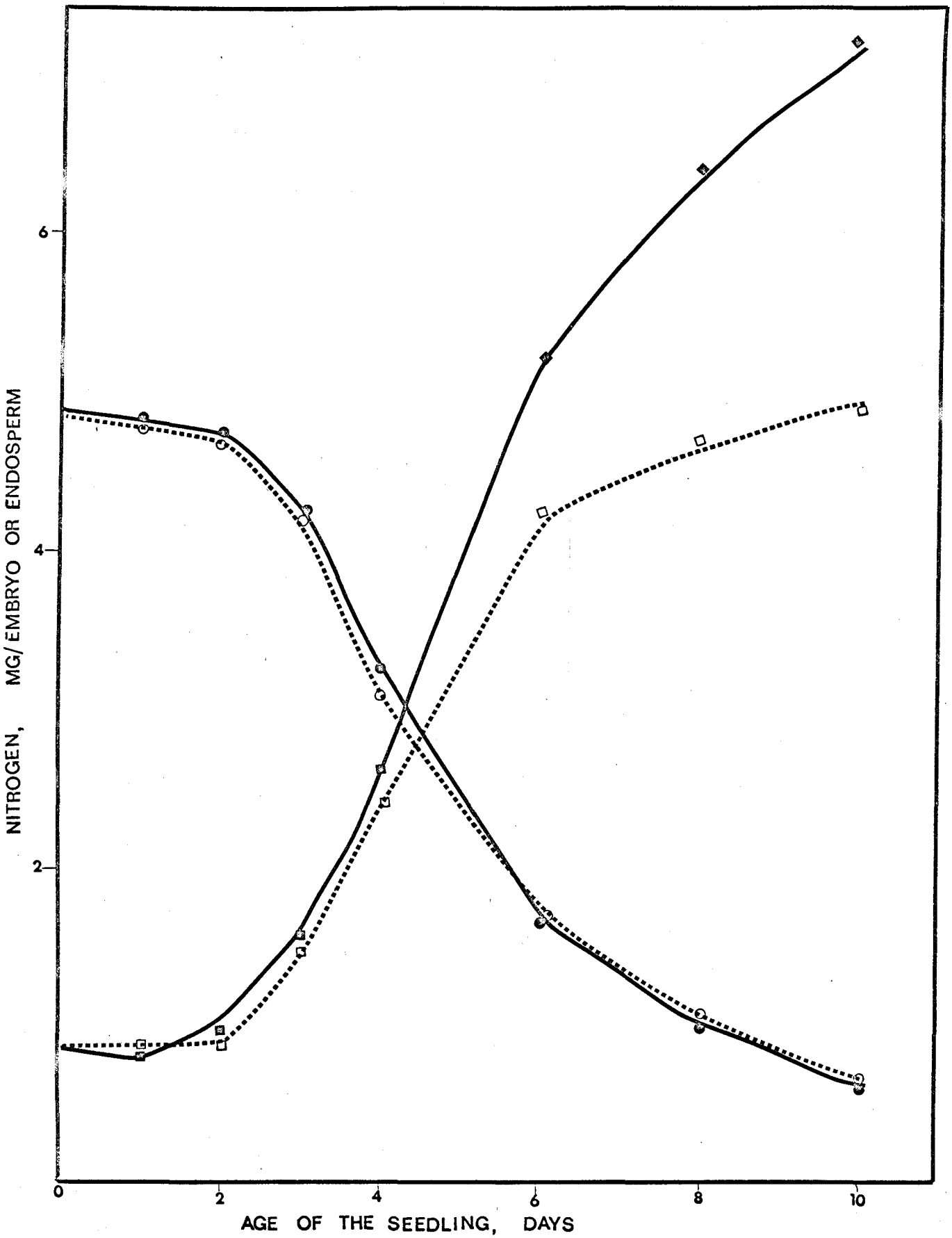
After 6 days, growth of the embryo is slow. There is very slow increase in the total nitrogen of the embryo between 6 and 10 days. There is no increase in the total or protein nitrogen after 10 days. On the other hand, soluble nitrogen levels off, after 6 days. At the end of third phase, soluble nitrogen of the embryo is only about 20 percent of the total nitrogen.

Figure 3 shows that, when exogenous nitrate is supplied during the growth of the seedling, there is no increase in the total nitrogen during the initial phase. Also during the rapid increase in nitrogen

Figure 3. Effect of nitrate on the transport of nitrogen from endosperm to the embryo and total nitrogen of the embryo.

Plant material and the growth conditions are same as in figure 1.

In the figure, dotted lines represent minus nitrate condition and the solid lines plus nitrate condition. Loss of nitrogen from the endosperm is shown by circles and gain in embryo + scutellum by squares. Similar results were obtained in three experiments.


NITROGEN, MG/EMBRYO OR ENDOSPERM
6
4
2
0
2
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10
AGE OF THE SEEDLING, DAYS

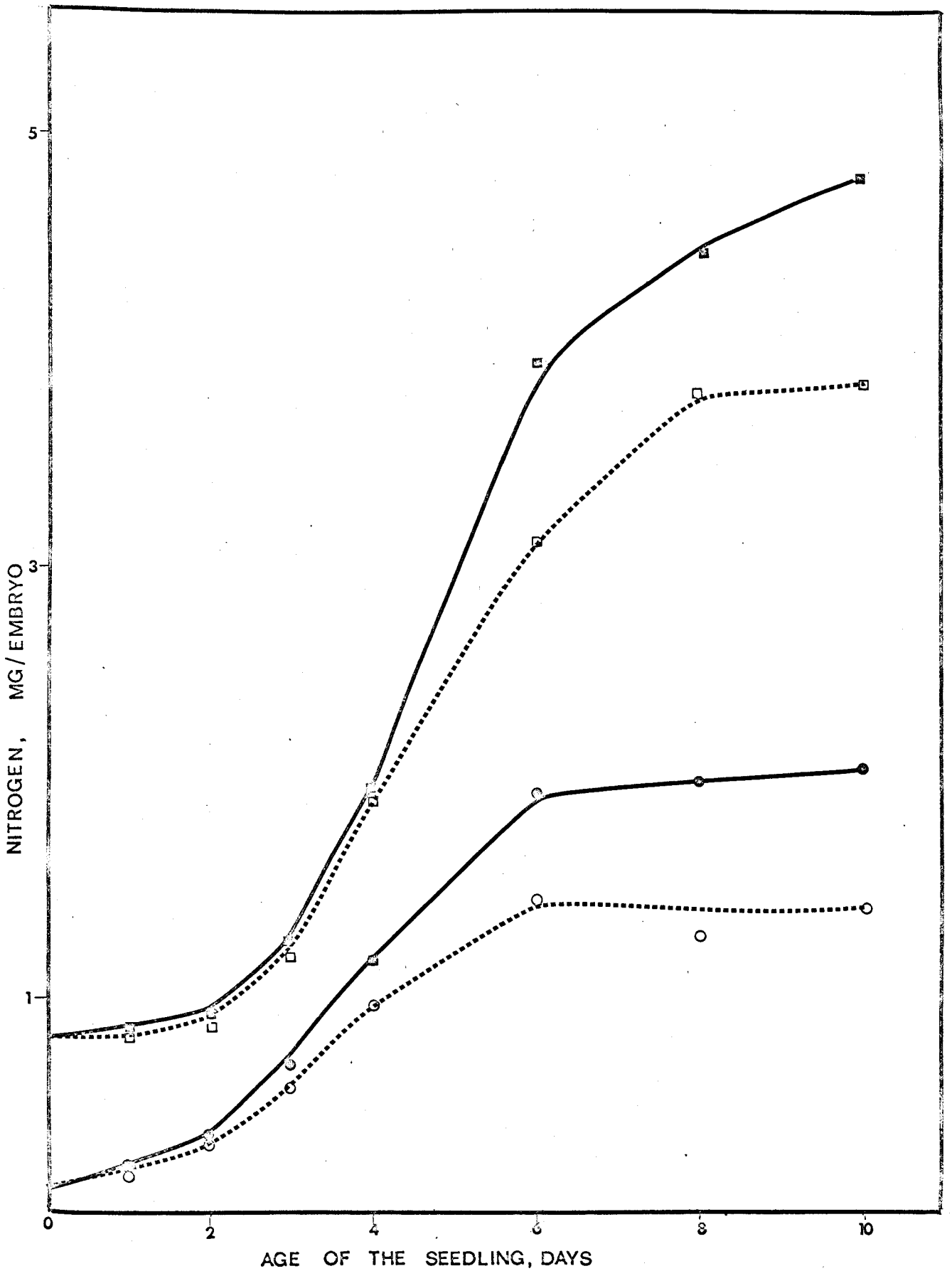
(phase 2), added nitrate causes a slight further stimulation of the total nitrogen content. As shown in figure 4, this increase is mainly in the soluble fraction. The results do show, however, that after the period of rapid increase, there is a considerable enhancement of both total and protein nitrogen caused by the addition of nitrate. Although the total and protein nitrogen of the nitrate supplied embryo increase during the third phase of growth, there is progressive decrease in the rate of increment. Soluble nitrogen increases very little with added nitrate up to 8 days and after that it is almost constant. Thus during this period, the exogenous nitrate is actively incorporated into protein. During this period, the embryo is a very complex organism. Some of the older roots and leaves start senescing and supply organic nitrogen to the younger regions. From our experiment, it is difficult to determine, whether the increase in embryo protein is actually due to new synthesis from exogenous nitrate or due to prevention of protein degradation in the senescent organs.

3. Nitrogen contribution of the Scutellum - The scutellum is the modified single cotyledon of monocots. In maize, the dormant embryo is embedded in the scutellar tissue. The scutellum plays an important role in the transfer of nitrogen from endosperm to the embryo. It contains about 12 percent

Figure 4. Effect of nitrate on the soluble and protein nitrogen levels of the growing embryo.

Plant material and the growth conditions are same as in figure 1.

In the figure, dotted lines represent minus nitrate condition and solid lines plus nitrate condition. Circles represent the soluble component while the squares represent the protein nitrogen. Similar results were obtained in three experiments.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NITROGEN, MG/EMBRYO
AGE OF THE SEEDLING, DAYS

of the total nitrogen of the maize seed. The embryo contains 3.4 percent and the remainder of the nitrogen is in the endosperm. As about 95 percent of the scutellar nitrogen is in the protein form, it is possible that during very early growth of the embryo, the scutellum may support the nitrogen requirement.

The nitrogen balance of the scutellum was studied for 12 days during the seedling growth (figure 5). There is a slight increase in the soluble nitrogen of the scutellum during the first 2 days. This is correlated to an almost similar decrease in the protein nitrogen. After the second day both components increase a little bit up to 4 days. At 4 days, there is approximately a 23 percent increase in the total nitrogen of the scutellum. This increase in the scutellum is apparently due to transport from the endosperm (table 6). Ingle et al., (1964) also observed that the total nitrogen content of maize scutellum increases slightly after 2 days, and it is maintained for at least 5 days. After 4 days, there is rapid decline in the total nitrogen of the scutellum and at 12 days, there is only about 27 percent of the original nitrogen left in the scutellum. This loss of nitrogen is predominantly from the protein fraction of the scutellum. At 12 days, the amount of nitrogen in protein and soluble fractions are almost the same. During the loss of nitrogen from the scutellum, there is increase in the embryo nitrogen (table 6). Thus

Figure 5. Nitrogen balance of the scutellum in a growing maize seedling and effect of nitrate.

Plant material and the growth conditions are same as in figure 1.

Solid lines in the figure represent plus nitrate condition and dotted lines minus nitrate condition. The lines represent:

1. Total nitrogen
2. Protein nitrogen
3. Soluble nitrogen

Similar results were obtained in two experiments.

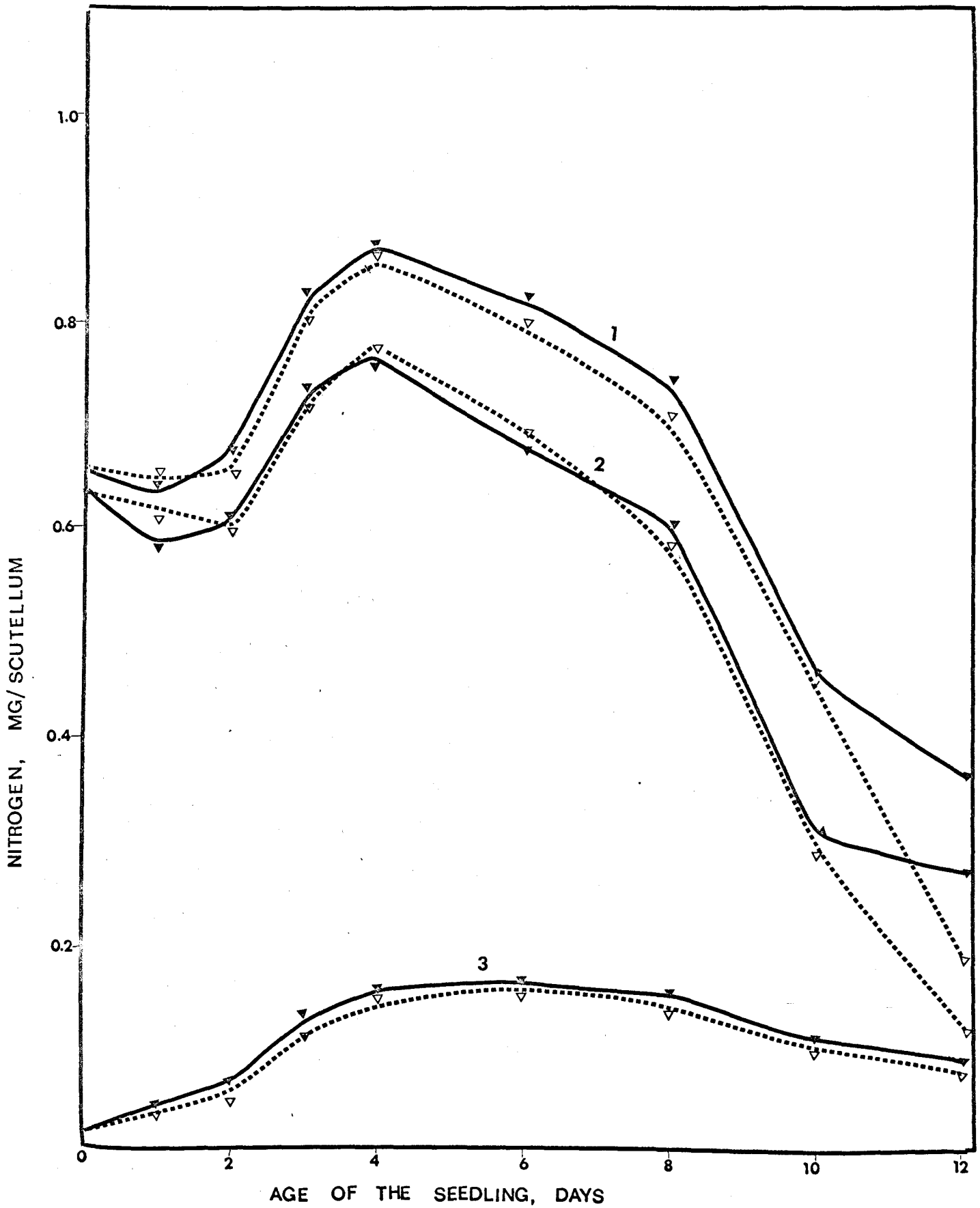

1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
1
2
3
NITROGEN, MG/SCUTELLUM
AGE OF THE SEEDLING, DAYS

TABLE 6

Nitrogen Contribution of the Endosperm to the Growing Embryo

Age of the Seedling days	Milligrams nitrogen per part		
	Total loss of nitrogen from the endosperm	Change in the nitrogen level of the scutellum	Gain in the total nitrogen of the embryo
2	0.041	+0.002	0.01
4	1.63	+0.23	1.41
8	3.67	+0.14	3.60
12	4.32	-0.57	4.56

