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An investigation of a nitrate reductase inhibitor in the roots of Zea mays.

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AN INVESTIGATION OF A NITRATE REDUCTASE INHIBITOR IN THE ROOTS OF *ZEA MAYS*

A Dissertation Presented

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INTRODUCTION

Growth and development of plant tissue is accompanied by changes in the complement of enzyme activities within the cell (30). Therefore, it follows that elucidation of the mechanisms that regulate enzyme activity is essential for a comprehensive understanding of the processes of growth and development. The level of activity of an enzyme is dependent upon both the rate at which it is synthesized and the rate at which it is inactivated. A majority of effort to date has been devoted to studies of enzyme synthesis. It is known that enzyme activity may decrease but the reasons are not always clear. Theoretically, enzyme activity can be envisioned to decrease because of two possibilities: first, the enzyme molecule may be inherently unstable and prone to spontaneous inactivation; second, the enzyme may be inactivated by factors independent of the enzyme molecule itself. As will be shown, very little is known about the factors which control the disappearance or the inhibition of enzyme activity in vivo.

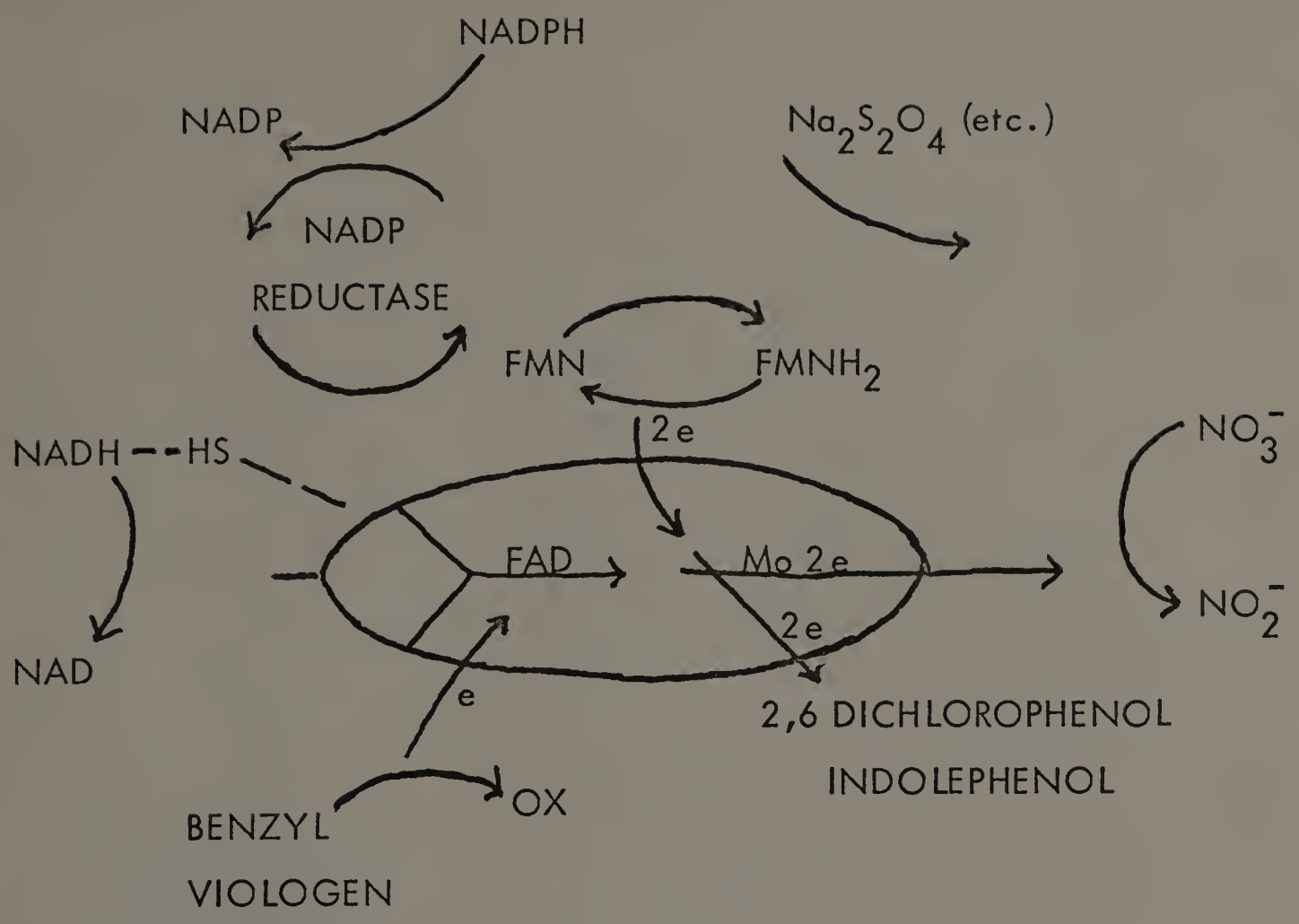
Nitrate reductase mediates the first step in the reduction of nitrate to the biologically functional amine level in bacteria, fungi and plants (17, 52). The majority of recent studies on nitrate metabolism in higher plants have been made with leaf tissue because of the abundance of enzyme and ready availability of material. It was demonstrated that in many plants root extracts contained nitrate reductase, however, activity usually was very low (3, 4, 11, 17, 23,

48, 66). In preliminary experiments it was observed that extracts from roots of corn seedlings 10 days or older did not show nitrate reductase activity and strongly inactivated nitrate reductase extracted from leaf blades. Apparently, these root extracts contained some inhibitors. There is essentially no information available on the inactivation system which is involved in controlling nitrate reductase activity in corn seedlings. This investigation constitutes a study of the factors which function in this inactivation system.

LITERATURE REVIEW

I. Nitrate Reductase

Knowledge of the mode of regulation of inorganic nitrogen metabolism in higher plants is important because of the central role of nitrogen in growth. Nitrate is the major inorganic form of nitrogen available to plants. The fact that nitrate is reduced to ammonia before being assimilated into the plant constituents has been known for a long time. Since nitrate reductase catalyzes the initial reaction in this process, it is considered to play a major role in regulating nitrogen metabolism. The existence of nitrate reductase was established almost two decades ago. Evans and Nason (17) first isolated and characterized the enzyme from soybean leaves and Neurospora. Nevertheless, although the enzyme has been known for such a long time, it has not been fully purified or characterized from higher plants. The properties of nitrate reductase were reviewed recently by Beevers and Hageman (5). It has been established that nitrate reductase contains molybdenum (2, 16), Fe^{++} (2), FAD (2, 16) and requires NADH or NADPH (4, 66, 76, 84) as its electron donor. The electron transport scheme of nitrate reductase can be diagramed as following (29, 76):



1. Location. Most studies of nitrate reductase in higher plants have been carried out with leaves because of the high level of enzyme activity in this tissue and ready availability of material. Sanderson and Cocking (66) demonstrated appreciable nitrate reductase activity in extracts from roots of several plant species but the activity of the root extracts was consistently lower than that in leaf extracts. They concluded that the properties of nitrate reductase extracted from root tissue was similar to that of the leaf enzyme.

It was initially suggested (13, 17) that nitrate reductase was located in chloroplasts. However, Ritenour et al. (63), using nonaqueous and aqueous techniques for chloroplast isolation, concluded that nitrate reductase was an exochloroplastic enzyme although the possibility was not excluded that it might

be associated with the external chloroplast membrane. Further indirect evidence for the cytoplasmic as opposed to the chloroplastic location of nitrate reductase was provided by the observation of Schrader et al. (74), which demonstrated that chloramphenicol inhibited the synthesis of nitrite reductase but not that of nitrate reductase. (Chloramphenicol inhibits chloroplast and mitochondrial, but not cytoplasmic, protein synthesis (44).)

2. Control Factors. A characteristic feature of nitrate reductase in higher plants is its susceptibility to a range of environmental conditions (5).

(a) Nitrate. Nitrate appears to induce nitrate reductase activity in many plants (1, 6, 11, 19, 23, 28, 37, 79, 85). Beevers et al. (6) found that the increase in nitrate reductase activity was roughly proportional to the amount of nitrate present in the tissue, but different levels of nitrate were required for optimal induction in different species. They concluded that this difference was attributable to different rates of nitrate uptake. De novo synthesis of the enzyme is believed to be involved in the increase in activity because inhibitors of protein synthesis prevented the increase (6, 85). However, the newly synthesized protein is not necessarily nitrate reductase. Ingle (32) suggested that the increase in activity of nitrate reductase in radish cotyledons involved only a very small percentage of the protein, either on the basis of total proteins present or of the protein synthesized during the induction period. Therefore, he suggested that the requirement of protein synthesis for induction, as determined by inhibitor studies, was not directly for nitrate reductase protein but might involve the synthesis of an effector necessary for nitrate reductase activity.

(b) Light. In green plants the strong stimulatory effect of light on nitrate reductase activity has long been recognized. Several investigators (17, 23) observed that a decrease in nitrate reductase activity occurred when plants were placed in the dark. The activity was quickly restored when plants were exposed to light. The level of nitrate reductase activity in the leaves of many species varies diurnally (24, 80) and seasonally (25, 64, 96). The quality and duration of light also may influence nitrate reductase activity in wheat (26). A greater activity of nitrate reductase occurred under blue light and long photoperiods; light intensity had no consistent effect on nitrate reductase activity.

The mechanism by which light controls nitrate reductase activity still is not clear. Beevers et al. (6) suggested that the effect of light might be indirect by enhancing the uptake of nitrate into plant tissue by promoting transpiration. A light dependence for nitrate uptake also has been reported by Chen et al. (12). This premise that light promotes nitrate reductase activity by promoting nitrate uptake is further supported by the observation that illumination increased the permeability of cell membranes (31, 45).

The interaction of light and nitrate in the induction of nitrate reductase activity is a matter of controversy. Travis et al. (93) found that reductase activity in etiolated leaves in darkness stayed at a low level even though large amounts of nitrate were accumulated. They suggested that both nitrate and light were independently required for significant induction of nitrate reductase activity in barley leaves. Recently, Travis et al. (91, 94) reported that the light effect was due to its influence upon the development of an active protein synthesizing apparatus, the polyribosome, and not for enzyme induction per se.

When leaf tissue containing nitrate was illuminated in a CO_2-free atmosphere, no detectable nitrate reductase was found. Kannangara et al. (37) believe that the synthesis of nitrate reductase was dependent on active photosynthesis. It was postulated that an increasingly negative redox potential in leaf tissue following illumination might be the explanation for the light requirement for nitrate reductase activity.

(c) Metals. Nitrate cannot induce the development of nitrate reductase in molybdenum deficient cauliflower (1) but the activity can be restored by the in vivo addition of Mo (1, 61). No increase in enzyme activity occurred upon the addition of Mo to cell-free extracts. It was concluded, therefore, that Mo also can serve as an inducer of nitrate reductase (1, 11, 28).

(d) End Product. Feedback inhibition is an important control mechanism for regulating enzyme activity in many organisms. Nitrate reductase has been observed to be repressed by ammonia in Neurospora and Chlorella (42, 51, 89), but not in higher plants (1, 6, 33, 75). However, Smith et al.(82) recently reported that the induction of nitrate reductase by nitrate was partially prevented by ammonia in excised barley roots. Ammonia also repressed the nitrate-induced synthesis of the reductase in Lemna minor (36).