Absolute values of nitrogen on 0 times are:

Endosperm = 4.715

Scutellum = 0.657

Embryo = 0.190

when the endosperm nitrogen has been used up, the scutellum supplies nitrogen to the embryo.

Figure 5, also shows that the nitrogen balance of the scutellum is not affected by exogenous nitrate up to 10 days. When nitrate is supplied, the loss of nitrogen from the scutellum after 10 days, is prevented. This may indicate that the scutellum serves as nitrogen source for the embryo, only when there is no alternate source of nitrogen.

4. Effect of Nitrate on Protein Content of Primary Leaves

For studying the effect of concentrations of nitrate on protein, primary leaves were selected as models of embryo behaviour. Like the embryo, they have a period of rapid increase in nitrogen and then a period of slow increase. On the other hand, they are easier to handle because of the size. In their development also, they are not as complex as the embryo.

The effect of nitrate on the protein levels of the primary leaf is presented in the figure 6. In the control experiment the soluble and the protein nitrogen of the primary leaf increases rapidly between 4 to 7 days of seedling growth. During this period there is almost a 9 fold increase in the soluble nitrogen. The protein nitrogen increases as much as 8 fold during the same 3 day period. Between 7 and 8 days, both nitrogenous components of the leaf

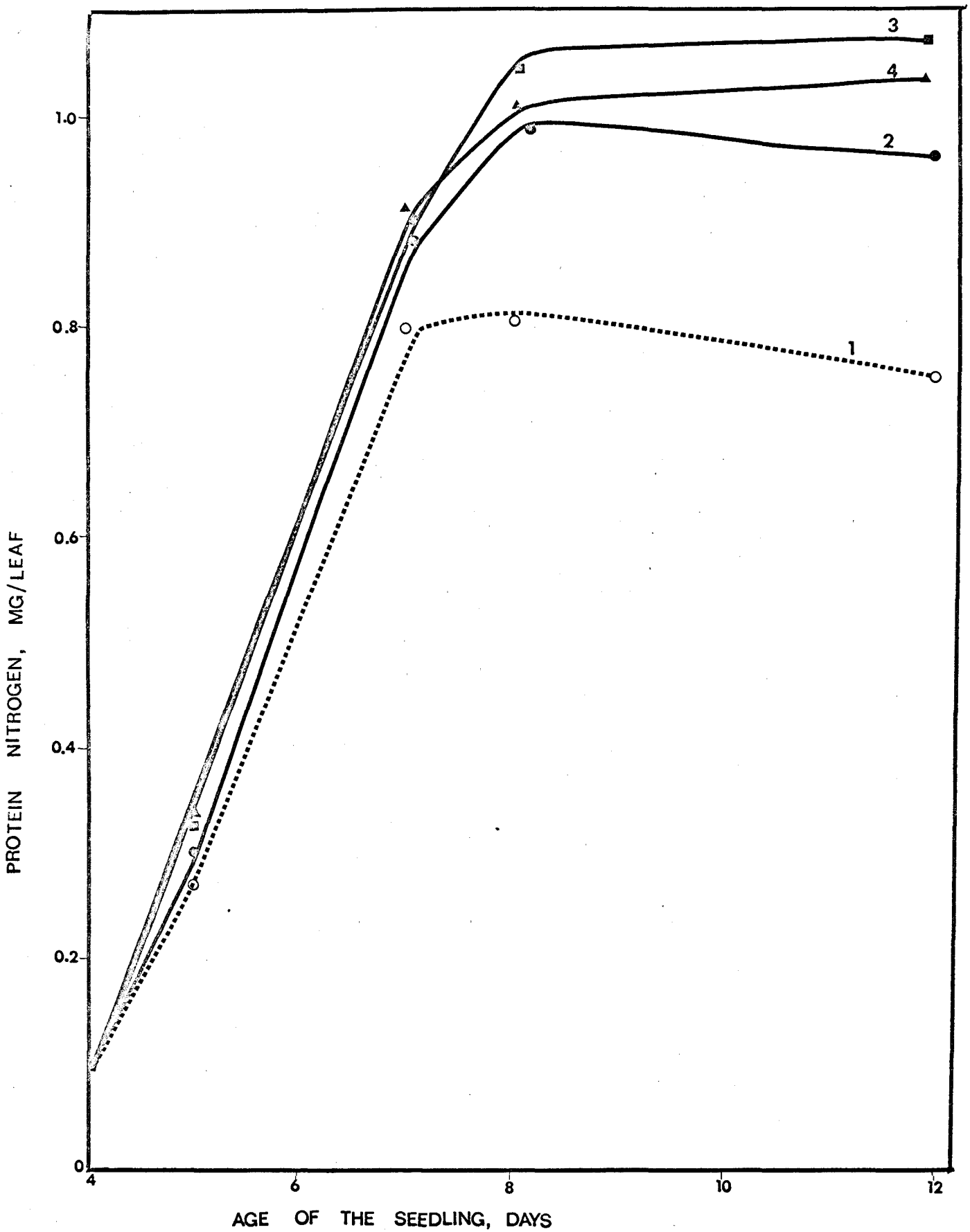
Figure 6. Effect of nitrate concentration on the protein levels of developing primary leaf.

Plant material was the hybrid W64A x W182E. Seedlings were grown without any nitrate up to 3 days. Desired concentrations of nitrate were supplied with 1/10 strength Hoagland's solution to the seedlings on the fourth day and onwards. Growth conditions are the same as described in the text.

In the figure lines represent:

1. No nitrate
2. 10 mM nitrate
3. 25 mM nitrate
4. 50 mM nitrate

Each point in the figure is the average from three experiments.


3
4
2
1
1.0
0.8
0.6
0.4
0.2
0
4
6
8
10
12
PROTEIN NITROGEN, MG/LEAF
AGE OF THE SEEDLING, DAYS

increase by only 2 to 3 percent. After 8 days, the protein level of the leaf does not increase and in fact on the 12th day, there is a loss of approximately 10 percent. During this period, there is an almost similar increase in the soluble nitrogen of the leaf (table 7).

When exogenous nitrate is supplied to the seedling, it has no effect on the total protein of primary leaf, up to 7 days. On the 7th day, there is only a 10 to 15 percent increase over the control value. Table 7 shows that, the soluble nitrogen of the leaf increases considerably, one day after adding nitrate and continues to increase for the entire period. The protein content of the primary leaves from 8 day old seedlings is 23 to 27 percent higher than the control. This elevated level is maintained up to 12 days, at least. This indicates that nitrate increases the protein level of primary leaf only after the rapid increase in leaf nitrogen.

From figure 6, it is apparent that there is not much difference in the protein levels of leaves supplied with different concentrations of nitrate. The maximum effect on the protein level of the primary leaf is obtained with 10, 25 or 50 mM nitrate. With 10, 25 and 50 mM nitrate, soluble nitrogen of the leaf from 8 day old seedlings is increased up to 100, 117 and 167 percent respectively. As compared to increase in the protein levels of the leaves of same

TABLE 7

Effects of Nitrate Concentration on the Soluble Nitrogenous Component of the Primary Leaf

Concentration of NO_3 (mM)	mg Nitrogen per leaf at different ages of the seedlings Days				
	4	5	7	8	12
0	.019	.053	.181	.184	.219
10	-	.058	.297	.356	.453
25	-	.070	.319	.390	.565
50	-	.087	.350	.481	.580

Seedlings were grown without any nitrate up to 3 days. Desired concentrations of nitrate were supplied to the seedlings on the fourth day and onwards.

age, it is 4 to 6 times greater. This indicates that capacity to absorb and reduce exogenous nitrate in the primary leaf is quite adequate, but the incorporation of reduced nitrogen into the protein is restricted in some way.

The experiments described in this section suggest that rapid growth of maize embryo is entirely maintained by the nitrogen from the endosperm. During the rapid increase of the embryo nitrogen, nitrate has no effect on the nitrogen balance of the seedling. When increase in the nitrogen of the embryo slows down, nitrate increases the total and protein nitrogen of the embryo, without affecting the loss from the endosperm. Primary leaves also behave in the same way with the added nitrate. Nitrate does not increase the protein level of leaf during active growth. When the increase in the protein or total nitrogen of leaf is slow nitrate increases the protein level. Nitrate also delays the loss of protein in mature leaf.

IV. Effect of Nitrate on the Nitrogen Balance of the Opaque-2 Seedlings

Opaque-2 seeds have relatively less zein and relatively more glutelin than normal maize. Hence the distribution of amino acids supplied to the embryo is potentially abnormal. For example, the growing seedling will obtain relatively more lysine and tryptophan and relatively less leucine, alanine and proline. Embryo, under this stress may be able to

assimilate more nitrate to make up for deficits. To test this, the effect of nitrate on the nitrogen level of opaque-2 seedlings was studied. Control experiments were performed with W64A, an inbred line of maize which is isogenic with the opaque-2 mutant on hand.

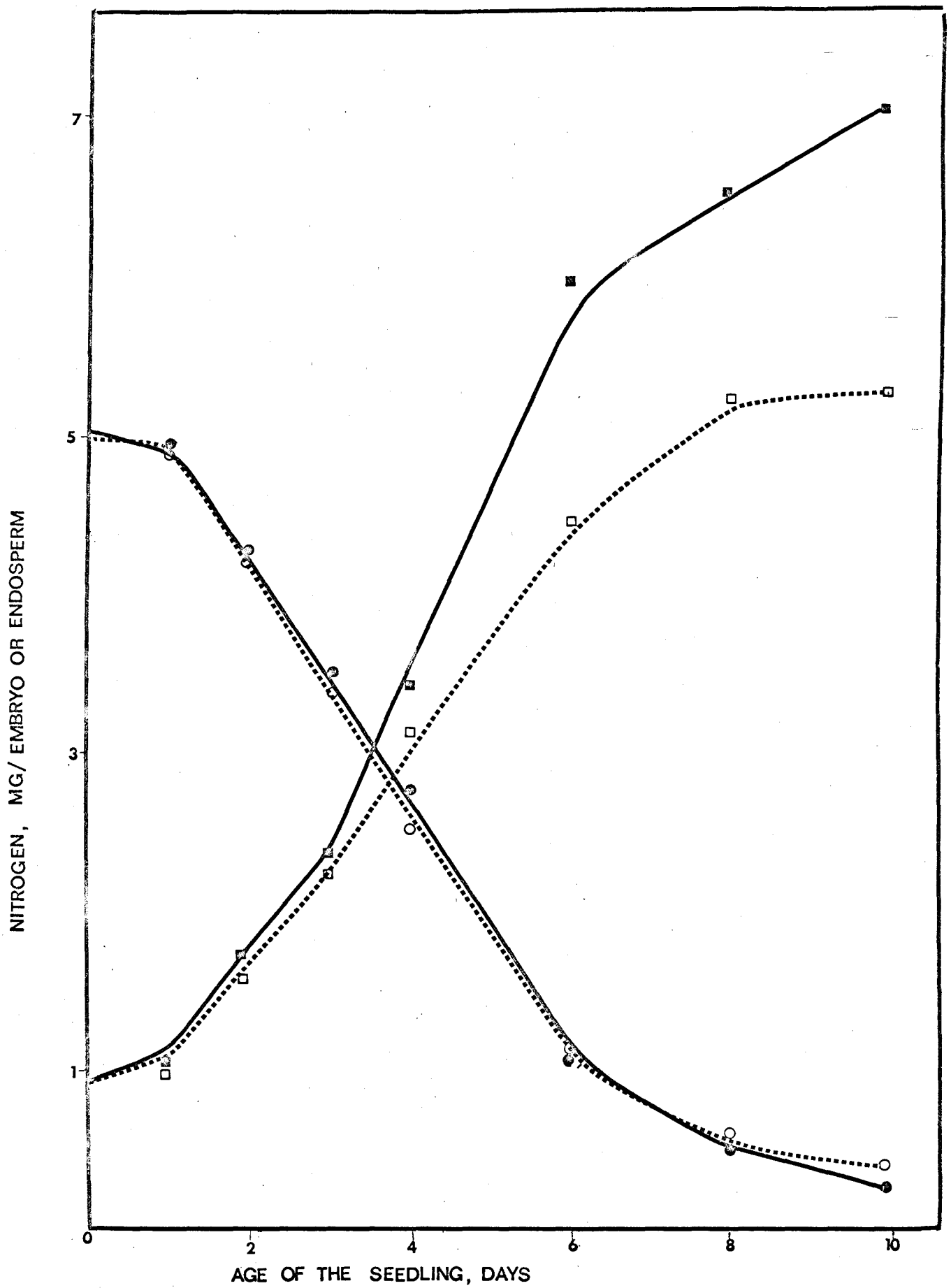
1. Loss of Nitrogen from the Endosperm - Figures 7 and 8 show the loss of nitrogen from the endosperm of W64A and opaque-2 mutant. The loss of nitrogen in both inbred lines is essentially similar. There is no decrease in the total nitrogen during the first day. The period of rapid loss is 1 to 8 days. After this time, about 80 percent of the endosperm nitrogen is lost, and there is very little further decrease. Except for the fact that the loss of nitrogen from the endosperm starts earlier than in the hybrid W64A x W182E, the general pattern of the loss is the same.

The distribution of nitrogen in the soluble and insoluble fractions of endosperm nitrogen in opaque-2 is different from the wild type, W64A. As shown in table 8, the initial soluble component of the opaque-2 endosperm is only about 20 percent of the total, whereas with the wild type it represents about 43 percent of the total. It increases slowly during the first 3 days in opaque-2, after which it starts declining. The insoluble nitrogen starts decreasing after the first day. A major portion of this is lost by the sixth day. By the tenth day only about 11 percent of the original insoluble nitrogen

Figure 7. Effect of nitrate on the transport of nitrogen from endosperm to the embryo and on total nitrogen of embryo.

Plant material was Zea mays L. var. W64A wild type. With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required.

Solid lines in the figure represent plus nitrate conditions and dotted lines minus nitrate conditions. Loss from endosperm is represented by circles and gain in embryo + scutellum by squares. Similar results were obtained in two experiments.


7
5
3
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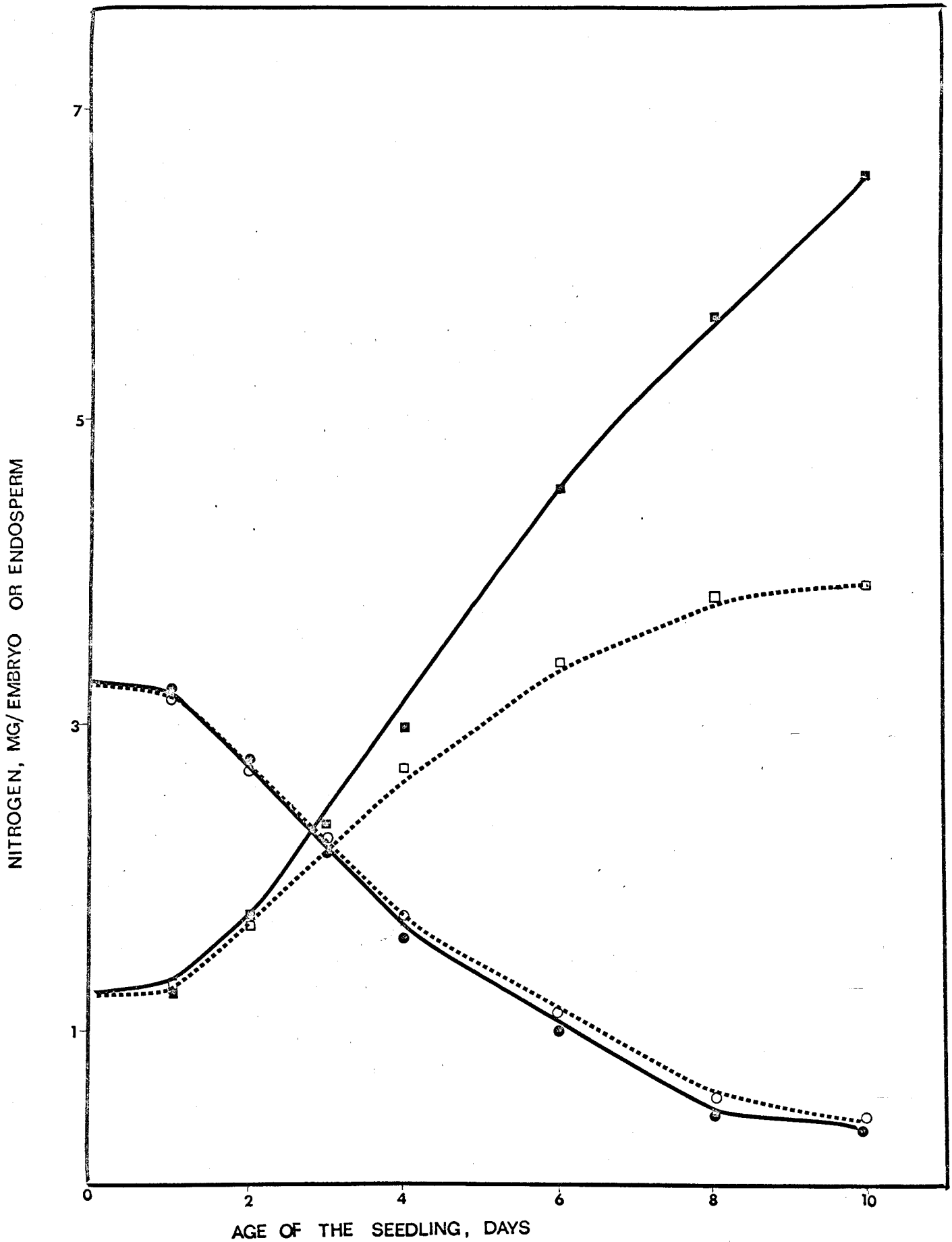
NITROGEN, MG/ EMBRYO OR ENDOSPERM
AGE OF THE SEEDLING, DAYS

Figure 8. Effect of nitrate on the transport of nitrogen from endosperm to the embryo and on total nitrogen of embryo.

Plant material was Zea mays L. var. W64A, opaque-2 mutant. With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required.

Solid lines in the figure represent plus nitrate conditions and dotted lines minus nitrate conditions. Loss from endosperm is represented by circles and gain in embryo + scutellum by squares. Similar results were obtained in two experiments.


NITROGEN, MG/ EMBRYO OR ENDOSPERM
7
5
3
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10
AGE OF THE SEEDLING, DAYS

TABLE 8

Transfer of Nitrogen from Endosperm to the Embryo of Growing Seedlings of Opaque-2 Maize

Age of the Seedling Days	Milligram of nitrogen per part							
	Endosperm				Embryo + Scutellum			
	Soluble	Insoluble	Total	Total loss each day	Soluble	Insoluble	Total	Total gain each day
0	0.70	2.56	3.26	-	0.14	1.11	1.25	-
1	0.72	2.53	3.25	0.01	0.22	1.04	1.26	0.01
2	0.82	2.02	2.84	0.41	0.46	1.24	1.70	0.44
3	0.86	1.42	2.28	0.56	0.61	1.60	2.21	0.51
4	0.79	0.99	1.78	0.50	0.79	1.87	2.66	0.45
6	0.44	0.68	1.12	0.66	0.88	2.43	3.31	0.65
8	0.20	0.40	0.60	0.52	0.68	3.14	3.82	0.51
10	0.13	0.30	0.43	0.17	0.66	3.24	3.90	0.08
		TOTAL		2.83				2.65

Seedlings were grown at 26°C with 1/10 strength Hoagland's solution without any nitrate.

is left in the endosperm.

Once again it is clear that an exogenous supply of nitrate has no effect on the solubilization or transfer of the endosperm nitrogen.

2. Gain of Nitrogen in the Embryo - As with the hybrid W64A x W182E, the increase in nitrogen of opaque-2 and its wild type embryo, may be divided into 3 phases. During the first day, there is no change in the total nitrogen of the embryo (figures 7 and 8). There is rapid increase in the nitrogen between 1 to 6 days. After 6 days, there is slow increase.

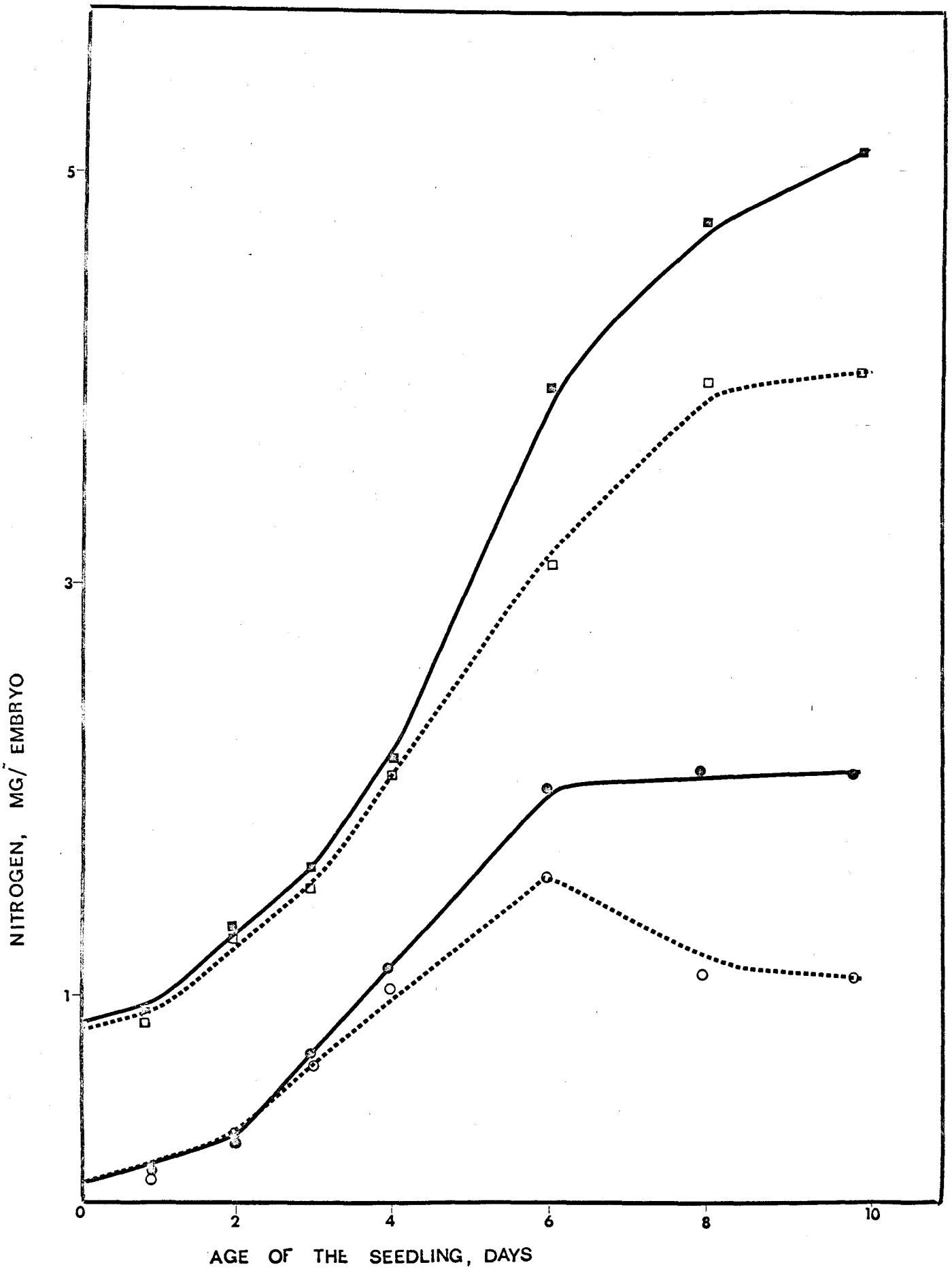
As shown in the figure 9, the soluble nitrogen of the W64A embryo starts increasing during the very first day of seedling growth. It continues to increase up to the sixth day and by that time it has increased approximately 14 times its original value. The soluble nitrogen of opaque-2 also increases between 1 and 6 days. By the sixth day, however, there is only a 6-fold increase in the soluble nitrogen of the opaque-2 embryo (table 8). In both inbred lines, the soluble nitrogen starts decreasing after 6 days. The protein nitrogen of W64A embryo does not change much during the first day. After that time it increases up to the eighth day. Increase in the protein nitrogen after 8 days is very slow. Increase in the protein nitrogen of the opaque-2 embryo is essentially similar to that of its control.

The effect of nitrate on the soluble and protein nitrogen of the embryos of the two inbred lines was also studied. The results are presented in figures 9 and 10. In W64A, nitrate has no effect on the nitrogen level of the embryo up to 4 days. The soluble and protein nitrogen of the embryo increases with the added nitrate after 4 days. When nitrate is supplied the total and protein nitrogen of the 6 day old embryo is 29 and 23 percent higher than the control. The level of protein and total nitrogen increases up to 10 days, but there is a progressive decrease in the daily increments after 6 days. Between 6 and 10 days, there is 1.3 fold increase in the protein nitrogen. In opaque-2 also, nitrate has no effect on the protein level up to 4 days. After that time, the positive effect of nitrate is better than the wild type, W64A. By the sixth day, the total and protein nitrogen of nitrate supplied embryos are 37 and 27 percent respectively higher than the control ones. Furthermore, the protein and total nitrogen of the opaque-2 embryo, increases linearly up to 10 days, when nitrate is supplied. There is approximately 2.4 fold increase in the protein nitrogen, between 6 and 10 days. The soluble nitrogen of the nitrate supplied embryo increases linearly up to 6 days. There is a further increase in this component up to 10 days, but at a slower rate. On the other hand, the soluble nitrogen of wild type, W64A, does not

Figure 9. Effect of nitrate on the soluble and protein nitrogen levels of growing embryo.