Filner (20) demonstrated that casein hydrolysate and 11 amino acids, added individually, would repress the synthesis of nitrate reductase in cultured tobacco pith cells. Both trans-cinnamate and trans-o-hydroxycinnamate inhibited the increase in nitrate reductase activity in corn seedlings (75). However, these compounds inhibited protein synthesis in general (5) and it was concluded that their effect was non-specific.

(e) Inactivation System. Nitrate reductase appears to be quite unstable in vivo (11, 23, 27). Schrader et al. (76) estimated that the half-life of nitrate reductase in excised corn seedlings was about four hours. As mentioned previously, a rapid loss in nitrate reductase activity was observed when plants containing nitrate were transferred to the dark (7, 23, 37) or to a nitrate-free medium (6, 76). Beevers and Hageman (5) suspected that the presence of an inactivation system might be responsible for the fast decline in nitrate reductase activity. Limited evidence currently available supports the premise that the loss of nitrate reductase may be under the control of an inactivation or degradation system. Recently, in vivo evidence for an inactivation system for nitrate reductase was reported for barley seedlings (54, 92). The disappearance of the reductase activity in the leaf tissue in the dark or under heat stress (41-43°C) could be prevented by cycloheximide which suggests that inactivation was dependent upon protein synthesis.

II. Inactivation of Enzyme

The level of enzyme activity in an organism is a function of its rate of synthesis and inactivation (62, 67, 71). On balance, inactivation is equally as important as protein synthesis in regulating the amount of enzyme activity. A simple formulation of this concept has been developed in several laboratories (8, 59, 78). Thus, a change in enzyme level can be expressed by the equation:

$$dE/dt = ks - kd\,E$$

Where E is the content of enzyme expressed as units per mass, ks is a zero-order rate constant of synthesis expressed as units time^{-1} mass^{-1} and kd is a first-order rate constant for degradation expressed as time. In the steady state, i.e. when $dE/dt = 0$, then $ks = kd\,E$ or $E = ks/kd$. Thus, in the steady state the amount of enzyme is a function both of the rate of synthesis and the rate of inactivation or degradation[1]. An alternation in either rate, therefore, can affect the level of enzyme activity. Thus, the inactivation rate constant of an individual enzyme species also is of significance in considering the nature of the response to agents which increase rates of enzyme synthesis (8). The fact an agent causes an increase in the activity of one enzyme relative either to total protein or to another enzyme at some finite time does not necessarily indicate a specific effect on that enzyme. The time required to approach a new steady state is solely a function of the rate constant of inactivation. Moreover, Schimke (69) suggested that the continual degradation of protein and other cell constituents could be considered as part of the continuing adaptation of an organism to variation in its environment. Such changes involve not only the synthesis of newly required proteins, but also their subsequent removal when no longer needed.

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1 When dealing with an individual enzyme, it is difficult to differentiate between degradation and inactivation. The loss of enzyme activity may in fact result only from a change in the conformation or aggregation state of the protein rather than a splitting of peptide bonds. Therefore, a loss of detectable enzyme activity may merely reflect a reversible inactivation or inhibition, rather than an irreversible degradation of the enzyme molecule. All data available at present for higher plants and animals are measurements of activity losses, not enzyme molecule losses.

Knowledge of events of protein synthesis has increased greatly in microorganisms due to the technique of genetic analysis coupled to biochemical measurements. Jacob and Monod (34) proposed a three-element regulatory system for the β-galactosidase gene in E. coli. Their proposal has been essentially established as fact for bacteria. However, in higher organisms such as plants, many questions as to control of enzyme activity remain unanswered since genetic analysis is not a useful tool for these organisms. A control mechanism at the gene level alone is not rapid enough for the regulation of enzyme activity in cells. Several investigators (21, 50, 95) suggested that the control by induction and repression was supplemented by feedback inhibition which was more immediately effective. However, the information about enzymes controlling inactivation or degradation is extremely limited in general and especially so in plants.

1. Enzyme Inactivation Systems. An understanding of the importance of enzyme inactivation systems has slowly developed in recent years. The use of specific inhibitors of steps involved in protein synthesis is the most generally used tool for analyzing the inactivation system. The phenomenon of the inactivation or degradation of an active enzyme dependent upon the synthesis of new protein has been suggested (3, 15, 40, 87, 98). Engelsma (15) and Zucker (97) found that cycloheximide, an inhibitor of protein synthesis (8, 39), could stop the decline of phenylalanine ammonia-lyase activity which followed the light induced elevation of this enzyme activity in gherkin hypocotyls and in potato tuber disks, respectively. This suggests that the fall in activity is dependent upon synthesis of protein or peptides. A similar phenomenon also has been suggested for other

enzymes in several organisms (40, 73, 86, 87). However, the identities of the proteins which control the decay of the enzymes remain unknown.

2. Proteolytic Enzymes. Proteolytic enzymes have been found in plants (22) but the physiological function of these proteinases is unknown. The random nature of the degradation by the proteolytic enzymes should be accounted for. Theoretically, protein molecules can exist in several different thermodynamic states. We can postulate, then, that a protein will be subject to degradation only when the molecule assumes one of a number of possible conformations (68). Such conformations could be altered by interactions with small molecules, similar or dissimilar peptides and subunits, or by association with various intracellular organelles (69). Presumably, the conformation of the enzyme-substrate complex is sufficiently different from that of the free enzyme that the enzyme is resistant to proteolysis (46, 47, 49, 88, 97). The stabilizing effect of the substrate or perhaps specific effectors could provide the basis for selective degradation of enzymes by proteinases. Rat liver tryptophan pyrrolase is stabilized *in vitro* by its substrate, tryptophan (70, 72) and yeast hexokinase is stabilized by glucose against trypsin attack (7). McClintock and Markus (49) have found that the proteolytic inactivation of aspartate transcarbamylase of *E. coli* was increased in the presence of aspartate but inhibited by the presence of CTP.

3. Inactivation by Specific Proteins. Many studies have been made on the inhibition of proteinases by specific proteins present in plants as well as animals (38, 43, 55, 60, 65, 90). In contrast to animals (3, 9, 10, 14, 18, 41), there are few reports of specific proteins which can inhibit or inactivate specific enzymes

in plants. One example of the activity of a protein enzyme being controlled by another protein is invertase (37, 77, 82). The level of this enzyme in potato tubers fluctuated reversibly with changes in the storage temperature (58). The change of invertase activity was found to be due to the presence of an endogenous inhibitor. Pressey (81, 82, 83) found that warmer temperatures increased the concentration of the inhibitor with a consequent decrease in the activity of invertase. The inhibitor was found to be a low molecular weight protein and its action was non-competitive. Jaynes and Nelson (35) also found that a protein present in the developing endosperm of maize caused a loss of invertase 1 activity. The inactivator decreased invertase 1 activity during a preincubation period before the addition of sucrose. The inactivator was non-competitive and relatively specific for invertase 1 of endosperm of maize.

LITERATURE CITED

1. Afridi, M. M. R. K. and E. J. Hewitt. 1964. The inducible formation and stability of nitrate reductase in higher plants. I. Effects of nitrate and molybdenum on enzyme activity in sulfhydryl mutants of Lycopersicon esculentum. J. Expt. Botany 15: 251-71.

2. Anacher, W. F. and V. Stoy. 1958. Proteinchromatographik an calciumphosphate. I. Reinigung van Nitrate-reduktase aus Weizenblattern. Biochem. Z. 330: 141-59.

3. Bechet, J. and J. M. Wiame. 1965. Indication of specific regulatory binding protein for ornithine transcarbamylase in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 21: 226-34.

4. Beevers, L., D. Flesher, and R. H. Hageman. 1964. Studies on the pyridine nucleotide specificity of nitrate reductase in higher plants and its relationship to sulfhydryl level. Biochem. Biophys. Acta. 89: 453-64.

5. Beevers, L. and R. H. Hageman. 1969. Nitrate reduction in higher plants. Ann. Rev. Plant Physiol. Vol. 20, pp 495-522.

6. Beevers, L., L. E. Schrader, D. Flesher, and R. H. Hageman. 1965. The role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. Plant Physiol. 40: 691-98.

7. Berger, L., M. W. Slein, S. P. Colowick, and C. F. Cori. 1946. Isolation of hexokinase from baker's yeast. J. Gen. Physiol. 29: 379-91.

8. Berlin, C. M. and R. T. Schimke. 1965. Influence of turnover rates on the responses of enzymes to cortisone. Mol. Pharmacol. 1: 149-56.

9. Blobel, G. and V. R. Potter. 1966. Relation of ribonuclease and ribonuclease inhibitor to the isolation of polysomes from rat liver. Proc. Natl. Acad. Sci. 55: 1238-88.

22. Greenberg, D. M. 1955. Plant proteolytic enzymes. In: S. P. Colowick and N. O. Kaplan (Eds.), Methods in Enzymology, Vol. 2, Academic Press, New York, pp 54-64.

23. Hageman, R. H. and D. Flesher. 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35: 700-08.

24. Hageman, R. H., D. Flesher, and A. Gitter. 1961. Diurnal variation and other light effects influencing the activity of nitrate reductase and nitrogen metabolism in corn. Crop Sci. 1: 201-04.

25. Harper, J. E. and R. H. Hageman. 1972. Canopy and seasonal profiles of nitrate reductase in soybeans (Glycine maxl. merr.). Plant Physiol. 49: 146-154.

26. Harper, J. E. and G. M. Paulsen. 1968. Influence of intensity, quality and duration of light on nitrogen reduction and assimilation in wheat. Crop Sci. 60: 537-539.

27. Hewitt, E. J. 1957. Some aspects of micronutrient element metabolism in plants. Nature 180: 1020-22.

28. Hewitt, E. J. and M. M. R. K. Afridi. 1959. Adaptive synthesis of nitrate reductase in higher plants. Nature 183: 57-58.

29. Hewitt, E. J. and D. J. D. Nicholas. 1964. Enzymes of inorganic nitrogen metabolism. In: H. F. Linskens, B. D. Sanwall and M. V. Tracey (Eds.), Modern Methods of Plant Analysis, Vol. 7, Springer Verlag, Berlin, pp 67-172.

30. Heyes, J. K. and R. Brown. 1965. Cytochemical changes in cell growth and differentiation in plants. In: W. Ruhland (Ed.), Encyclopedia of Plant Physiology, Vol. 15/1, Springer Verlag, Berlin, Heidelberg, New York, pp 189-212.

31. Hope, A. B. and N. A. Walker. 1960. Ionic relations of cells of Chara australis. III. Vacuolar fluxes of sodium. Aust. J. Biol. Sci. 13: 277-91.

32. Ingle, J. 1968. Nucleic acid and protein synthesis associated with the induction of nitrate reductase activity in radish cotyledons. Biochem. J. 108: 715-23.

10. Bowman, D. E. 1945. Amylase inhibitor of navy beans. Science 102: 358-59.

11. Candela, M. I., E. C. Fisher, and E. J. Hewitt. 1957. Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. Plant Physiol. 32: 280-88.

12. Chen, T. M. and S. K. Ries. 1969. Effect of light and temperature on nitrate uptake and nitrate reductase activity in rye and oat seedlings. Can. J. Bot. 47: 341-43.

13. Del Campo, J. M., A. Paneque, J. M. Ramirez, and M. Losada. 1963. Nitrate reductace in the light by isolated chloroplasts. Biochim. Biophys. Acta. 66:450-52.