Plant material was Zea mays L. var. W64A wild type. With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required.

Solid lines in the figure represent plus nitrate conditions and dotted lines minus nitrate conditions. Circles indicate the soluble component while squares indicate the protein nitrogen. Similar results were obtained in two experiments.


NITROGEN, MG/ EMBRYO
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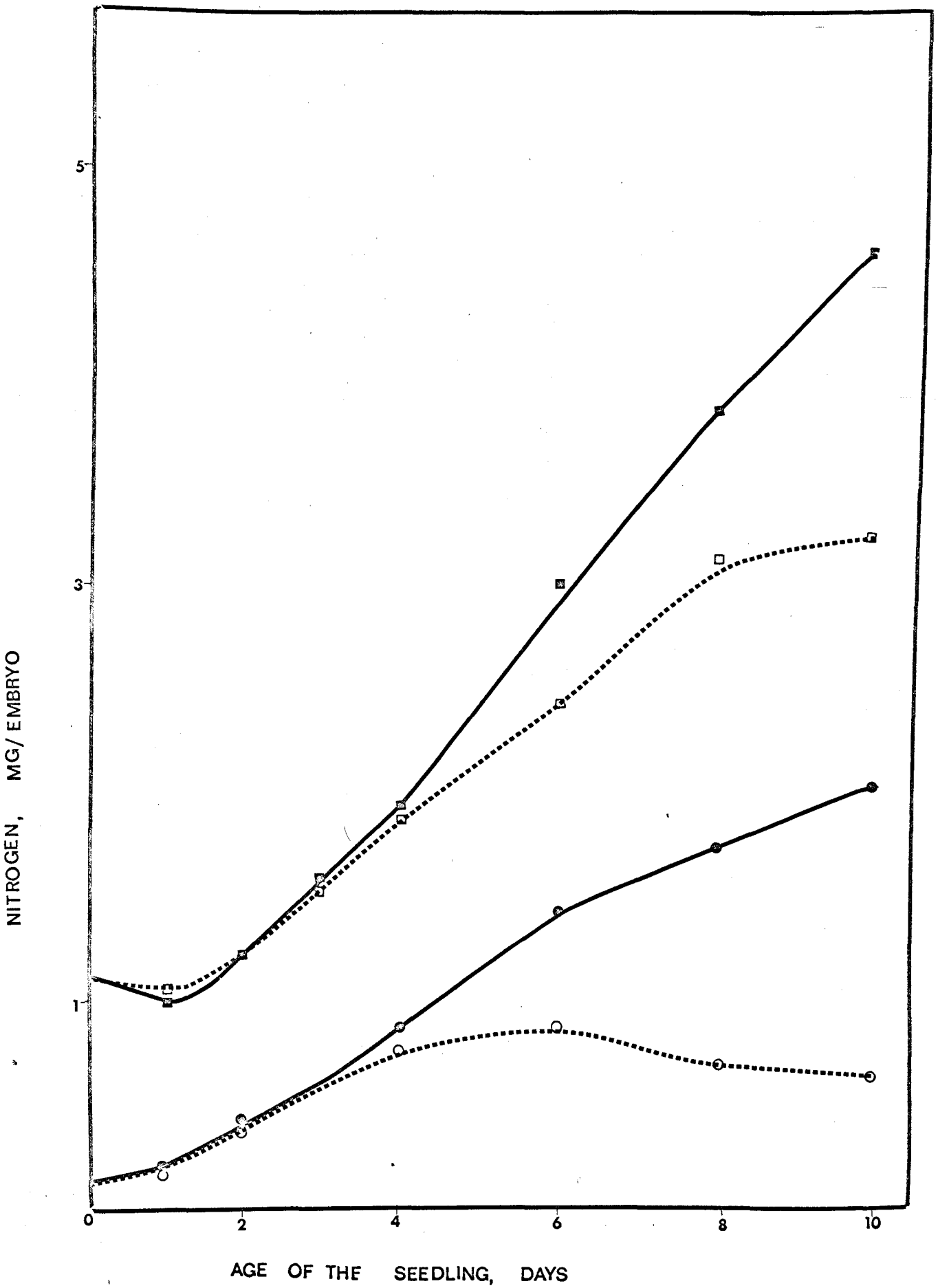
AGE OF THE SEEDLING, DAYS

Figure 10. Effect of nitrate on the soluble and protein nitrogen levels of growing embryo.

Plant material was Zea mays L. var. W64A opaque-2. With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required.

Solid lines in the figure represent plus nitrate conditions and dotted lines minus nitrate conditions. Circles indicate the soluble component while the squares indicate the protein nitrogen. Similar results were obtained in two experiments.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NITROGEN, MG/ EMBRYO
AGE OF THE SEEDLING, DAYS

increase after 6 days in similar treatments.

3. Chlorophyll Contents of the Primary Leaves -

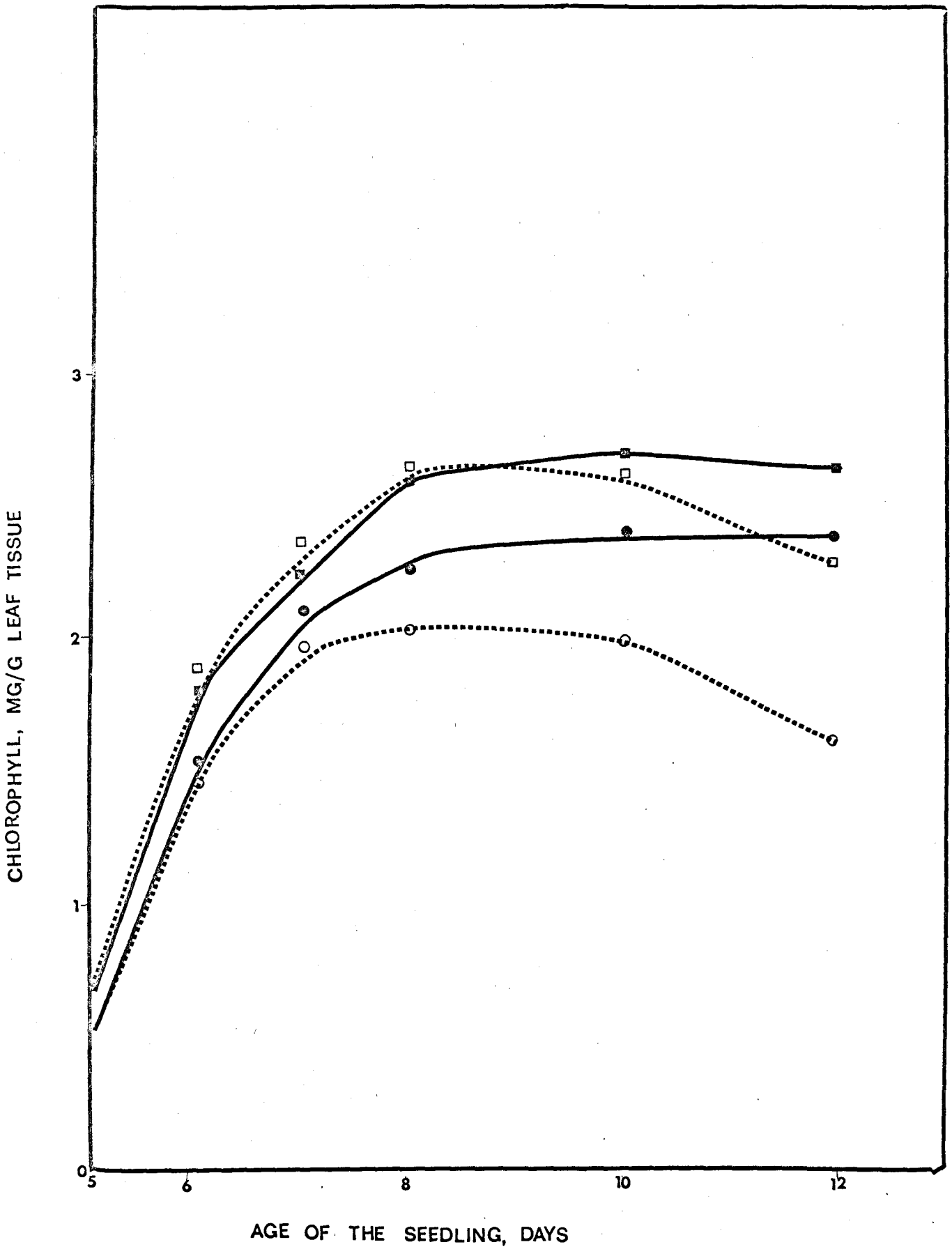
The studies of the chlorophyll content of primary leaves were undertaken as another parameter of the amino acid requirement of the opaque-2 embryo. If the essential amino acids are lower in amount, in this mutant, the chlorophyll content of the leaves may be lower than the wild type, W64A. Furthermore, if nitrate is being assimilated during the development of leaf, addition of nitrate may increase the chlorophyll content.

The effect of nitrate on the chlorophyll content of the primary leaves of two inbred lines is shown in figure 11. The chlorophyll content of the primary leaves of wild type, W64A increases up to 8 days. There is no further change in the amount of chlorophyll up to 10 days. Between 10 and 12 days, about 13 percent of the chlorophyll is lost. The amount of chlorophyll does not increase after 7 days in the leaves of opaque-2. There is no change between 7 and 10 days and after 10 days, it starts decreasing. About 20 percent of chlorophyll is lost between 10 and 12 days. The total amount of chlorophyll in the leaves of W64A is higher than opaque-2. On the eighth day, the primary leaves of W64A possess 31 percent more chlorophyll than those of opaque-2.

Figure 11. Effect of nitrate on the chlorophyll content of primary leaves of maize.

With 1/10 strength Hoagland's solution, 10 mM nitrate was supplied as required.

Solid lines represent plus nitrate conditions and dotted lines minus nitrate conditions. Changes in the chlorophyll content of opaque-2 is shown by circles, while that in W64A, wild type by squares. Each point is the average of two duplicate samples.


CHLOROPHYLL, MG/G LEAF TISSUE
0
1
2
3
5
6
8
10
12
AGE OF THE SEEDLING, DAYS

An exogenous supply of nitrate does not affect the development of chlorophyll in the wild type. However, it does prevent the loss between 10 and 12 days. In opaque-2, the chlorophyll content of primary leaves is not affected up to 7 days. After 7 days, addition of nitrate increases the level of chlorophyll. The chlorophyll content of nitrate supplied primary leaves from 8 and 10 day old seedlings are 15 and 21 percent higher than the control leaves of the same ages. There is no loss of chlorophyll between 10 to 12 days in nitrate treated leaves. Thus it appears that when chlorophyll level in the primary leaves of opaque-2 has plateaued nitrate increases the amount. The prevention of chlorophyll loss by nitrate in the mature primary leaves is observed in both inbred lines.

These experiments indicate that nitrate does not affect the nitrogen levels of opaque-2 embryo, during the initial phase or during the period of rapid increase. Unlike the hybrid, W64A x W182E or its wild type W64A, the increase in the total and protein nitrogen of the opaque-2 embryo, with added nitrate is linear up to 10 days. Thus nitrate has better positive effect during the period of the slow increase in nitrogen. Nitrate also increases the amount of chlorophyll in fully developed primary leaves of opaque-2. It prevents the loss of chlorophyll in the leaves from both inbred lines.

V. The Development of Nitrate Reductase in the Seedling

1. In Embryonic Axis - Assimilation of exogenous nitrate by the embryonic axis may be inhibited by amino acids coming from the endosperm. They may inhibit the uptake and/or reduction of the nitrate. A study of nitrate reductase was undertaken to determine whether the embryonic axis is capable of reducing exogenous nitrate or not.

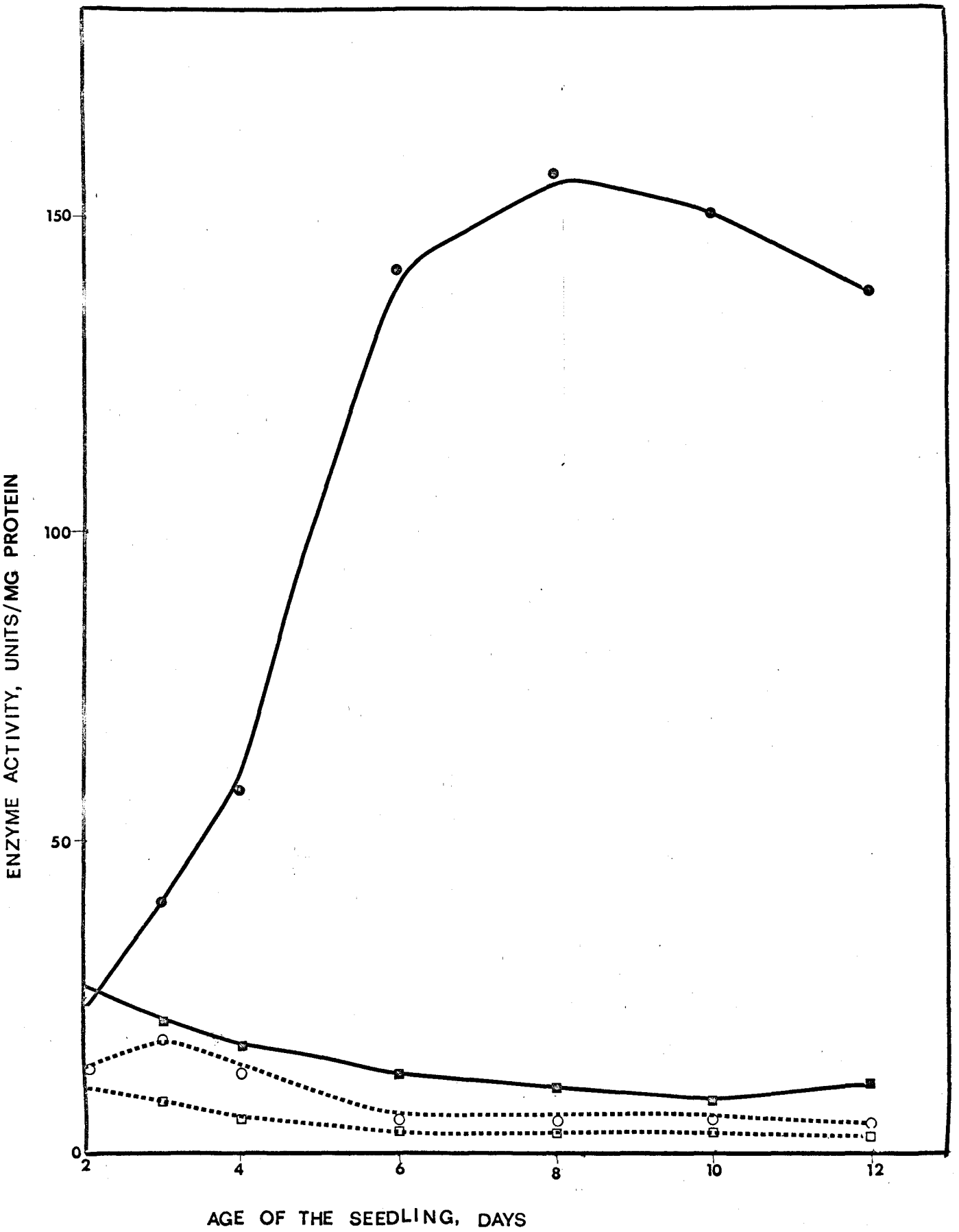
Maize seeds have very little endogenous nitrate (McNamara et al., 1971). Figure 12 shows that the initial activity in the shoot is about 2 times higher than in the root. This is probably because endogenous nitrate is preferentially translocated to the shoot. In the nitrate supplied seedlings, the enzyme activity in the shoot increases up to 8 days. In absolute values, the enzyme activity increases from 26 units to 157 units per milligram protein between 2 and 8 days. After 8 days, the enzyme activity on the second day is almost equal to the shoot of the same age. The activity in roots, however, starts decreasing on the third and subsequent days. After 12 days, its level is equal to that in the root and shoot of nitrate untreated seedlings. This trend in the induction of nitrate reductase in the seedling may suggest that most of the nitrate absorbed by the roots is translocated to the shoots, where it is reduced.

Figure 12. Development of nitrate reductase in the root and shoot of growing maize seedling.

Nitrate (10 mM) was supplied with additional molybdenum at 0.05 ppm in 1/10 strength Hoagland's solution. The enzyme was extracted and assayed according to method I. The reaction was terminated by adding 1 percent sulphanilimide in 1.0N HCl. Malic dehydrogenase and oxalacetic acid to oxidize residual NADH were not used in this assay.

In the figure, dotted lines show minus nitrate conditions and solid lines plus nitrate conditions. Circles represent the enzyme level in shoot while squares in the root. Each point is the average of two duplicate samples.

One unit of enzyme activity is equal to one n mole of nitrite produced per hour.


ENZYME ACTIVITY, UNITS/MG PROTEIN
150
100
50
0
2
4
6
8
10
12
AGE OF THE SEEDLING, DAYS

Since nitrate reductase is a substrate inducible enzyme, the development of nitrate reductase in nitrate treated seedlings, may suggest that nitrate is being absorbed. Furthermore, with an enzyme activity of 40 units in 3 day old shoots, approximately 72 n moles of protein may be synthesized per hour. As this activity was obtained, with the poor assay, a much more active enzyme in the shoot is expected. Thus it is possible that nitrate reductase may not be limiting in the overall assimilation of the nitrate.

2. In Intact Primary Leaves - Instead of whole root or shoot, the primary leaves were used for studying the induction of nitrate reductase in the embryonic tissue. They are less complex than the whole shoot or root. Studies were performed with young and mature leaves to evaluate the possible role of endosperm amino acids in the induction of nitrate reductase. If the amino acids coming from the endosperm, inhibit the induction of nitrate reductase, we may expect a lower induction in the younger than the older leaves.

Induction of nitrate reductase in the primary leaves from 5 day and 9 day old seedlings is shown in figures 13 and 14 respectively. The enzyme is inducible in both young and mature primary leaves. The enzyme is induced about 3-fold in 3 hours in both leaves. Although the enzyme activity increases up to 24 hours, the rate of induction after 3 hours is lower. In young leaves, with 10 mM nitrate, the

Figure 13. Induction of nitrate reductase in attached primary leaves from 5 day old seedlings.

The seedlings were watered with desired concentrations of nitrate in 1/10 strength Hoagland's solution. The solution was also supplemented with 0.05 ppm of molybdenum. Growth conditions are described in the text. The dark periods during the induction, were between 13 and 21 hours and again between 37 and 45 hours. The enzyme was extracted and assayed by method I. Malic dehydrogenase and oxalacetic acid were added routinely to oxidize residual NADH.

In figure the lines represent:

1. No nitrate
2. 10 mM nitrate
3. 25 mM nitrate
4. 50 mM nitrate

Unit of enzyme activity is defined in figure 12.

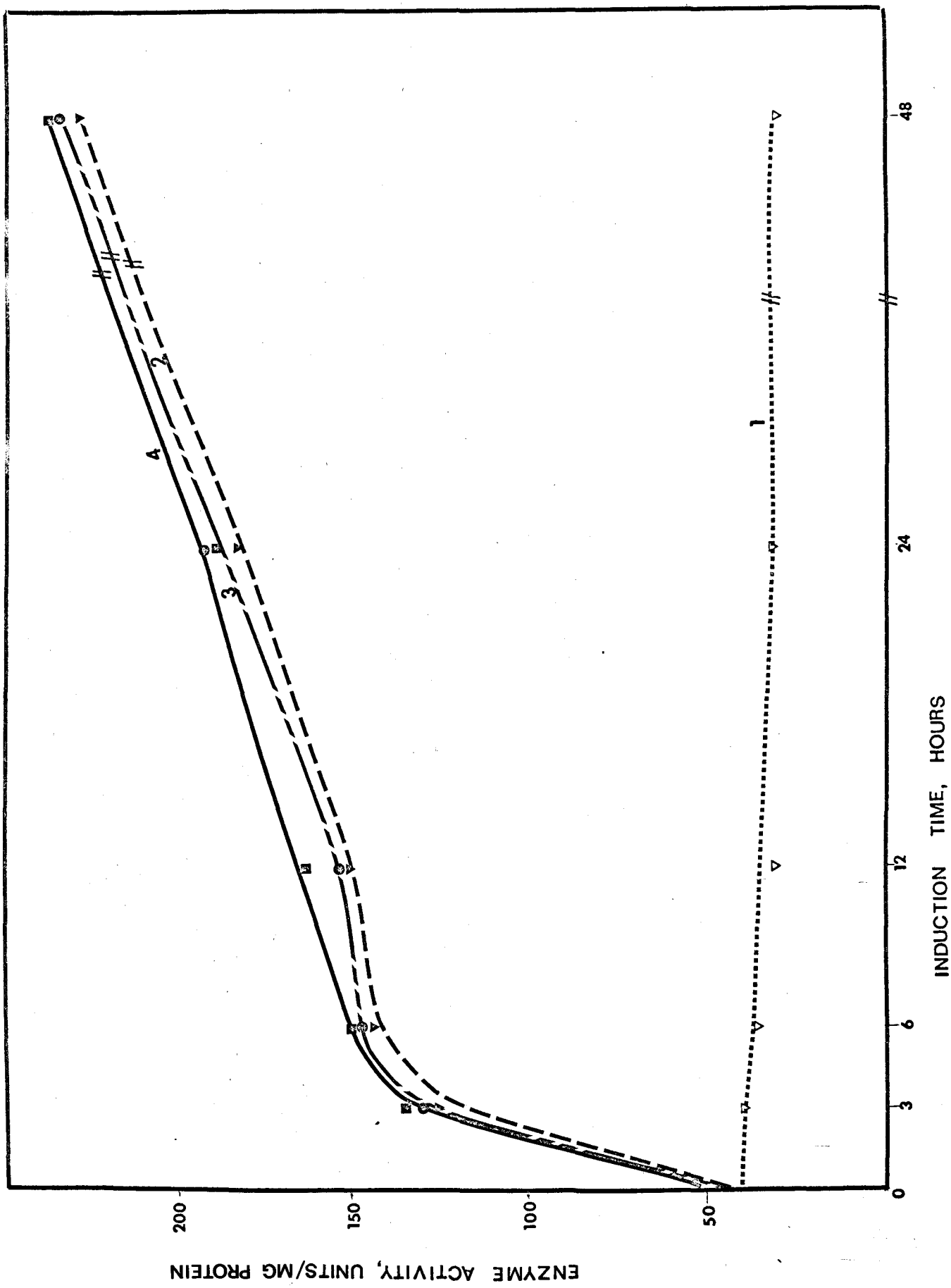

ENZYME ACTIVITY, UNITS/MG PROTEIN
200
150
100
50
0
3
6
12
24
48
INDUCTION TIME, HOURS
1
2
3
4

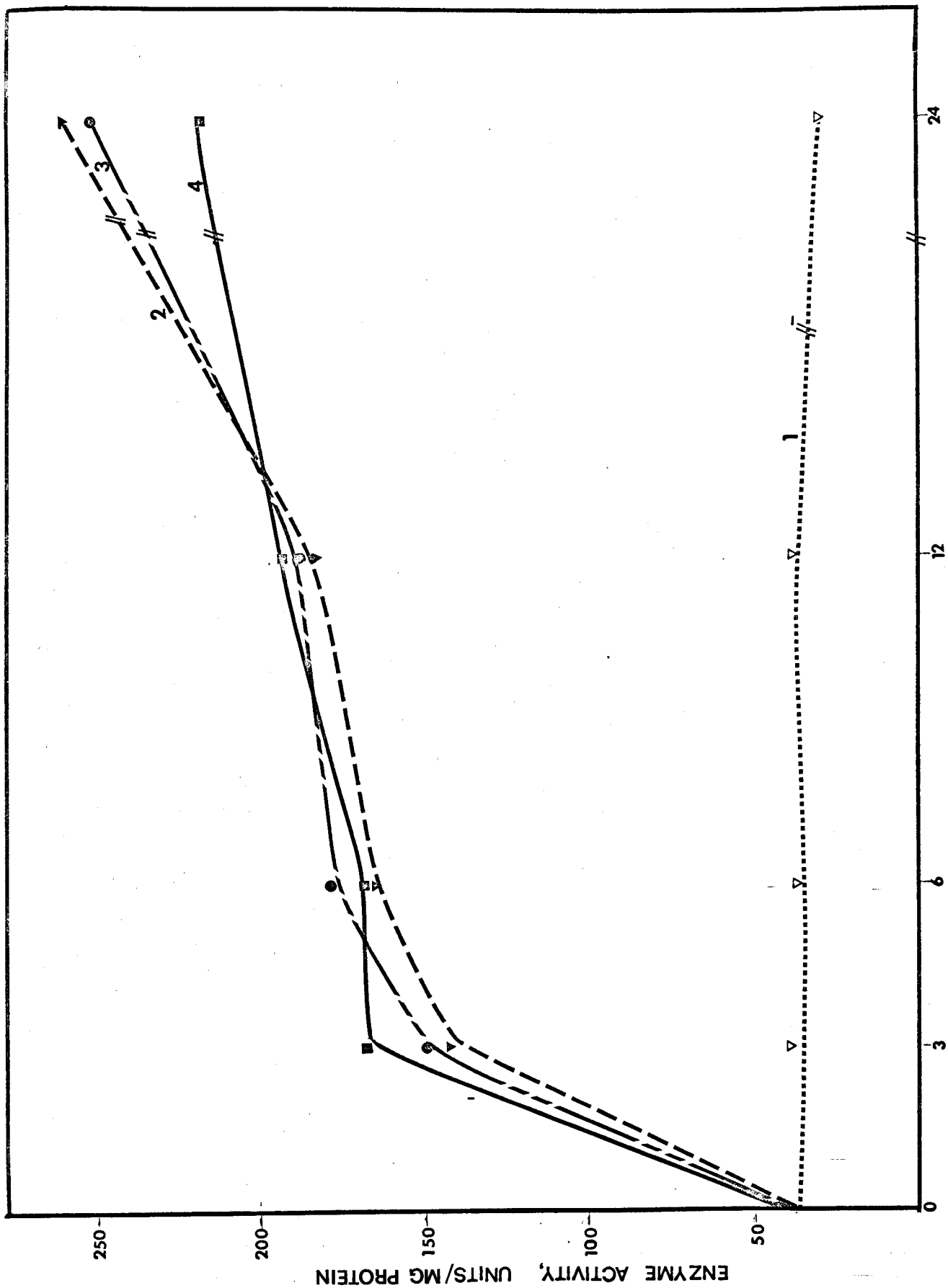
Figure 14. Induction of nitrate reductase in attached primary leaves from 9 day old seedlings.

The induction conditions and the extraction and assay procedures are same as described in figure 13. The dark period was between 13 and 21 hours.

In figure, the lines represent:

1. No nitrate
2. 10 mM nitrate
3. 25 mM nitrate
4. 50 mM nitrate

Unit of enzyme activity is defined in figure 12.


ENZYME ACTIVITY, UNITS/MG PROTEIN
250
200
150
100
50
0
3
6
12
24
INDUCTION TIME, HOURS
1
2
3
4

enzyme is induced from 115 units to 193 units between 3 and 24 hours. In mature leaves the enzyme increases from 130 units to 237 units during the same period, with same concentration of nitrate. The initial induction in mature leaf is slightly higher than the younger leaf. With increase in the concentration of nitrate, there is very little increase in the enzyme activity. A maximum induction is obtained with 10 mM nitrate. During the induction period, lights were switched off between 13 and 21 hours. This 8 hour dark period had no effect on the induction of the enzyme.