14. Dvorak, H. F., Y. Anrabu, and L. A. Heppel. 1966. The occurrence of a protein inhibitor for 5'-nucleotidase in extracts of E. coli. Biochem. Biophys. Res. Commun. 24: 628-32.

15. Engelsma, G. 1967. Effect of cycloheximide on the inactivation of phenylalanine deaminase in gherkin seedlings. Naturwissenschaften. 54: 319-320.

16. Evans, H. J. and H. S. Hall. 1955. Association of Mo with nitrate reductase from soybean leaves. Science 122: 922-23.

17. Evans, H. J. and A. Nason. 1953. Pyridine nucleotide nitrate reductase from extracts of higher plants. Plant Physiol. 28: 233-54.

18. Ferguson, J. J., Jr., M. Boll, and H. Holzer. 1967. Yeast malate dehydrogenase: Enzyme inactivation in catabolite repression. European J. Biochem. 1: 21-25.

19. Ferrari, T. E. and J. E. Varner. 1969. Substrate induction of nitrate reductase in barley aleurone layers. Plant Physiol. 44: 85-88.

20. Filner, P. 1965. Regulation of nitrate reductase in cultured tobacco cells. Biochem. Biophys. Acta. 118: 299-310.

21. Gerhart, J. C. and A. B. Pardee. 1962. The enzymology of control by feedback inhibition. J. Biol. Chem. 237: 891-96.

33. Ingle, J., K. W. Joy, and R. H. Hageman. 1966. The regulation of activity of the enzyme involved in the assimilation of nitrate by higher plants. Biochem. J. 100: 577-88.

34. Jacob, F. and J. Monod. 1961. On the regulation of gene activity. In: Cold Spring Harbor Symposium on Quant. Biol., Vol. 26, pp 193-211.

35. Jaynes, T. A. and O. E. Nelson. 1970. An inactivator in maize endosperm and factors affecting inactivation. Plant Physiol. 47: 629-34.

36. Joy, K. W. 1969. Nitrogen metabolism of Lemna minor. II. Enzymes of nitrate assimilation and some aspects of their regulation. Plant Physiol. 44: 849-53.

37. Kannangara, C. G. and H. W. Woolhouse. 1967. The role of carbon dioxide, light and nitrate in the synthesis and degradation of nitrate reductase in leaves of Perilla frutescens. New Phytologist 66: 553-61.

38. Kazal, L. A., D. S. Spicer, and R. A. Brahinsky. 1948. Isolation of a crystalline trypsin inhibitor-anticogulant protein from pancreas. J. Am. Chem. Soc. 70: 3034-40.

39. Kerridge, D. 1958. The effect of acti-dione and other anti-fungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis. J. Gen. Microbiol. 19: 497-506.

40. Kenney, F. T. 1967. Turnover of rat liver tyrosine transaminase: Stabilization after inhibition of protein synthesis. Science 156: 525-28.

41. Kern, M. and R. Natale. 1958. A diphosphopyridine nucleotidase and its protein inhibitor from Mycobacterium butyricum. J. Biol. Chem. 231: 41-51.

42. Kinsky, S. C. 1961. Induction and repression of nitrate reductase in Neurospora crassa. J. Bacteriol. 82: 898-904.

43. Kunitz, M. 1945. Crystallization of a trypsin inhibitor from soybean. Science 101: 668-69.

44. Lehninger, A. L. 1971. Biochemistry. Worth Publishers, New York, p 704.

45. MacRobbie, E. A. C. and J. Dainty. 1958. Sodium and potassium distribution and transport in the seaweed Rhodymenia palmata L. grev. Physiol. Plantarum 11: 782-801.

46. Markus, G. 1965. Protein substrate conformation and proteolysis. Proc. Natl. Acad. Sci. 54: 253-58.

47. Markus, G., D. K. McClintock, and B. A. Castellani. 1967. Ligand-stabilized conformations in serum albumin. J. Biol. Chem. 242: 4402-08.

48. Maretzki, A. and A. D. Cruz. 1967. Nitrate reductase in sugar cane tissues. Plant and Cell Physiol. 8: 605-11.

49. McClintock, D. and G. Markus. 1968. Conformational changes in aspartate transcarbamylase. I. Proteolysis of the intact enzyme. J. Biol. Chem. 243: 2855-62.

50. Monod, J., J. P. Changeux, and F. Jacob. 1963. Allosteric proteins and cellular control system. J. Mol. Biol. 6: 306-29.

51. Morris, I. and P. J. Syrett. 1963. The development of nitrate reductase in chlorella and its repression by ammonium. Arch. Mikrobiol. 47: 32-41.

52. Nason, A. and H. J. Evans. 1953. Triphosphopyridine nucleotide nitrate reductase in neurospora. J. Biol. Chem. 202: 655-73.

53. Oji, Y. and G. Izawa. 1972. Quantitative changes of free amino acids and amides in barley plants during ammonia and nitrate assimilation. Plant and Cell Physiol. 13: 249-59.

54. Onwueme, I. O., H. M. Laude, and R. C. Huffaker. 1970. Nitrate reductase activity in relation to heat stress in barley seedlings. Crop Science 11: 195-200.

55. Peanasky, R. J. and M. Laskowski. 1953. Partial purification of the trypsin inhibitor from blood plasma. J. Biol. Chem. 204: 153-57.

56. Pressey, R. 1966. Separation and properties of potato invertase and invertase inhibitor. Arch. Biochem. Biophys. 113: 667-74.

57. Pressey, R. 1967. Invertase inhibitor from potato: purification, characterization and reactivity with plant invertase. Plant Physiol. 42: 1780-86.

58. Pressey, R. and R. Shaw. 1966. Effect of temperature on invertase, invertase inhibitor and sugars in potato tubers. Plant Physiol. 41: 1657-61.

59. Price, V. E., W. R. Steerling, V. A. Tarantola, R. W. Hartley, Jr., and M. Rechcigel, Jr. 1962. The kinetics of catalase synthesis and destruction in vivo. J. Biol. Chem. 237: 3468-75.

60. Ramirez, J. S. and H. L. Mitchell. 1960. The trypsin inhibitor of alfalfa. J. Agr. Food Chem. 8: 393-95.

61. Randall, P. J. 1969. Changes in nitrate and nitrate reductase levels on restoration of molybdenum to molybdenum-deficient plants. Aust. J. Agr. Res. 20: 634-42.

62. Rechcigel, M., Jr., and W. E. Heston. 1967. Genetic regulation of enzyme activity in mammalian systems by the alternation of the rates of enzyme degradation. Biochem. Biophys. Res. Commun. 27: 119-24.

63. Ritenour, G. L., K. W. Joy, J. Bunning, and R. H. Hageman. 1967. Intracellular localization of nitrate reductase, nitrite reductase and glutamic acid dehydrogenase in green leaf tissue. Plant Physiol. 42: 233-37.

64. Roth-Bejerano, N. and S. H. Lips. 1970. Seasonal characteristics of the daily fluctuation in nitrate reductase activity. Physiol. Plantarum 23: 530-35.

65. Ryan, C. A. 1966. Chymotrypsin inhibitor I from potatoes: Reactivity with mammalian, plant, bacterial, and fungal proteinases. Biochem. 5:1592-96.

66. Sanderson, G. W. and E. C. Cocking. 1964. Enzymic assimilation of nitrate in tomato plants. I. Reduction of nitrate to nitrite. Plant Physiol. 39: 416-22.

67. Schimke, R. T. 1964. The importance of both synthesis and degradation in the control of arginase levels in rat liver. J. Biol. Chem. 239: 3808-17.

68. Schimke, R. T. 1969. On the roles of synthesis and degradation in regulation of enzyme levels in mammalian tissues. In: B. L. Horecker and E. R. Stadtman (Eds.), Current Topics in Cellular Regulation, Vol. 1, Academic Press, New York and London, pp 77-124.

69. Schimke, R. T. 1970. Regulation of protein degradation in mammalian tissues. In: H. N. Munro (Ed.), Mammalian Protein Metabolism, Vol. 4, Academic Press, New York and London, pp 177-228.

70. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1964. An analysis of the kinetics of rat liver tryptophan pyrrolase induction: The significance of both enzyme synthesis and degradation. Biochem. Biophys. Res. Commun. 15: 214-19.

71. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1965. The role of synthesis and degradation in the control of rat liver tryptophan pyrrolase. J. Biol. Chem. 240: 322-31.

72. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1965. Studies of the stability in vivo and in vitro of rat liver tryptophan pyrrolase. J. Biol. Chem. 240: 4609-20.

73. Schlessinger, D. and F. Ben-Hamida. 1966. Turnover of protein in E. coli starving for nitrogen. Biochim. Biophys. Acta. 119: 171-82.

74. Schrader, L. E., L. Beevers, and R. H. Hageman. 1967. Differential effects of chloramphenicol on the induction of nitrate and nitrite reductase in green leaf tissue. Biochem. Biophys. Res. Commun. 26: 14-17.

75. Schrader, L. E. and R. H. Hageman. 1967. Regulation of nitrate reductase activity in corn (Zea mays L.) seedlings by endogenous metabolites. Plant Physiol. 42: 1750-56.

76. Schrader, L. E., G. L. Ritenour, G. L. Eilrich, and R. H. Hageman. 1968. Some characteristics of nitrate reductase from higher plants. Plant Physiol. 43: 930-40.

77. Schwimmer, S., R. U. Makower, and E. S. Rorem. 1961. Invertase and invertase inhibitor in potato. Plant Physiol. 36: 313-16.

78. Segal, H. and Y. S. Kim. 1963. Glucocorticoid stimulation of the biosynthesis of glutamic-alanine transaminase. Proc. Natl. Acad. Sci. 50: 912-18.

79. Shen, T. C. 1969. The induction of nitrate reductase and the preferential assimilation of ammonium in germinating rice seedlings. Plant Physiol. 44: 1650-55.

80. Shibata, M., M. Kobayashi, and E. Takahashi. 1969. The possiblity of photo-induced induction of nitrate reductase in rice seedlings. Plant and Cell Physiol. 10:337-48.

81. Siegel, M. R. and H. S. Sisler. 1964. Site of action of cycloheximide in cells of Saccharomyces pastorianus. I. Effect of the antibiotic on cellular metabolism. Biochim. Biophys. Acta. 87: 70-82.

82. Smith, F. W. and J. F. Thompson. 1971. Regulation of nitrate reductase in excised barley roots. Plant Physiol. 48: 219-23.

83. Sohonie, K. and K. S. Ambe. 1955. Crystalline inhibitors of trypsin from potato. Nature 176: 972.

84. Spencer, D. 1959. A diphosphopyridine nucleotide-specific nitrate reductase from germinating wheat. Aust. J. Biol. Sci. 12: 181-91.

85. Stewart, G. R. 1968. The effect of CH on the induction of nitrate and nitrite reductase in Lemna minor L. Phytochem. 7: 1139-42.

86. Sussman, M. 1965. Inhibition by acti-dione of protein synthesis UDP-Gal polysaccharide transferase accumulation in Dictyostelium discoideum. Biochem. Biophys. Res. Commun. 18: 763-67.

87. Sussman, M. and R. R. Sussman. 1965. The regulatory program for UDP Galactose polysaccharide transferase activity during slime mold cyto-differentiation: requirement for specific synthesis of ribonucleic acid. Biochim. Biophys. Acta. 108: 463-73.

88. Trayser, K. A. and S. P. Colowick. 1961. Properties of crystalline hexokinase from yeast. III. Studies on glucose-enzyme interaction. Arch. Biochem. Biophys. 94: 169-76.