3. In Detached Leaves - When a leaf is detached from the shoot axis, the supply of amino acids from the reserve proteins is cut off. The amount of amino acids in the detached leaf may be enough for induced synthesis, but not enough for effecting the induction of nitrate reductase as an end product. Thus, if the detached leaf has got all the essential requirements for an induced enzyme synthesis, a more active nitrate reductase is expected.

Figure 15 shows the induction of nitrate reductase in a detached leaf from 5 day old seedlings. The enzyme is inducible in young detached leaf. With 10 mM nitrate, the enzyme activity increases from 42 to 178 units in the first 3 hours. After that the increase in activity is much slower. The rate between 3 and 24 hours is about 7 units per hour with 10 mM

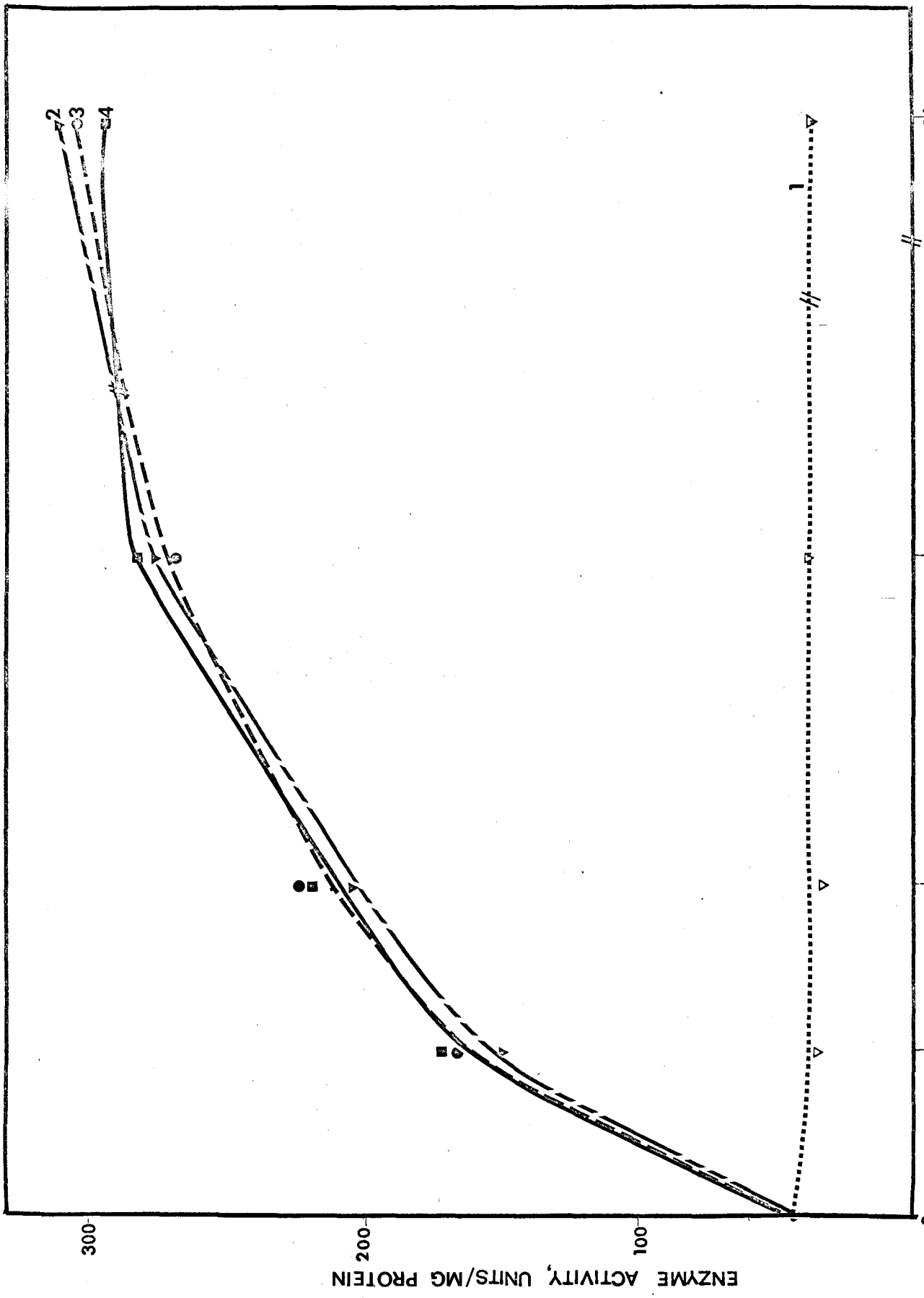
Figure 15. Induction of nitrate reductase in the detached primary leaves from 5 day old seedlings.

The detached leaves were induced in a 500 ml. flask. The extraction and the assay procedures are same as in figure 13.

In the figure, lines represent:

1. No nitrate
2. 10 mM nitrate
3. 25 mM nitrate
4. 50 mM nitrate

Unit of enzyme activity is defined in figure 12.


ENZYME ACTIVITY, UNITS/MG PROTEIN
300
200
100
0
3
6
12
24
INDUCTION TIME, HOURS
1
2
3
4
5

nitrate. Increasing the concentration of nitrate from 10 to 50 mM has no effect on the induction pattern.

Although the induction pattern in the detached leaf is similar to that in the intact leaf, the initial rate of induction in the detached leaf is higher. With 50 mM nitrate the enzyme in the attached leaf increases from 42 units to 124 units during the first 3 hours. Whereas in the detached leaf the enzyme increases from 42 units to 152 units in the same period with the same concentration of nitrate. After 3 hours, increases in the detached leaf are similar to attached leaf.

Induction of nitrate reductase in the mature detached leaf is presented in figure 16. In mature detached leaf, the enzyme is not induced up to 6 hours. At 12 hours, there is almost a 3 fold increase over the control value. There is a slow increase in the enzyme activity between 12 and 24 hours. With 10 mM nitrate, the rate of increment during this period is 8 units per hour. At 12 and 24 hours, 25 and 50 mM nitrate induced the higher levels of enzyme than 10 mM.

The experiments described in this section indicate that nitrate reductase is inducible in the shoot, root, attached primary leaves and detached young primary leaves. The enzyme is inducible in detached mature leaves only after 12 hours with nitrate. The experiments also indicate that amino acids coming to leaf may inhibit nitrate reductase to some extent.

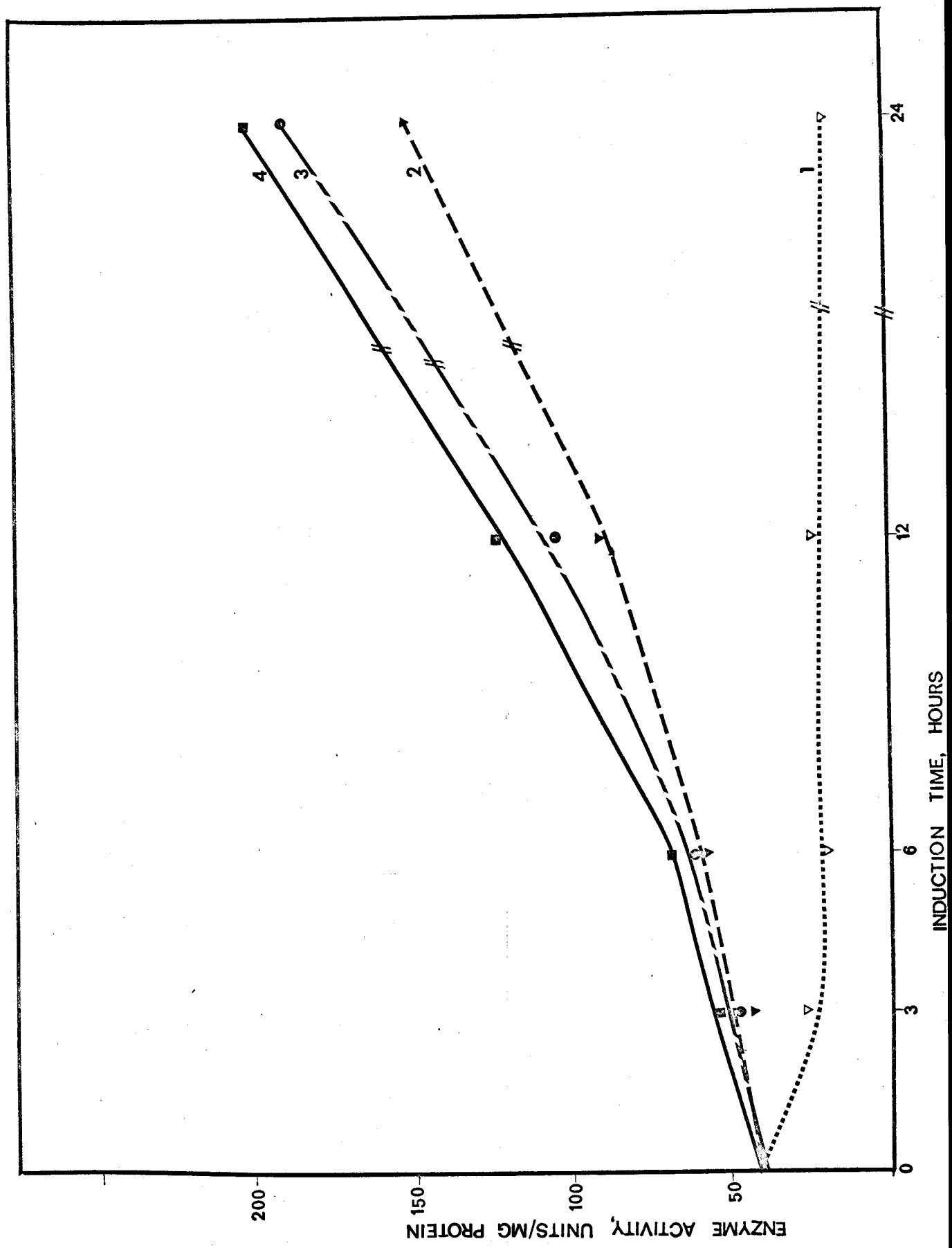
Figure 16. Induction of nitrate reductase in the detached primary leaves from 9 day old seedlings.

The detached leaves were induced in a 500 ml. flask. The extraction and assay procedures are same as in figure 13.

In the figure, lines represent:

1. No nitrate
2. 10 mM nitrate
3. 25 mM nitrate
4. 50 mM nitrate

Unit of enzyme activity is defined in figure 12.


ENZYME ACTIVITY, UNITS/MG PROTEIN
200
150
100
50
0
3
6
12
24
INDUCTION TIME, HOURS
1
2
3
4

VI. The Role of Amino Acids in the Regulation of Nitrate Reductase in Root Tips

1. Effect of Exogenous Addition of Amino Acids -

Ammonium compounds or amino acids, potential end products of nitrate reduction, can inhibit the induction of nitrate reductase. The work with cultured tobacco cells has shown that some amino acids inhibit the induction of nitrate reductase while others do not (Filner, 1966). Alanine, asparagine, glycine, nethionine, proline, threonine, valine, aspartate, glutamate, histidine and leucine repress. Arginine and lysine 'derepress' in the presence of any of these repressors. Actively metabolising root tips get a variety of amino acids from the endosperm proteins. To see whether amino acids also affect the level of nitrate reductase different amino acids were supplied to the roots, during induction.

In the induction studies with root tips, Stevens observed that after the lag of about 0.5 hour, the enzyme increased linearly up to 4 hours and then it plateaued. Maximum induction could be obtained with 10 mM nitrate (figure 17). Because of this observation of Stevens an induction time of 3 hours was chosen for studying the effect of amino acids.

Of all the amino acids tested, only arginine and lysine, inhibited the induction (table 9). Proline, leucine, alanine, glutamate, aspartate and casamino acids have no effect on the induction of nitrate reductase. Since the pH of all induction media was

Figure 17. Induction of nitrate reductase in the root tips of Zea mays L. var. W64A x W182E.

The roots were induced as described in the text. The extraction and assay were performed by method II.

In the figure, the lines represent:

1. 1 mM nitrate
2. 10 mM nitrate
3. 25 mM nitrate

Unit is defined in figure 12.