89. Syrett, P. J. and I. Morris. 1963. The inhibition of nitrate assimilation by ammonium in Chlorella. Biochim. Biophys. Acta. 67: 566-75.

90. Tauber, H., B. B. Kreshaw, and R. D. Wright. 1949. Studies on the growth inhibitor fraction of lima beans and isolation of a crystalline heat-stable trypsin inhibitor. J. Biol. Chem. 179: 1155-61.

91. Travis, R. L., R. C. Huffaker, and J. L. Key. 1970. Light induced development of polyribosomes and the induction of nitrate reductase in corn leaves. Plant Physiol. 46: 800-05.

92. Travis, R. L., W. R. Jordan, and R. C. Huffaker. 1969. Evidence for an inactivating system of nitrate reductase in Hordeum vulgare L. during darkness that requires protein synthesis. Plant Physiol. 44: 1150-56.

93. Travis, R. L., W. R. Jordan, and R. C. Huffaker. 1970. Light and nitrate requirements for induction of nitrate reductase in Hordeum vulgare L. Physiol. Plant. 23: 678-85.

94. Travis, R. L. and J. L. Key. 1971. Correlation between polyribosome level and the ability to induce nitrate reductase in dark-grown seedlings. Plant Physiol. 48: 617-20.

95. Zieserl, J. F., W. L. Rivenbark, and R. H. Hageman. 1963. Nitrate reductase activity, protein content and yield of four maize hybrids at varying plant populations. Crop Sci. 3: 27-32.

96. Zito, R., E. Antonini, and J. Wyman. 1964. The effect of oxygenation on the rate of digestion of human hemoglobins by carboxypeptidases. J. Biol. Chem. 239: 1804-08.

97. Zucker, M. 1968. Sequential induction of phenylalanine ammonia-lyase and a lyase-inactivating system in potato tuber disks. Plant Physiol. 43: 365-74.

AN INHIBITOR OF NITRATE REDUCTASE FROM MAIZE (ZEA MAYS)

PART I

OCCURRENCE OF A PROTEIN-LIKE INHIBITOR OF POSSIBLE PHYSIOLOGICAL SIGNIFICANCE IN MAIZE ROOTS

ABSTRACT

The activity of nitrate reductase in root extracts prepared from young corn (Zea mays L.) seedlings decreased with age until 10 days after germination when it was no longer detectable. A factor which inhibited nitrate reductase developed in the extracts coincident with the disappearance of the reductase activity. The inhibitor, which could be precipitated by acetone or ammonium sulfate and redissolved in buffer, was nondialyzable and was not affected by Polyclar-AT, indicating that it was not a phenolic compound or other small molecule. Heating the inhibitor at 60°C for 10 min led to a partial loss of its action and heating at 80°C for 10 min caused a complete loss of inhibitory action. The inhibitor was also completely inactivated when held at pH 2 at 0°C for 30 min but was relatively stable at higher pH's. Passage of the inhibitor preparation through a Bio-Gel P-100 column indicated that all of the inhibitor was associated with one large molecular weight component. All of these observations considered together indicate that the corn root inhibitor of nitrate reductase is probably a protein.

The inhibitor appears to be relatively specific for nitrate reductase. It had no effect on phenylalanine ammonia-lyase, invertase, glutamate dehydrogenase, malate dehydrogenase or glucose oxidase. The action of the inhibitor did not appear to be through an effect on the flavin nor molybdenum components of the enzyme nor on the NADH electron donor. Also, the inhibitor had no effect on exogenous nitrite.

Nitrate reductase activity could be detected in the roots by an in vivo assay, but the level was less than 15% of that found in the leaves by the same assay. It was concluded that the inhibitor may play a physiological role in corn by causing a shift in the site of nitrate reduction from the roots to the leaves.

The level of activity of an enzyme is a function of both its rates of synthesis and inactivation. The significance of the inactivating process has been recognized for some time in animal systems (33, 37, 38). For plants, however, knowledge of enzyme inactivation has developed only in recent years. The apparently active degradation or inhibition of several plant enzymes recently has been reported (11, 17, 26, 32, 43, 46). It was demonstrated that inhibitors of protein synthesis prevented the decline of phenylalanine ammonia-lyase activity which followed the light- or wounding-induced elevation of the activity of this enzyme in a number of plants (11, 46). Recently, Travis et al. (43) and Onwueme et al. (26) also found that the disappearance of nitrate reductase activity in the leaves of barley was inhibited by cycloheximide. A low molecular weight protein inhibitory to invertase occurs in potato tubers (30, 31). Pressey (32) found that warmer temperatures increased the concentration of this inhibitor and consequently decreased invertase activity in the tuber. Similarly, Jaynes et al. (17) reported a protein in the developing endosperm of maize which caused a loss of invertase activity. Inhibitors of proteolytic enzymes are also widespread in plants (19, 41).

Thus there are some examples in plants of a process which may well prove to be as significant in the control of plant metabolism as enzyme induction and protein synthesis.

Nitrate reductase has been intensively studied since its discovery by Evans and Nason (12). It is an enzyme known to turn over quickly, and factors which cause the level of its activity to increase have been studied intensively (3). However, except for the work with barley (26, 43) there is little information relative to its inactivation.

Except for those plants which grow symbiotically with nitrogen fixing micro-organisms, the majority of plants probably obtain most of their nitrogen by absorbing it through their roots in the form of nitrate. Therefore, it would be logical that roots would constitute a primary site for the process of nitrate reduction to occur. It has been reported that most of the nitrogen transported in the cell sap of both dicotyledons and monocotyledons was in the organic form (4, 44) which implies that most of the incoming nitrogen is incorporated directly into organic compounds in the root. However, the significance of the role of plant roots in the reduction of nitrate is controversial. The majority of nitrate reductase activity in many plants is found in the foliage instead of the roots (12, 15, 36), and the level of nitrate reductase activity in root extracts is frequently low (6, 15, 22, 36). Also, some plants, such as corn, accumulate large quantities of nitrate in their leaves. On the other hand, Boutard (5) and Coupe et al. (8) extracted nitrate reductase from barley roots and concluded that the roots were more efficient than the leaves in nitrate assimilation. Mifflin (24), on the basis of his work with

barley roots, suggested that no or low activity in root extracts was not due to the absence of the enzyme from roots but rather to a failure to obtain optimal conditions for extraction.

Young corn seedlings, like most other plants, have a low level of nitrate reductase activity in the root extracts (15). In our preliminary experiments, however, it was observed that extracts from roots of corn seedlings older than 10 days were devoid of nitrate reductase activity and further, that they apparently contained a factor capable of inactivating nitrate reductase extracted from leaves. This paper describes some of the physiological aspects of this inhibitor which appears to be a protein specific for nitrate reductase.

MATERIALS AND METHODS

Plant Material. Seeds of the dominant tall form of the d_1 dwarf of maize (Zea mays L.) (34) were soaked in tap water for three days prior to planting. Seedlings were grown in a mixture of soil, sand, and peat moss in the greenhouse. They were watered daily and fertilized weekly with 20:20:20 (N, P_2O_5, K_2O) soluble fertilizer. Except where noted otherwise, the plants were three to five weeks old when harvested.

Preparation of Nitrate Reductase and Inhibitor. The mid-portion of leaf blades of maize seedlings was used as the source of nitrate reductase. Chilled tissue was weighed and cut into small pieces. The tissue (5 to 10 g) was ground with an equal weight of hydrated Polyclar-AT powder (GAF Corporation) in a "Virtis 45" homogen-

izer at high speed for 1.5 minutes in five volumes of 0.1 M tris-(hydroxymethyl)-aminomethane-HCl buffer (tris-HCl buffer), pH 7.6, containing 0.1 M cysteine (L-cysteine hydrochloride· H_2O) and 0.003 M ethylenedinitrilotetraacetic acid (EDTA). The homogenate was squeezed through two layers of cheesecloth and centrifuged for 10 minutes at 22,000 X g. The supernatant fluid in some instances was used directly as the enzyme. In most cases, however, a limited purification was accomplished by ammonium sulfate fractionation. The material insoluble between 25 and 50% saturation with respect to $(NH_4)_2SO_4$ was dissolved in 0.1 M phosphate (K^+) buffer (pH 7.6) and used as the enzyme.

A fraction inhibitory to nitrate reductase was prepared from root tissue by a procedure analogous to that for nitrate reductase. Root tissue was washed, blotted, weighed, and then ground with an equal weight of hydrated Polyclar-AT in 0.1 M tris-HCl buffer (pH 7.6) (1 : 2 $^{w}/_{v}$) in a "Virtis 45" homogenizer for 1.5 minutes at high speed. The homogenate was strained through cheesecloth and clarified by centrifugation at 22,000 X g for 10 minutes. The supernatant fluid was referred to as the "crude root extract". The protein in the crude root extract was precipitated by the addition of 1.5 volumes of cold (-10 °C) acetone. After thorough mixing, the aqueous acetone solution was stored at -10° C for 20 to 30 minutes to allow for flocculation of material which was then sedimented by centrifugation at 12,000 X g for 10 minutes. The precipitated material was washed with distilled water and resuspended in 0.1 M phosphate (K^+) buffer (pH 7.6). This material was referred to as the "acetone-precipitated fraction of the root extracts" and in most experiments was used as the source of inhibitor of nitrate reductase from maize roots.

The acetone fraction of the root extract was also used as the source of several enzymes, including invertase, phenylalanine ammonia-lyase, malate dehydrogenase and glutamate dehydrogenase.

Nitrate Reductase Assay. Nitrate reductase was assayed *in vitro* with NADH as electron donor according to the procedure of Hageman and Flesher (15). A standard reaction mixture contained the following in 3.0 ml: 40 µmoles KNO_3, 100 µmoles potassium phosphate (pH 7.6), an appropriate amount of NADH (0.13 to 0.63 µmoles) and 0.2 to 0.5 ml of enzyme preparation. The reaction mixture was incubated at 30 °C for 10 to 15 minutes. An *in vivo* assay of nitrate reductase was performed according to the method described by Mulder *et al*. (25). Enzyme activity was expressed as the number of µmoles of NO_2^- formed/g fresh wt/hr.

Other Enzyme Assays. Each enzyme was assayed under optimal conditions as recorded in the literature. L-phenylalanine ammonia-lyase (PAL) was assayed as described by Reid and Marsh (35). One unit of activity for PAL was defined as the amount of enzyme which catalyzed the production of 1 µmole of *trans*-cinnamic acid in 1 min under standard conditions (30° C). Invertase was assayed as described by Pressey (30). A unit of invertase activity is defined as that amount of enzyme which catalyzes the liberation of 1 µmole of reducing hexose (glucose and fructose) per hour under the condition of the assay. The decrease in absorbance at 340 nm due to NAD production was used for assays of malate dehydrogenase activity according to Danner and Ting (9) and of glutamate dehydrogenase activity as described by Pahlich and Joy (27). One unit of both malate dehydrogenase and glutamate dehydrogenase activity is that amount of enzyme catalyzing the oxidation

of 1 μmole of NADH per minute. Glucose oxidase (purchased from Nutritional Biochem. Corp., Cleveland, Ohio) was assayed by following the rate of oxygen uptake as measured manometrically (40). Enzyme activity was expressed as micromoles of oxygen uptake per minute.

Protein Determination. Protein was estimated by the biuret method using bovine serum albumin as a standard (13).

RESULTS AND DISCUSSION

Hageman and Flesher (15) reported that the roots of young corn seedlings (about 8 days old) contained 20% of the nitrate reductase activity found in the shoots by a standard in vitro assay method (15). However, using similar methods we could not detect nitrate reductase activity in extracts prepared from the roots of two-week old corn seedlings. It seemed possible that this discrepancy might be due to the age of the seedlings and therefore the nitrate reductase activity in the roots and shoots of seedlings of increasing age was determined. From the data presented in Table I it appears that the presence of extractable nitrate reductase in root tissue is influenced by the age of the seedlings. A relatively high level of activity, nearly equal to that found in leaf tissue, was found in crude extracts prepared from very young roots (4 days old). In contrast to leaf tissue where the amount of activity increased with age, the level of nitrate reductase activity in the crude root extracts rapidly decreased with age. No nitrate reductase activity could be detected in root extracts of seedlings older than 10 days. The leaf sheath

Table 1. Changes with Age in Nitrate Reductase Activity in Corn Seedlings

Seedlings were grown in a growth chamber (14 hr light at 29.5°C and 10 hr dark at 24.0°C). The standard deviations are given.

Age	Activity		
	Root	Sheath*	Leaf
days	μmoles NO_2^-/g fresh wt/hr		
4	0.70 ± 0.06	...	0.91 ± 0.06
6	0.20 ± 0.02	...	1.86 ± 0.17
7	0.24 ± 0.01	1.27 ± 0.23	6.34 ± 0.22
8	0.24 ± 0.02	1.37 ± 0.03	6.05 ± 0.19
10	0	0.61 ± 0.11	6.43 ± 0.36
14	0	0.31 ± 0.03	5.39 ± 0.48

* Includes encompassed leaf tissue.

sections contained a significant amount of nitrate reductase activity, but the point was not ascertained whether this activity was actually associated with the leaf sheaths or the leaf blades enclosed within the sheaths. The activity in the leaf sheath sections declined with age for unknown reasons, although preliminary investigations did not reveal any inhibitor in the tissue.

The slow decrease in nitrate reductase activity in corn roots with age could be due to a reduced rate of enzyme synthesis coupled with a constant rate of enzyme inactivation. Possibly, in seedlings older than 10 days, there would be no more new enzyme synthesis in the root tissue. This assumption is consistent with the suggestion of Wallace and Pate (44) who thought that the site of nitrate reduction shifted from the root to the leaf with plant maturation. This concept, however, provides no explanation for why synthesis of active nitrate reductase should cease in the roots while at the same time the level of activity increases in the leaves (Table I).

The absence of detectable nitrate reductase activity in the root extracts also could be due to the presence of an inhibitor. To test this possibility mixing experiments were performed. Root extracts devoid of nitrate reductase activity were added to leaf extracts which contained active nitrate reductase, and the activity was determined. The results (Table II) indicated that crude extracts of roots from 10-day old seedlings partially inhibited nitrate reductase from leaf tissue. Moreover, preincubation of the reductase with the root extracts for 30 min increased the inhibition from an initial 36% to 75%. (This preincubation effect will be discussed in more detail in the companion paper (29).) Thus, it is reasonable to

Table II. Effect of Preincubation on the Degree of Inhibition by the Nitrate Reductase Inhibitor

Nitrate reductase (crude extracts prepared from the leaves of 10-day old seedlings) was preincubated either with water or inhibitor (crude extracts prepared from the roots of 10-day old seedlings, 0.8 mg protein) for 0 to 30 minutes at 25°C before initiating the reaction by the addition of NO_3^- and NADH. The reaction was run 10 minutes at 30°C. The standard deviations are given.

Preincubation	Activity		Inhibition
	Leaf	+ Root Extracts	
min	μmoles NO_2^-/g fresh wt/hr		%
0	8.83 ± 0.19	5.66 ± 0.34	36
30	8.01 ± 0.46	2.02 ± 0.13	75

postulate that the disappearance of nitrate reductase activity from the root extracts with the growth of the seedling is due to the development of an inhibitor in the roots.

Crude extracts prepared from the roots of 4- to 8-day old seedlings contained active nitrate reductase (Table I). However, when the protein in these extracts was precipitated with 1.5 volumes of acetone and then resuspended in buffer (cf Materials and Methods), all or most of the nitrate reductase activity originally present in the extract was lost. While the acetone precipitation treatment led to the loss of nitrate reductase activity from the root extracts, it also served to reveal the presence of a nitrate reductase inhibitor in these same extracts. Examples of these points can be seen by comparing the data presented in Tables I and III. The data in Table III were obtained using acetone precipitated preparations of the crude extracts of the roots and shoots for which the nitrate reductase activities are reported in Table I. The data (Table III) indicate that a factor which inhibits nitrate reductase develops in the roots of growing maize seedlings. Extracts prepared from both the roots and leaves of 4-day old seedlings contained a low but significant level of nitrate reductase activity. When the two extracts were combined, the results were additive. Six days after germination nitrate reductase activity in the leaf extract had increased more than 3-fold while it decreased in the acetone-precipitated root extract to a barely detectable level. Direct mixing of the leaf and shoot extracts suggested the possibility of an inhibitor in the extracts. A 30 min preincubation of the two extracts further indicated the presence of an inhibitor. The inhibitory action of the acetone precipitated root extract became much more pronounced

Table III. Development of the Nitrate Reductase Inhibitor in Root Extracts with Growth of the Corn Seedlings

Seedlings were the same material as used in Table I. Nitrate reductase and inhibitor were precipitated from the crude extracts by 1.5 volumes of acetone. The preincubation (30 min) and reactions were run as described in Table II. The standard deviations are given.

Age of Seedlings	Extract Source	Nitrate Reductase Activity	
		Without Preincubation	With Preincubation
days		µmoles NO_2^-/ g fresh wt/hr	
4	Roots	0.16 ± 0.03	
	Leaves	0.51 ± 0.02	
	Leaves and Roots	0.67 ± 0.06	
6	Roots	0.05 ± 0.02	0
	Leaves	1.73 ± 0.01	1.39 ± 0.01
	Leaves and Roots	1.58 ± 0.03	1.09 ± 0.01
7	Roots	0	0
	Leaves	1.05 ± 0.05	1.06 ± 0.08
	Leaves and Roots	0.96 ± 0.10	0.48 ± 0.07
8	Roots	0	0
	Leaves	1.78 ± 0.08	1.63 ± 0.21
	Leaves and Roots	1.54 ± 0.25	1.05 ± 0.10
10	Roots	0	0
	Leaves	3.55 ± 0.08	3.17 ± 0.05
	Leaves and Roots	2.98 ± 0.14	1.63 ± 0.08

with age until, 10 days after germination, a 30 min preincubation of root and leaf extracts gave 50% inhibition of nitrate reductase activity.

The presence of a system in the root extract which removed the NO_2^- formed by nitrate reductase or an NADH oxidase which removed the NADH required for the reduction of NO_3^- to NO_2^- could lead to the erroneous conclusion that an inhibitor of nitrate reductase occurred in the root extracts. These two possible explanations for the inhibitor were eliminated by the following experiments. Incubation of the root or leaf preparations and combinations of the two with varying amounts of NO_2^- under the same experimental conditions as used for the nitrate reductase assays did not lead to any detectable loss of the added NO_2^-. NADH oxidase activity was assayed in both the root and leaf extracts by following the decrease in NADH concentration spectrophotometrically. The data from Experiment I, Table IV, show that 126 mμ moles of NADH/3 ml were sufficient to support maximum nitrate reductase activity under our experimental conditions. From Experiment 2, Table IV, it can be seen that there was twice as much NADH oxidized by the leaf extract as there was NO_2^- produced, which indicated the presence of an NADH oxidase in this extract. However, the level of NADH did not fall in 10 min below the level required to support maximum nitrate reductase activity. Simularly, in the root extract, which contained the nitrate reductase inhibitor, and the leaf plus root preparations, there was NADH oxidation, but again, it did not reduce the level of NADH enough to affect reductase activity. The observation that preincubation of the inhibitor with nitrate reductase in the absence of its substrates led to a marked increase in the degree of inhibition

Table IV. Evidence that the Nitrate Reductase Inhibitor is not an NADH Oxidase

Enzyme Source	Concentration of NADH in Reaction Mixture			Nitrate Reductase Activity
	Zero Time	10 Min	Difference	
	mμ moles/3 ml			mμ moles NO_2^-/ 10 min/3 ml
Experiment I				
Leaf	126	...	...	45
	252	...	...	43.5
Experiment II				
Leaf	252	179	73	36
Root	252	226	26	0
Leaf + Root	252	187	65	18

(Table II) (29) is another line of evidence which indicates that the inhibitor is not effective by removing either the substrates (NADH and NO_3^-) or the reaction product (NO_2^-).

Inhibitors of nitrate reductase have been observed in numerous plants (10, 14, 18). Usually, these compounds are heat- and acid-stable and dialyzable. Frequently they appear to be phenolic in nature. It has proven difficult to establish whether or not in vivo they are of any physiological significance. As part of our efforts to characterize the nitrate reductase inhibitor from corn roots, aliquots of the acetone-precipitated extracts were either boiled for 10 min or acidified to pH 2 for 30 min. Both acidification and boiling completely removed the efficacy of the inhibitor from the root extracts (Table V). On the other hand, unlike inhibitors from some other tissues (18), dialysis overnight of the corn root extract against 0.1M phosphate (K^+), pH 7.6, did not eliminate the inhibitor. The sensitivity of the inhibitor in the corn roots to acidification and boiling, considered in conjunction with the observation that the inhibitory action is non-dialyzable, suggests that the inhibitor is associated with a protein-like molecule. In support of this concept is the observation that the inhibitor can be precipitated by acetone and by $(NH_4)_2SO_4$ (25 to 75% saturation). Further evidence that the inhibitor is associated with a large molecule is the observation that it is eluted near the solvent front, although trailing a predominant protein fraction, in passage through a Bio-Gel P-100 column (Figure 1). The fact that the inhibitor is associated with but one peak in the elution profile from the Bio-Gel P-100 column rather than being spread over the entire column is considered significant, for it minimizes the

Table V. Effect of Boiling, Acidification or Dialysis on the Action of the Nitrate Reductase Inhibitor Fraction from Corn Roots

An acetone-precipitated inhibitor preparation was either (1) boiled for 10 minutes and then quickly cooled to 0°C; (2) acidified to pH 2 with 5 N HCl and held for 30 minutes at 0°C and then readjusted back to pH 7.6 with 5 N NaOH; or (3) dialyzed overnight at 4°C against 0.1 M phosphate (K^+) buffer, pH 7.6. The treated inhibitor preparation was preincubated for 30 minutes at 25°C with nitrate reductase prior to running the enzyme assay.

Additives	Activity	Inhibition	Stimulation
	μmoles NO_2^-/g fresh wt/hr	%	
None	4.40	...	...
Inhibitor	0.16	96	...
Boiled inhibitor	5.50	...	25
Acidified inhibitor	5.12	...	16
Dialyzed inhibitor	0.24	94	...