(After D. Stevens)

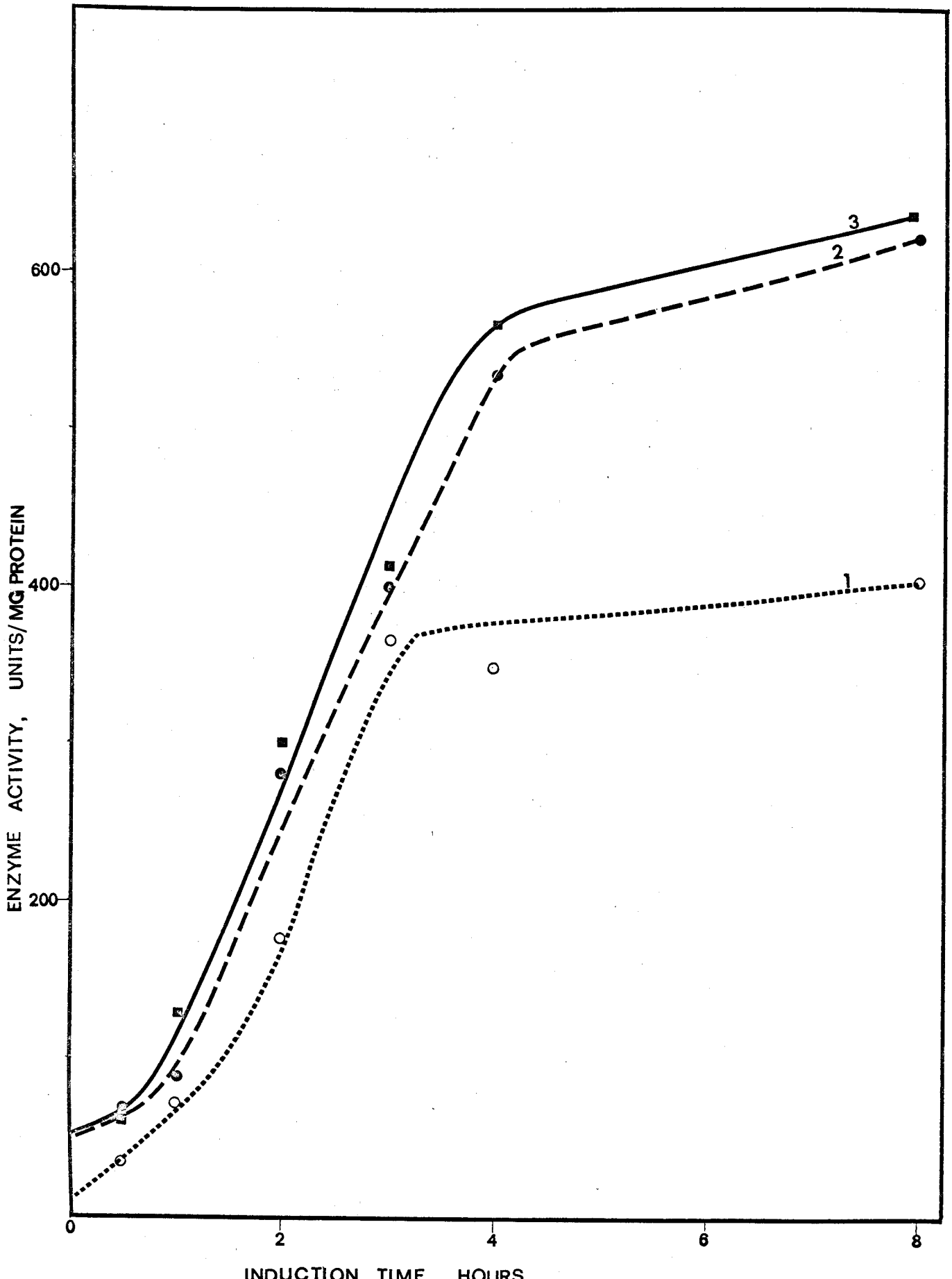

ENZYME ACTIVITY, UNITS/MG PROTEIN
600
400
200
0
2
4
6
8
INDUCTION TIME, HOURS
1
2
3

TABLE 9

Effect of Amino Acids on the Induction of Nitrate Reductase in the Root Tips of Maize var. W64A x W182E

Amino acid added	Fresh weight of root tips mg/tip	Enzyme activity units/mg protein	Percentage of control
None	9.10	319.00	100
Arginine	8.90	226.50	70
Lysine	8.85	162.25	51
Aspartic acid	8.95	310.86	97
Glutamic acid	8.90	320.70	100
Alanine	9.00	334.95	104
Proline	8.90	331.76	103
Leucine	9.10	315.15	98
Casamino acids	8.90	307.80	96

Seedlings were grown in the dark at 26°C for 65 hours. Before 3 hours induction with 10 mM nitrate in the presence of amino acids, the roots were pretreated with same amino acids for two hours. Concentration of amino acids was 20 mM. Induction and pretreatments were done in the dark at 26°C.

adjusted to 6.0 inhibition caused by arginine and lysine is not the effect of pH. These amino acids also did not affect the growth of root during the induction period.

Ammonium chloride, ammonium sulphate, ammonium tartrate and urea at the concentrations of 1, 2 and 5 mM have no effect on the induction under similar conditions (see appendix).

2. Effect of Amino Acid Composition of the Endosperm - In the previous experiment, arginine and lysine were shown to inhibit nitrate reductase in maize root tips. If this is the case, the induction of the enzyme in the root tip of opaque-2 could be lower than in the wild type, W64A. On the other hand, opaque-2 is deficient in certain amino acids, for example alanine, leucine and proline. In order to make those amino acids, opaque-2 needs more reduced nitrogen and thus a more active nitrate reductase is expected. To test this, the kinetics of the induction of nitrate reductase in opaque-2 and its wild type W64A, have been studied.

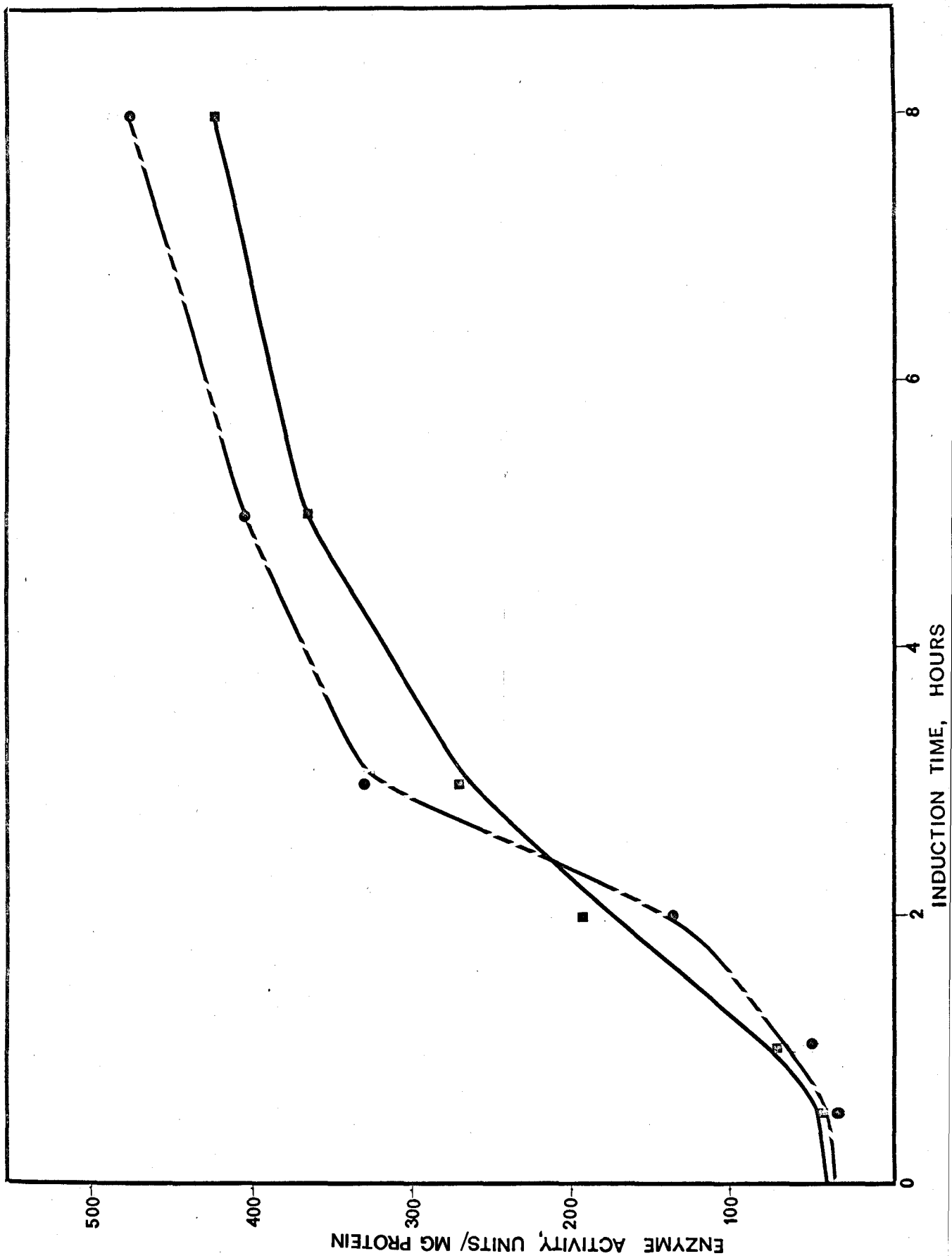
Figure 18 shows that, there is approximately a 0.5 hour lag in the induction of enzyme in both inbred lines. This lag may represent the time required for the synthesis of specific m-RNA and protein. The absorption and transport of nitrate to the site of action may also take some time. After 0.5 hour, the enzyme activity in the wild type, increases up to 3 hours at a rate of 83 units per hour. Although,

Figure 18. Effect of induction time on the induction of nitrate reductase in the root tips of opaque-2 and its wild type W64A.

Growth and induction conditions are described in the text. Concentration of nitrate was 10 mM. Assay and extraction were performed by method II.

In the figure, the circles represent enzyme activity in opaque-2 and the squares in wild type. Similar results were obtained in three experiments.

Unit of enzyme activity is defined in figure 12.


ENZYME ACTIVITY, UNITS/ MG PROTEIN
500
400
300
200
100
0
2
4
6
8
INDUCTION TIME, HOURS

the activity increases further, during the next 5 hours, the rate of increase, 33 units per hour is much less. In opaque-2, on the other hand, the rate from 0.5 to 2 hours is 67 units per hour and during the subsequent 1 hour period it increases to 198 units per hour. At 3 hours, the enzyme activity in opaque-2 is higher than the wild type and this elevated level is maintained for at least 8 hours.

The effect of different concentrations of nitrate, on a 3 hour induction of nitrate reductase in the root tips from both inbred lines was studied next (figure 19). In each the activity of nitrate reductase without any nitrate is very low. In a concentration range of 1 to 50 mM, opaque-2 has higher activity than the wild type, W64A. At 5 mM nitrate, the enzyme activity in opaque-2 is 300 units as against only 213 units in W64A. With increasing concentration of nitrate the enzyme activity in opaque-2, increases up to 5 mM. There is no further increase with higher concentrations of nitrate. On the other hand, the maximum induction in the wild type is obtained with 10 mM nitrate. Higher concentrations have no further effect.

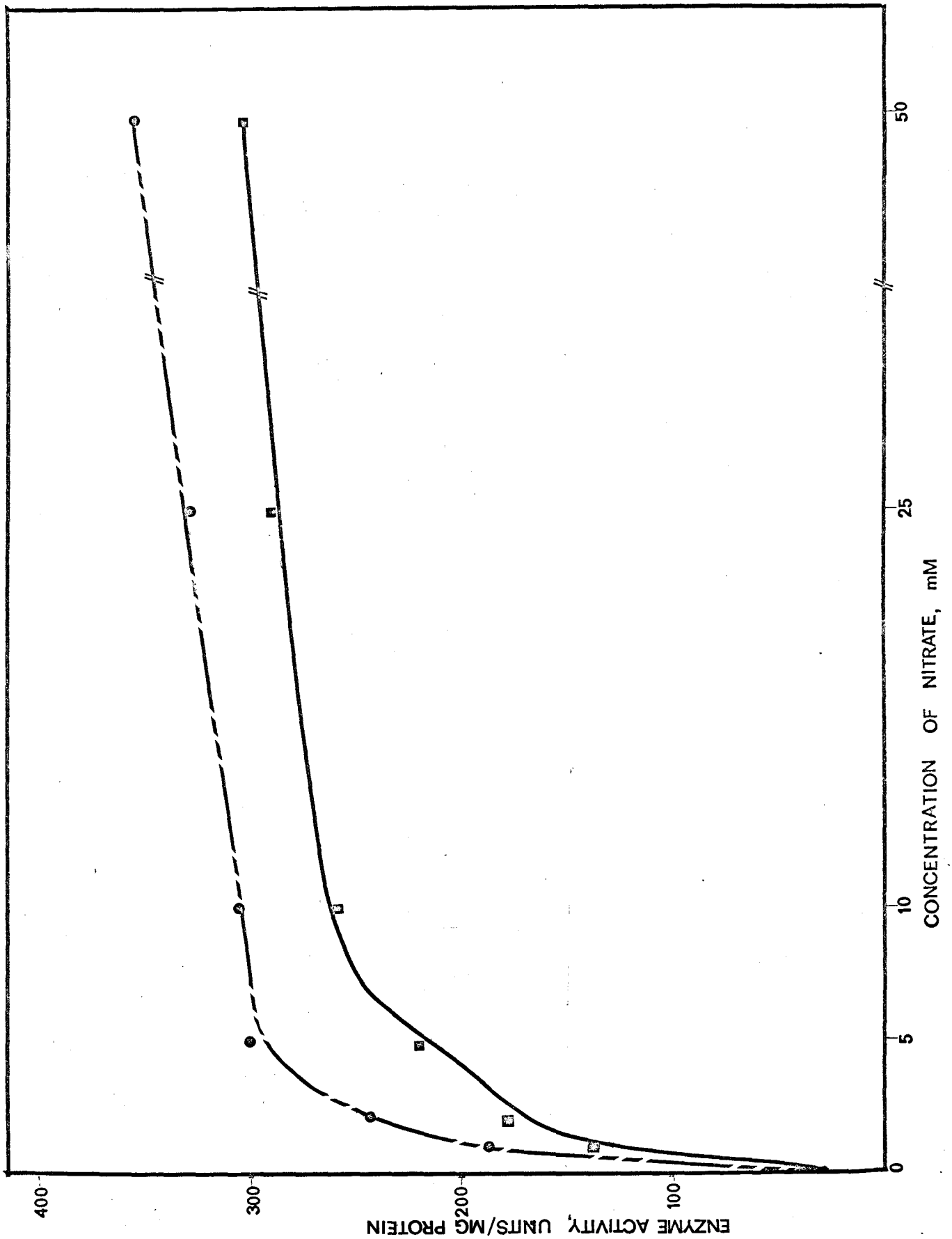
These experiments suggest that amino acid composition of the endosperm, may effect the induction of nitrate reductase in the root tips.

Figure 19. Effect of concentration of nitrate on the induction of nitrate reductase in the root tips of opaque-2 and its wild type W64A.

Growth and induction procedures are described in the text. The roots were induced with desired concentrations of nitrate for 3 hours. Extraction and assay were performed by method II.

In the figure, circles show enzyme activity in opaque-2 and the squares in W64A wild type. Similar results were obtained in three experiments.

Unit of enzyme activity is defined in figure 12.