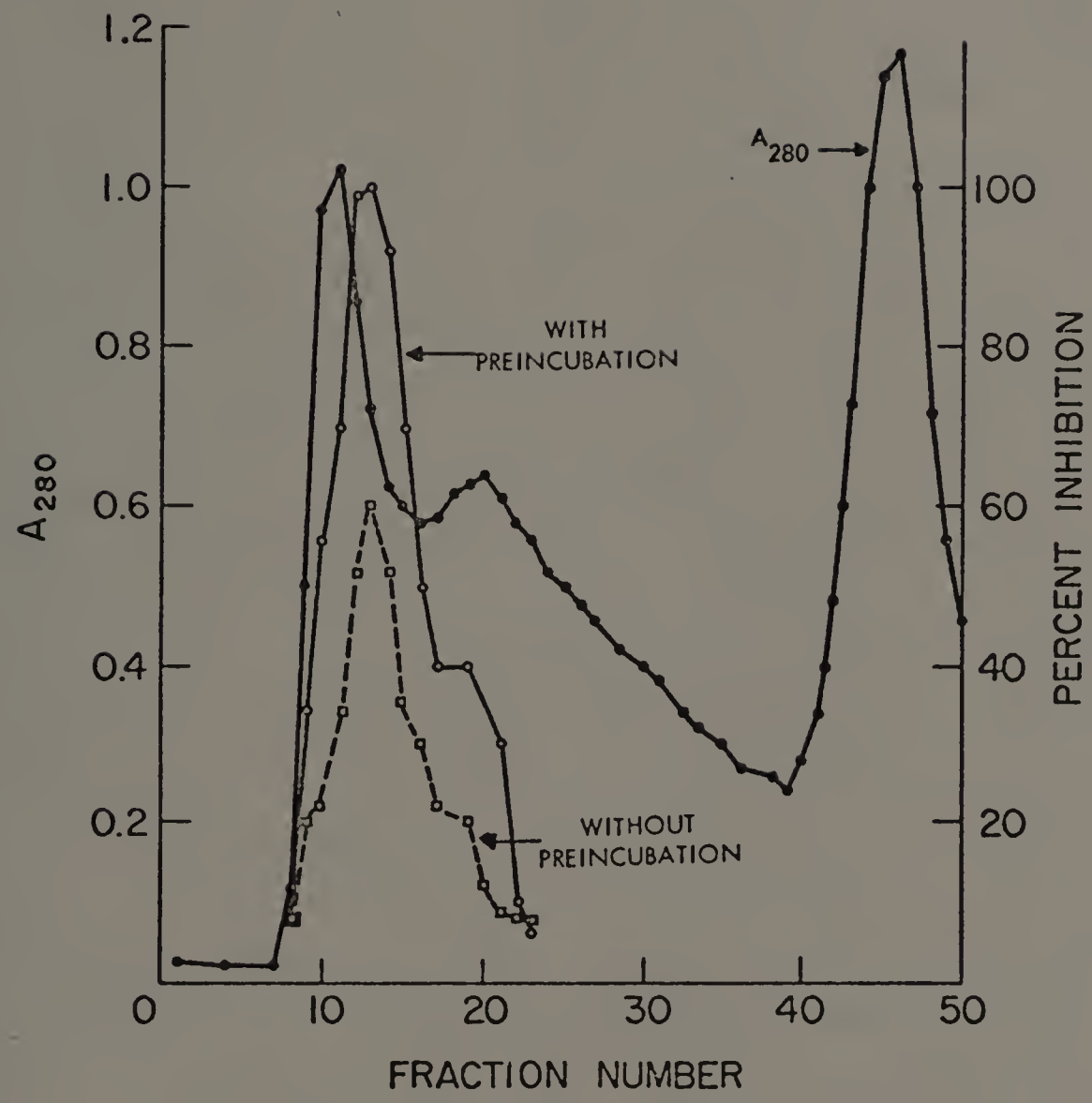

Figure 1. Elution Profile of a Nitrate Reductase Inhibition Preparation from a Bio-Gel P-100 Column

The sample was eluted from a 2.5 X 40 cm column at 4°C with 0.1 M phosphate (K^+), pH 7.6. The flow rate was adjusted to 3 ml per 10 min and 3 ml fractions were collected.

possibility that we are dealing here with a small inhibitory substance, such as a phenolic compound, bound indiscriminately to all proteins.

The sensitivity of the inhibitor to the hydrogen ion concentration and temperature was investigated further. Inhibition, measured both with and without preincubation of inhibitor and reductase, was relatively insensitive to changes in hydrogen ion concentration except at very acid conditions (pH 2) (Figure 2). To determine the effect of temperature on the inhibitor, the acetone-precipitated root extract was heated to temperatures between 40°C and 100°C for 10 min and then quickly cooled to 0°C. The inhibitor was relatively stable at temperatures below 60°C (Figure 3). However, the effectiveness of the inhibitor decreased steadily in proportion to the increase in temperature. It will be noted here and also in Table III that the heat-denatured inhibitor preparation and, to a lesser extent the acidified extract, actually stimulated the apparent nitrate reductase activity. This stimulation was not invariably observed in all experiments of this nature and the explanation for the phenomenon is not apparent; addition of exogenous flavin and MoO_3^- indicated that these two cofactors were not limiting.

In plants phenolic compounds are widespread and frequently occur in very high concentrations (16). The presence of phenolic compounds has made it impossible to isolate active enzymes by ordinary techniques from many plant tissues. Phenols combine reversibly with proteins by hydrogen bonding and irreversibly by oxidation of the phenol to the quinone followed by covalent condensation (21). Recently, insoluble polyvinylpyrrolidone (Polyclar-AT), which forms insoluble complexes with polyphenols and tannins, has been used to obtain active, soluble enzymes (1, 20).

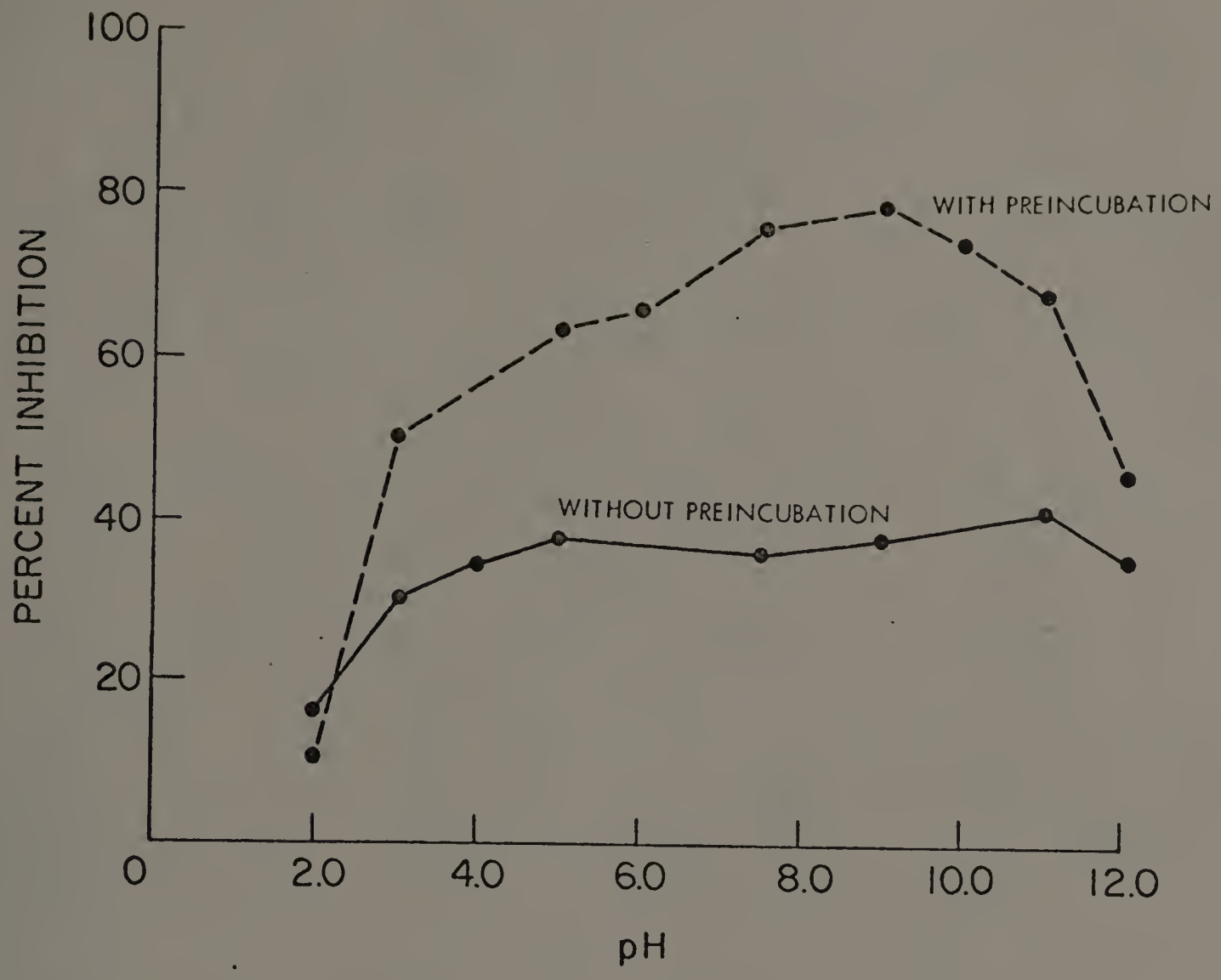

Figure 2. Effect of pH on the Stability of the Nitrate Reductase Inhibitor

The pH of aliquots of the root extracts was adjusted to the indicated values with either 2 N HCl or 2 N NaOH. After holding the samples for 10 minutes at 0°C, the pH was readjusted back to the original value of 7.6. The final volume of each treated extract was brought to the same volume with 0.1 M phosphate (K^+), pH 7.6. The inhibition assays were carried out under standard conditions (cf Table II).

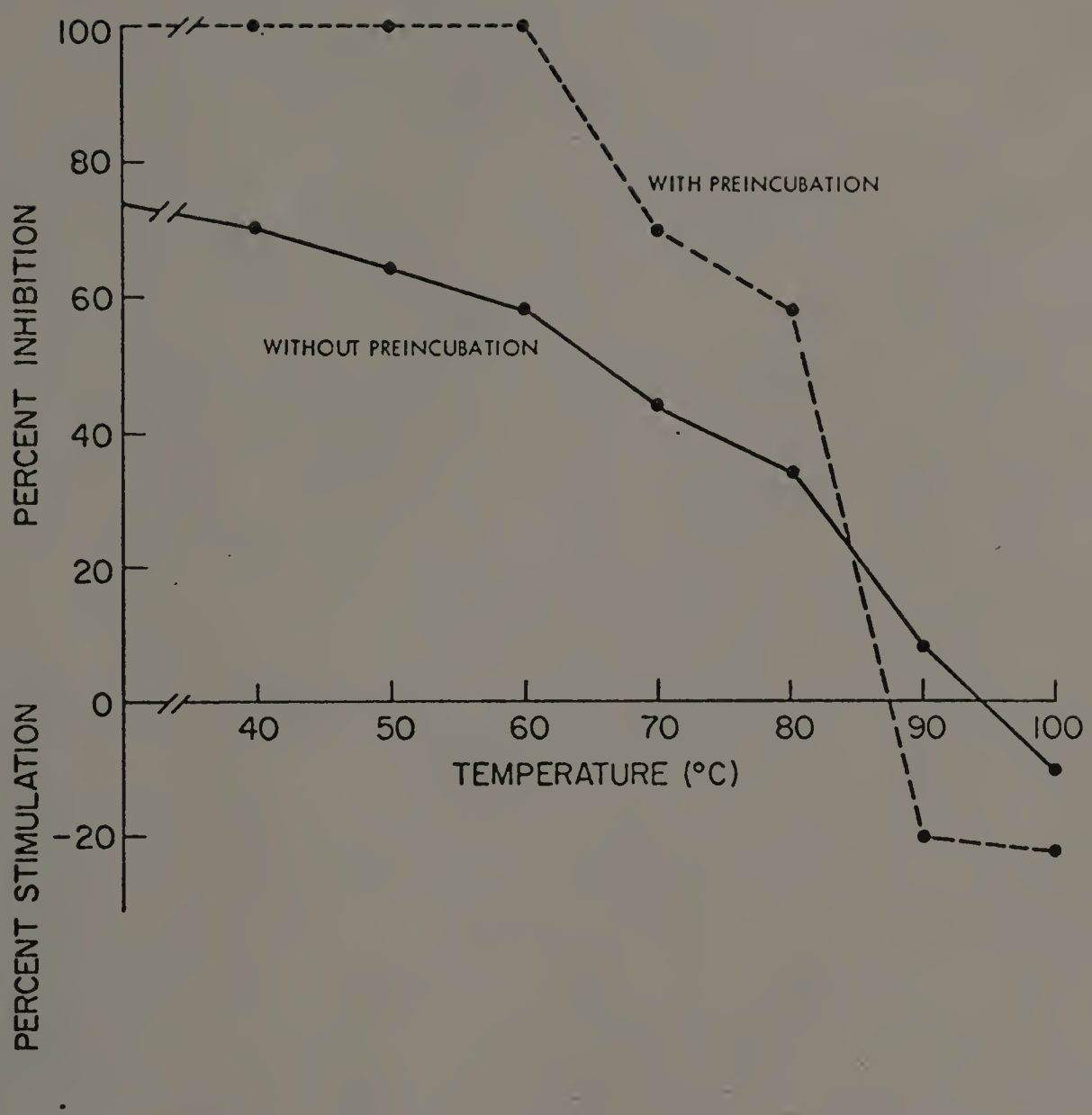

Figure 3. Effect of Temperature on the Stability of the Nitrate Reductase Inhibitor

Root extracts were heated in a water bath to temperatures varying from 40 to 100°C for 10 minutes. Following this heat treatment, the root extracts were cooled and then added to nitrate reductase preparation for inhibition study. The inhibition assays were carried out under standard conditions (cf Table II).

For example, Klepper and Hageman (18) successfully extracted nitrate reductase from apple leaves, tissue which previously had been considered to lack this enzyme. The effect of Polyclar-AT on nitrate reductase and its inhibitor from corn seedlings was investigated. Polyclar-AT increased the amount of nitrate reductase activity extracted from the leaves of three-week old plants but had no significant effect on the amount of activity obtained from the foliage of young (4- to 8-day old) seedlings. It has been reported elsewhere that nitrate reductase activity in leaf tissue decreased with age (39, 45). The results presented in Table VI imply that the low activity in older tissue may be partially due to the accumulation of phenolic compounds in the tissue.

The absence of detectable nitrate reductase activity in the extracts of corn roots could be due to the accumulation of phenolic compounds in this tissue. However, the addition of Polyclar-AT to the extraction medium did not result in the recovery of nitrate reductase activity from the root extracts (Table VI). Moreover, Polyclar-AT had no apparent effect on the inhibitor as the same percentage inhibition was observed when the reductase preparation was mixed with the inhibitor prepared with or without Polyclar-AT. These data, then, are further evidence that the inhibitor from corn roots is not simply a phenolic compound.

Although Polyclar-AT increased the activity of nitrate reductase in the corn leaf extracts, presumably by removing inhibitory phenolic compounds, we could not exclude the possibility that reduced forms of phenolic compounds remained in the leaf extracts. It is conceivable that such phenolic compounds could lead to the inhibition of nitrate reductase if a polyphenol oxidase was added to the leaf

Table VI. Effect of Polyclar-AT in the Extracting Medium on Nitrate Reductase and Inhibitor Activities

Tissue was ground either with or without an equal weight of hydrated Polyclar-AT powder.

Tissue	Activity
	μmoles NO_2^-/g fresh wt/hr
Leaf	
1) Young (8 days)	
Control	6.60
Treated with Polyclar-AT	6.30
2) Old (3 weeks)	
Control	3.36
Treated with Polyclar-AT	5.12
Root (3 weeks)	
Control	0
Treated with Polyclar-AT	0
Leaf (3 weeks) + Root (3 weeks)	
Leaf (Polyclar-AT) + Root	1.11
Leaf (Polyclar-AT) + Root (Polyclar-AT)	1.17

extracts (23, 42). The data reported in Table V and Figure 1 indicated that the inhibitor in the root extracts was a protein-like macromolecule. Therefore, the inhibition from the root extracts could be due to a polyphenol oxidase oxidizing phenolic compounds in the leaf extract. Cysteine, ascorbic acid and 2-mercapto-benzothiazole are effective inhibitors of polyphenol oxidase action (2, 7, 28). As shown in Table VII cysteine and 2-mercaptobenzothiazole alone had no significant effect on nitrate reductase activity. Ascorbic acid by itself, on the other hand, was slightly inhibitory. 2-Mercaptobenzothiazole had no effect on the action of the inhibitor and cysteine even enhanced the effectiveness of the inhibitor. The inhibitory action of ascrobic acid and the root extract appeared to be additive. Failure of these compounds to prevent or reduce the inhibition by the root extract indicates that the inhibitor is probably not a polyphenol oxidase.

It has been established that the corn root extracts contain a strong inhibitor of nitrate reductase. A logical question is whether the inhibitor is specific for nitrate reductase or is a general inhibitor of many diverse enzymes. To test this point the effect of the inhibitor on several different enzymes was examined. If the inhibitor in the root extract was universal for other enzymes then no enzyme activity should be detectable in the extract. However, phenylalanine ammonia-lyase, invertase, malic dehydrogenase and glutamate dehydrogenase were all active in the root extract (Table VIII). Since nitrate reductase is a metaloflavo-protein, it is logical to suspect that the inhibitor may be effective via its action on the FAD component of the enzyme. Although we cannot unequivocally exclude this possibility at this time, the inhibitor had no effect on the activity of another FAD dependent enzyme,

Table VII. Effect of Ascorbic Acid, Cysteine, and 2-Mercaptobenzothiazole on the Action of the Nitrate Reductase Inhibitor

Treatment	Nitrate Reductase Activity	Inhibition
	μmoles NO_2^-/g fresh wt/hr	%
None	4.26	...
+ Inhibitor	2.34	45
+ Ascorbic Acid (57 mM)	3.51	18
+ Cysteine (57 mM)	4.14	3
+ 2-Mercaptobenzothiazole (23 mM)	3.98	6
+ Inhibitor and Ascorbic Acid (57 mM)	1.08	75
+ Inhibitor and Cysteine (57 mM)	1.08	75
+ Inhibitor and 2-Mercaptobenzothiazole (23 mM)	2.27	47

Table VIII. Effect of Nitrate Reductase Inhibitor on the Activities of Several Enzymes

Both invertase and L-phenylalanine ammonia-lyase (PAL) were prepared from the leaf sheath of corn seedlings. Glucose oxidase was purchased from the Nutritional Biochemical Corporation.

Treatment	Activity			
	Nitrate Reductase	Invertase	PAL	Glucose Oxidase
	μmoles NO_2^-/g fresh wt/hr	mU/mg protein	mU/mg protein	mU/mg protein
Enzyme alone	4.34	0.5	9.0	1.25
Inhibitor alone	0	0.3	8.7	...
Enzyme + Inhibitor	1.30	0.8	18.3	1.38

glucose oxidase (Table VIII). The fact that glutamic dehydrogenase and malic dehydrogenase, both of which utilize NADH as the electron donor, were not affected by the inhibitor also indicates that the inhibition is not mediated via NADH. Adding MoO_3 to the reaction mixture afforded no protection against the inhibitor (unpublished observations). However, when the nitrate reductases of several other species were tested, they were all inhibited by the corn root inhibitor (29). These observations led us to believe that the inhibitor was relatively specific for nitrate reductase.

The recently developed in vivo assay for nitrate reductase has revealed the presence of this enzyme in tissues heretofore considered devoid of the enzyme (8, 10, 39). The in vivo assay minimizes the possibility of releasing compartmentalized inhibitors which could inactivate the enzyme during extraction. Trace amounts of nitrate reductase were demonstrable in the roots by the in vivo assay (Table IX). However, the activity in the roots was only 15% of that found in the leaf tissue by the same assay. The standard in vitro method, which requires homogenization of the tissue, showed, as usual, good activity in the leaves and none in the roots.

The intracellular location of the inhibitor is not known. The fact that a low level of nitrate reductase activity could be demonstrated by the in vivo method (Table IX) suggests that the inhibitor is at least not complexed with all of the nitrate reductase in the roots. On the other hand, the very low level of the reductase activity in the roots suggests that much of its activity may indeed be inhibited in this tissue. The fact that the inhibitor is relatively specific for

Table IX. Nitrate Reductase Activity in Corn Seedlings Obtained by the *In Vitro* (15) and *In Vivo* (25) Assays

The seedlings were 5 weeks old.

Tissue	Activity	
	In Vivo	*In Vitro*
	μmoles NO_2^-/g fresh wt/hr	
Leaf	0.27 ± 0.04	3.93 ± 0.12
Root	0.04 ± 0.01	0

nitrate reductase (Table VIII) strongly suggests that it should have some physiological significance in the control of this enzyme in root tissues. The question(s) of why an inhibitor of nitrate reductase should develop in the roots and what, if any, survival value such an inhibitor would have is an intriguing one. The reducing power required for the conversion of NO_2^- to ammonia appears to be photosynthetically generated in chloroplastic tissue. On the other hand, in the roots the source of the reducing power for the reduction is not clear, but since the nitrite reductases studied appear to require ferredoxin and NADPH, presumably the hexose monophosphate shunt would be the source of electrons. It conceivably could be of some advantage to the plant (i.e., by conserving NADPH for other purposes) for most of the NO_2^- reduction to occur in the tissue which contained the photosynthetic apparatus, that is, the leaf tissue. Therefore, we suggest that this inhibitor of nitrate reductase reported here may serve the very important function of turning off the nitrate reductase in the roots and shifting the bulk of the nitrate reduction reactions to the leaves in corn plants.

LITERATURE CITED

1. Anderson, L. W. 1968. Extraction of enzymes and subcellular organelles from plant tissues. Phytochemistry 7: 1973-98.

2. Baruah, P. and T. Swain. 1953. The effect of L-ascorbic acid on the in vitro activity of polyphenol oxidase from potato. Biochem J. 55: 392-99.

3. Beevers, L. and R. H. Hageman. 1969. Nitrate reduction in higher plants. Ann. Rev. Plant Physiol. Vol. 20, 495-522.

4. Bollard, E. G. 1956. Nitrogenous compounds in plant xylem sap. Nature 178: 1189-90.

5. Boutard, J. 1966. Effects de la lumiere et de alimentation en nitrate sur la variations de l'activite nitrate reductase de plantulis d'orge. Physiol. Veg. 4: 105-25.

6. Candela, M. I., E. C. Fisher, and E. J. Hewitt. 1957. Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. Plant Physiol. 32: 280-88.