ENZYME ACTIVITY, UNITS/MG PROTEIN
400
300
200
100
0
5
10
25
50
CONCENTRATION OF NITRATE, mM

CHAPTER III

DISCUSSION

I. Nitrogen Balance of the Seedling.

During the early growth of the seedlings, the protein and the other stored macromolecules in the endosperm or cotyledons, support the embryo. While observing the translocation of soluble nitrogen from endosperm to the embryo of barley seedlings, Folkes and Yemm (1958) advanced the idea that the amino acids liberated by the hydrolysis of endosperm proteins are translocated to the embryo. In barley and maize seedlings, the endosperm nitrogen starts decreasing after a period of 1 to 2 days. There is a parallel increase in the embryo nitrogen after the initial lag period. In their experiments, Folkes and Yemm observed that the transfer of endosperm nitrogen was almost complete in 8 days of seedling growth at 22.5°C. The movement of metabolites from endosperm to embryo in growing maize seedlings was studied by Dure (1960) and Ingle et al., (1964). Dure observed that transfer of endosperm nitrogen at 20°C is almost complete by 10 days. Ingle et al. studied the growth of maize seedlings for 5 days at 25°C. About two thirds of the endosperm nitrogen was transported to the embryo axis between 1 and 5 days. The disappearance of total nitrogen from the endosperm was parallel to an increase in the total nitrogen of

the axis. The trend of loss of nitrogen from the endosperm and a parallel increase in the embryo, in our experiments is also essentially similar to that observed by Ingle et al. At 26°C the major amount of nitrogen is transported between 2 and 8 days. During the period 2 to 6 days the protein nitrogen of the embryo increases at a rate of 597 µgm per day and the total nitrogen 835 µgm a day. This was correlated to a similar loss in the total nitrogen of the endosperm. After 6 days the nitrogen components of the embryo increase slightly. About 6-7 percent of the nitrogen lost from the endosperm over a period of 10 to 12 days is not recovered in the embryo axis. This loss of nitrogen may be partly due to leaching and partly due to damage to the roots while uprooting the seedlings. It was also observed that the scutellum does not contribute any nitrogen to the growing embryo while the endosperm was able to support it.

It appears that endosperm nitrogen is able to support the requirement of the embryo for a period of 6 to 8 days. On the other hand, isolated embryos can be grown on nitrate alone as a nitrogen source (Harris, 1956). Thus when exogenous nitrate is supplied to the young maize seedlings it may cooperate with the organic nitrogen coming from the endosperm. However, if the amino acids alone are able to meet the growth requirements of the growing embryo, the assimilation of nitrate during this period could be

unnecessary. This was realized in our experiments when an exogenous supply of nitrate did not increase the protein level of the embryo before 6 days. After this time the supply of nitrogen from the endosperm is low and the addition of nitrate does, in fact, increase the protein and total nitrogen of the embryo. It seems, therefore, that during the period when the endosperm nitrogen is able to meet the growth requirements of the embryo, assimilation of exogenous nitrate into the protein is restricted. In this respect, the maize embryo appears to be essentially similar to the tobacco suspension culture. Filner (1966) observed that when tobacco cells have access to both casein hydrolysate and nitrate, they preferentially use casein hydrolysate. When there was insufficent casein hydrolysate to support the maximum growth rate the cells assimilated just enough nitrate required for maximum growth rate.

Filner (1966) also found that inhibition of nitrate assimilation in the presence of casein hydrolysate is due to inhibition of nitrate reductase. It is possible that the assimilation of exogenous nitrate by a young maize seedling is restricted in a similar way. Our experiments with the induction of nitrate reductase in the seedling and the root tips show that enzyme is readily induced. The presence of an active enzyme, however, may not be a true indication of what is happening in vivo. In Spirodella

oligorhiza leaves, the utilization of ammonia prevented the assimilation of nitrate even by plants that contained high levels of both nitrite and nitrate reductases (Ferguson, 1969). It was concluded that ammonium or other end products of assimilation inhibit the in vivo activity of nitrate reductase. In barley aleurone layers, a comparison of nitrate reduction rates in cell free and in vivo assay methods indicated that only a small fraction of the total enzyme activity induced in response to nitrate is functional in the tissue (Ferrari and Varner, 1970). Klepper et al., (1971) also observed that the level of nitrate reductase activity obtained with the in vitro assay is 2.5 to 20-fold higher than the in vivo assay for most plant species.

Since nitrite and ammonia do not normally accumulate in plants, the primary product of nitrate reduction may be glutamate or amides. A significant synthesis of glutamate or amides will increase the soluble component of the embryo nitrogen. Our experiments show that up to 4 days of seedling growth nitrate causes only minor increases in the soluble nitrogen. Thus it is possible that the reduction of nitrate in maize seedlings is limiting.

In addition to this, the availability of carbohydrates to accept reduced nitrate for the synthesis of amino acids may also limit the reduction and assimilation of nitrate. During the course of our

studies, it was found that the dry weight of the endosperm decreases at a rate of 13 mg per day per endosperm up to 4 days. After that period, the rate was accelerated to 16 mg per day. The primary leaves develop by 5 days and they may supplement the carbohydrate of the embryo by assimilating carbon dioxide into sugars. This supplemented level of carbohydrate may be enough to aid in the assimilation of exogenous nitrate in addition to supporting the usual growth of the embryo.

II. Role of Nitrate in the Growth of the Embryo.

It was observed that nitrate induces the germination of maize seeds without affecting the nitrogen balance of the seed and the young seedling. The effect of nitrate on germination is specific. Other salts such as sodium and potassium chlorides have no effect on germination.

Protein turnover is a general feature of plant tissues (Chibnall, 1954 and Bidwell et al., 1964). Chibnall also pointed out that the growth of younger tissues in mature seedlings is maintained by the degradation products of protein in mature tissues. During the period from 2 to 6 days of seedling growth, a linear increase in the protein content of the maize embryo, is the result of high relative rates of protein synthesis. Apparently after 6 days, the rate of protein synthesis is

almost equal to protein degradation. This is probably because at this time some of the older parts of the plant start senescing. When exogenous nitrate is supplied to the seedling, the protein level of the embryo increases only after the period of net synthesis of protein. This increase in protein may be due to either increased synthesis or decreased degradation.

The primary leaves are simpler than the whole embryo axis in their development. The increase in the protein level of the leaf with added nitrate, however, is similar to that of the embryo. A significant increase in the protein is seen only when the normal increases in protein are slight. Nitrate also protects against further loss of protein and chlorophyll. In this respect, nitrate delays the senescence of the mature leaves. In some other cases it has been observed that repeated spraying of attached leaves with ammonium nitrate will delay yellowing or cause regreening (Woolhouse, 1967). Wangerman and Lacey (1955) also reported that nitrate reduced the rate of ageing of Lemna minor fronds. From our experiments, it appears that nitrate delays senescence of primary leaves by preventing the protein loss. It is possible that the primary role of nitrate in the growth of the seedlings is to prevent the protein loss.

III. Role of Amino Acids in the Induction of Nitrate Reductase.

For an induced enzyme synthesis a minimal level of amino acids and other factors involved in protein synthesis is required. When the leaves are attached to the shoot, they receive these factors from the plant. Induction of nitrate reductase in the detached young leaves shows that they have the required minimum of amino acids and other factors. In the mature detached leaves, however, there is no significant induction of enzyme before 12 hours. Thus in mature detached leaves, it appears that the essential prerequisites involved in the induction of nitrate reductase are not met. The enzyme is readily induced in the attached leaves of both ages. In the leaves maximum induction is achieved with 10 mM nitrate. In the roots the saturating concentration was either 5 mM (opaque-2) or 10 mM (W64A wild type). The saturating concentrations of nitrate differ in different systems. In Lolium perenne, the induction is apparently maximum at 0.5 mM nitrate (Bowerman and Goodman, 1971). In Neurospora crassa (Kinsky, 1961) and Lemna minor (Stewart, 1972) concentrations above 1 mM have little effect in increasing the nitrate reductase activity. On the other hand, in radish cotyledons 10 mM nitrate was required to induce the enzyme maximally (Beevers et al., 1965). In barley aleurone layers, the enzyme activity

increased upto 100 mM nitrate (Ferrari and Varner, 1969). These differences probably indicate differences in the rate of uptake between species. In any system there is a saturating level of nitrate due to following reasons: 1. As described by Beevers et al. (1965), higher concentrations of nitrate may suppress the activity of nitrate reductase in maize leaves. In Neurospora crassa also higher concentrations of nitrate inhibit nitrate reductase activity (Kinsky, 1961). Thus the enzyme levels obtained with higher concentrations of nitrate may be actually a balance between the suppressing and inducing action of nitrate. 2. It is probable that relatively small concentrations of nitrate are able to induce the activity of all nitrate reductase specific genes. 3. Ferrari et al., (1970) have described two types of nitrate pools in the plant systems, namely a metabolically active pool and an inactive pool. If the nitrate lies in the inactive pool it may not work as an inducer for nitrate reductase. Probably with higher concentrations, most of the nitrate absorbed goes to the inactive pool.

Kanangara and Woolhouse (1967) demonstrated that enzyme synthesis in senescent leaves of Perilla frutescence was lower than in the mature leaves. The capacity to synthesize nitrate reductase by a 70 day old (senescent) leaf was only 32 percent of the incompletely expanded 15 day old leaf. Recently, it

has been shown that the differences in the capacity to induce nitrate reductase between tissues of two ages is due to the difference in their protein synthesizing capacity (Travis and Key, 1971). In our experiments, the pattern of induction of nitrate reductase in the leaves of 5 day and 9 day old seedlings is the same. In contrast to Perilla frutescence, however, the initial rate of induction in the mature leaf is higher than the young leaf (table 10). This may be due to a difference in the two systems. On the other hand, we believe that the lower rate of induction in the younger leaf, may be due to the effect of amino acids coming from the storage proteins. The level of enzyme after 24 hours is similar in the leaves of both ages. At this time probably the reduced products of nitrate in the older leaves are affecting the induction of nitrate reductase in a way similar to incoming nitrogenous compounds in the younger leaf. Furthermore, when the leaves from 5 day old seedlings are detached, the rate of induction increases. In the first 3 hours of induction there is 3.8 fold increase in the enzyme in detached leaf while there is only 2.7 fold in the attached leaf. The initial activity in both leaves was the same. As detached leaves are not receiving the endosperm nitrogen, the higher rate of induction may again be interpreted as due to absence of an inhibitory concentrations of amino acids. In addition to this it is also possible

TABLE 10

Comparison of the Rate of Induction of Nitrate Reductase in Young and Mature Intact Leaf

Induction time Hr.	Activity of the enzyme			
	5 day old seedling		9 day old seedling	
	Units/mg protein	Percentage of original value	Units/mg protein	Percentage of original value
0	42.4	100	41.8	100
3	111.5	263	131.7	315
6	125.7	299	142.5	340
12	140.7	332	153.6	365
24	178.0	420	177.0	421

Concentration of nitrate in the induction medium was 50 mM. One unit of enzyme activity is equal to 1 n mole of nitrite produced per hour per milligram protein.

that during this initial period detached leaves are able to absorb more nitrate than the attached ones.

Studies with the induction of nitrate reductase in the root tips of opaque-2 and its wild type, W64A shows that in both the enzyme is induced after a lag of about 0.5 hour. This lag period is similar to many other systems. The lower rate of induction in opaque-2 between 0.5 to 2 hours may be either the effect of lysine or the deficiency of some zein amino acids. Once the seedling is able to buffer the inhibitory effect of lysine somehow or to synthesize deficient amino acids, the enzyme is induced at its maximum rate.

Fluctuations in the enzyme activity could be brought about by changes in the relative rates of synthesis or degradation of the enzyme. Thus the apparent inhibition of nitrate reductase by amino acids could be associated with either an increased degradation or inactivation or with a decreased synthesis. In Filner's experiment (Filner,1966) with tobacco cells the effect of amino acids on the activity of nitrate reductase was measured after 24 hours in the induction medium, when the enzyme level was at a steady state. This may not tell us, whether the amino acids are inhibiting the synthesis or accelerating degradation or inactivation of the enzyme. In our experiments with maize root tips, the inhibition of nitrate reductase by lysine and arginine was

measured after three hours in the induction medium. Since during this time, the enzyme level increases linearly, indicating the rate of synthesis far outweighs the rate of degradation, it is suggested that amino acids inhibit the activity of nitrate reductase in maize root tips by decreasing the synthesis. However, further experimentation is required to prove it.

CHAPTER IV

SUMMARY

1. Nitrate caused a 10-15 percent increase in the germination of several varieties of maize. The effect is specific and probably not related to the reduction of nitrate or the synthesis of protein.

2. During early seedling growth, there is a period of relatively minor change in total protein, a period of rapid increase and a second period of minor change. When nitrate is added to the system it has no effect on the protein content of the embryo during the period of rapid protein increase but it does cause a significant increase in protein nitrogen after this time.

3. Leaves are simpler than the embryo in their development. They have a period of rapid increase in protein and then a period of slow increase. When nitrate is supplied, the protein level of the leaf increases only after the period of rapid increase in protein. Nitrate also prevents the loss of protein and chlorophyll from mature leaves.

4. Lysine and arginine inhibit the induction of nitrate reductase in the root tips. A comparison of the rate of induction between opaque-2 and its wild type W64A suggests that amino acid composition of the endosperm may affect the induction of nitrate reductase in the root tips.

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APPENDIX

TABLE

Effect of Ammonium Salts on the Induction of Nitrate Reductase in the Root Tips of Maize.

Ammonium salt	Concentration mM	Enzyme Activity units/mg protein	Percentage of control
None	-	334.50	100
Ammonium Chloride	1	321.00	96.3
	2	318.50	95.5
	5	329.70	98.9
Ammonium Sulphate	1	316.50	95.0
	2	339.65	101.9
	5	320.00	96.0
Ammonium tartrate	1	340.80	102.2
	2	320.50	96.2
	5	319.85	95.9
Urea	1	352.65	105.8
	2	340.00	102.0

Seedlings were grown in the dark at 26°C for 65 hours. Before 3 hours induction with 10 mM nitrate in the presence of the ammonium salts, the roots were pretreated with same ammonium salt for two hours. Induction and pretreatments were done in the dark at 26°C.