7. Clayton, R. A. 1959. Properties of tobacco polyphenol oxidase. Arch. Biochem. Biophys. 81: 404-17.

8. Coupé, M., M. L. Champigny, and A. Mayse. 1967. Intracellular distribution of nitrate reductase in barley leaves and roots. Physiol. Veg. 5: 271-91.

9. Danner, J. and I. P. Ting. 1967. CO_2 metabolism in corn roots. II. Intracellular distribution of enzymes. Plant Physiol. 42: 719-24.

10. Dirr, M. A., A. V. Barker, and D. N. Maynard. 1972. Nitrate reductase activity in the leaves of the highbush blueberry and other plants. J. Amer. Soc. Hort. Sci. 97: 331-34.

11. Engelsma, G. 1967. Effect of cycloheximide on the inactivation of phenylalanine deaminase in gherkin seedlings. Naturwissenschaften 54: 319-320.

12. Evans, H. J. and A. Nason. 1953. Pyridine nucleotide nitrate reductase from extracts of higher plants. Plant Physiol. 28: 233-54.

13. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-62.

14. Grasmanis, V. O. and D. J. D. Nicholase. 1967. A nitrate reductase from apple roots. Phytochemistry 6: 217-218.

15. Hageman, R. H. and D. Flesher. 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35: 700-08.

16. Harborne, J. B. and N. W. Simmonds. 1964. The natural distribution of the phenolic aglycones. In: J. B. Harborne (Ed.), Biochemistry of Phenolic Compounds. Academic Press, London, pp 77-127.

17. Jaynes, T. A. and O. E. Nelson. 1971. An invertase inactivator in maize endosperm and factors affecting inactivation. Plant Physiol. 47: 629-34.

18. Klepper, L. and R. H. Hageman. 1969. The occurrence of nitrate reductase in apple leaves. Plant Physiol. 44: 110-14.

19. Kunitz, M. 1945. Crystallization of a trypsin inhibitor from soybean. Science 101: 668-69.

20. Leece, D. E., D. R. Dilley, and A. L. Kenworthy. 1972. The occurrence of nitrate reductase in leaves of prunus species. Plant Physiol. 49: 725-28.

21. Loomis, W. D. and J. Battaile. 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5: 423-38.

22. Maretzki, A. and A. D. Cruz. 1967. Nitrate reductase in sugar cane tissues. Plant and Cell Physiol. 8: 605-11.

23. Mason, H. S. 1955. Comparative biochemistry of the phenolase complex. In: F. F. Nord (Ed.), Advances in Enzymology, Vol. 16. Interscience Publishers, New York, pp 105-84.

24. Miflin, B. J. 1967. Distribution of nitrate and nitrite reductase in barley. Nature 214: 1133-34.

25. Mulder, E. G., R. Boxma, and W. L. VanVeen. 1959. The effect of molybdenum and nitrogen deficiencies on nitrate reductase in plant tissues. Plant and Soil 10: 335-55.

26. Onwuene, I. O., H. M. Laude, and R. C. Huffaker. 1970. Nitrate reductase activity in relation to heat stress in barley seedlings. Crop Sci. 11: 195-200.

27. Pahlich, E. and K. W. Joy. 1971. Glutamate dehydrogenase from pea roots: purification and properties of the enzyme. Can. J. Biochem. 49: 127-38.

28. Palmer, J. K. and J. B. Roberts. 1967. Inhibition of banana polyphenol-oxidase by 2-mercaptobenzothiazole. Science 157: 200-01.

29. Pan, Y. T. 1972. An investigation of a nitrate reductase inhibitor in the roots of Zea mays. Part II, Ph.D. Thesis. University of Massachusetts, Amherst.

30. Pressey, R. 1966. Separation and properties of potato invertase and invertase inhibitor. Arch. Biochem. Biophys. 113: 667-74.

31. Pressey, R. 1967. Invertase inhibitor from potato: purification, characterization and reactivity with plant invertase. Plant Physiol. 42: 1780-86.

32. Pressey, R. and R. Shaw. 1966. Effect of temperature on invertase, invertase inhibitor and sugars in potato tubers. Plant Physiol. 41: 1657-61.

33. Rechcigel, J., Jr. and W. E. Heston. 1967. Genetic regulation of enzyme activity in mammalian system by the alternation of the rates of enzyme degradation. Biochem. Biophys. Res. Commun. 27: 119-24.

34. Reid, P. H., E. A. Havir, and H. V. Marsh, Jr. 1972. L-phenylalanine ammonia-lyase (maize). Partial purification and response to gibberellic acid and cycloheximide of L-phenylalanine and L-tyrosine ammonia-lyase activities. Plant Physiol. (In Press).

35. Reid, P. D. and H. V. Marsh, Jr. 1969. Gibberellic acid promoted activity of L-phenylalanine ammonia-lyase in several plant species. Z. Pflanzenphysiol. 61: 170-72.

36. Sanderson, G. W. and E. C. Cocking. 1964. Enzymic assimilation of nitrate in tomato plants. I. Reduction of nitrate to nitrite. Plant Physiol. 39: 416-22.

37. Schimke, R. T. 1964. The importance of both synthesis and degradation in the control of arginase levels in rat liver. J. Biol. Chem. 239: 3808-17.

38. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1965. The role of synthesis and degradation in the control of rat liver tryptophan pyrrolase. J. Biol. Chem. 240: 322-31.

39. Streeter, J. G. and M. E. Bosler. 1972. Comparison of in vitro and in vivo assays for nitrate reductase in soybean leaves. Plant Physiol. 49: 448-50.

40. Swoboda, B. E. and V. Massey. 1965. Purification and properties of the glucose oxidase from aspergillus niger. J. Biol. Chem. 240: 2209-15.

41. Tauber, H., B. B. Kreshaw, and R. D. Weight. 1949. Studies on the growth inhibitor fraction of Lima beans and isolation of a crystalline heat-stable trypsin inhibitor. J. Biol. Chem. 179: 1155-61.

42. Thomson, R. H. 1964. Structure and reactivity of phenolic compounds. In: J. B. Harborne (Ed.), Biochemistry of Phenolic Compounds. Academic Press, London, pp 1-32.

43. Travis, R. L., W. R. Jordan, and R. C. Huffaker. 1969. Evidence for an inactivating system of nitrate reductase in Hordeum vulgare L. during darkness that requires protein synthesis. Plant Physiol. 44: 1150-56.

44. Wallace, W. and J. S. Pate. 1965. Nitrate reductase in the field pea (Pisum arvense L.). Ann. Botany 29: 655-71.

45. Wallace, W. and J. S. Pate. 1967. Nitrate assimilation in higher plants with special reference to the cocklebur (Xanthium pensylvanicum Wallr.). Ann. Botany 31: 213-28.

46. Zucker, M. 1968. Sequential induction of phenylalanine ammonia-lyase and a lyase-inactivating system in potato tuber disks. Plant Physiol. 43: 365-74.

AN INHIBITOR OF NITRATE REDUCTASE FROM MAIZE (ZEA MAYS L.)

PART II

AN INVESTIGATION OF THE MECHANISM OF INHIBITION

ABSTRACT

Some aspects of the mechanism of action of an inhibitor of nitrate reductase were examined. The inhibitor, a protein-like macromolecule obtained from corn roots, exhibited two types of inhibition towards nitrate reductase; one was little affected by temperature and was apparently instantaneous. The other was temperature sensitive, slow acting and caused a linear decrease in nitrate reductase activity when enzyme and inhibitor were incubated together in the absence of NO_3^- and NADH. The substrates did not afford any protection. A simple stoichiometric relationship did not exist between the inhibitor and enzyme. Instead, it appeared that some sort of equilibrium was established between the two. The inhibitor from corn roots appeared general for nitrate reductase for it inhibited the activity of this enzyme from several different species.

The possibility is discussed that the inhibitor is a binding protein which causes an immediate partial loss of nitrate reductase activity and at the same time makes the enzyme unstable. This latter would account for the linear loss of the balance of activity observed in preincubation experiments.

Much effort has been expended on the challenging problems involved in enzyme induction and synthesis, but comparable attention has not been paid to enzyme inactivation and degradation. The latter must be significant in the regulation of enzyme activity in higher organisms, for it is known that the life span of many enzymes is much shorter than that of the cells which contain them (6, 11, 22, 24). One such short-lived enzyme in plants is nitrate reductase with an estimated half-life of four hours in excised leaves (24). It is possible that this enzyme is inherently unstable and spontaneously breaks down. However, recent work (17, 26) has suggested that synthesis of a protein is required for the inactivation of nitrate reductase.

We reported in the companion paper (18) that extracts prepared from the roots of young maize seedlings contained a low but significant level of nitrate reductase activity. On the other hand, extracts from seedling roots 10 days old or older were devoid of nitrate reductase activity and instead were found to contain a protein-like macromolecule which inhibited nitrate reductase. The inhibitor was relatively specific for nitrate reductase having no effect on several other enzymes including NADH and FAD dependent ones. It appeared that the inhibitor was of some physiological significance since its appearance seemed to coincide with the disappearance of nitrate reductase from the roots and also because of its apparent specificity. We report here the results of investigations

on the mechanism of inhibition. The initial assumption in these investigations was that the inhibitor was either a binding protein which inhibited by forming a complex with the nitrate reductase molecule, or alternatively that the inhibitor was a proteolytic enzyme which specifically degraded nitrate reductase.

MATERIALS AND METHODS

The preparation of nitrate reductase and its inhibitor from maize (Zea mays L.) seedlings and the general method for assaying nitrate reductase have been described (18).

Nitrate Reductase Inhibitor Assay. There were two ways for studying the inhibition: 1) The "direct assay" in which the inhibitor (usually 0.2 to 0.3 mg protein) was added directly to a standard 3 ml reaction mixture for nitrate reductase and the amount of NO_2^- produced in 10 min at 30°C relative to the control was determined; 2) the "preincubation assay" in which the inhibitor was preincubated at 25°C with the nitrate reductase preparation in a standard 3 ml reaction mixture minus the substrates (NO_3^- and NADH) for varying time periods before initiating the nitrate reductase reaction by the addition of substrates.

Nitrate Reductase from other Plants. Nitrate reductase was prepared from the leaves of field grown spinach, tomatoes, peppers, radishes, and cucumbers the same as from maize except that the tissue was ground in a chilled mortar with a pestle and sand rather than in a "Virtis 45" homogenizer. Thereafter all manipulations and assays were the same as for the maize enzyme. Protein was determined by the biuret method (8).

RESULTS

A stoichiometric relationship should exist between the enzyme and inhibitor if the latter functioned as a protein which bound directly and irreversibly to the enzyme and thus caused inhibition. Under these conditions it should be possible to titrate the nitrate reductase activity by adding increasing amounts of inhibitor to a fixed amount of enzyme. The results of an experiment designed to test this possibility indicated that such a relationship did not exist (Figure 1). The reductase activity declined curvilinearly with increasing amounts of inhibitor. At low levels of inhibitor, the extent of inhibition appeared directly proportional to the amount of inhibitor added. However, the proportion of nitrate reductase activity which was inhibited decreased as the amount of inhibitor was increased.

As a corollary to the preceding experiment, an experiment was conducted in which a constant amount of inhibitor was added directly to varying amounts of enzyme (Table I). The actual amount of nitrate reductase activity lost due to the action of the inhibitor increased as the amount of enzyme in the reaction mixture was increased. However, the percent loss in activity was essentially the same for all cases. The results of these experiments suggested that the inhibitor probably was not simply a binding protein which, once associated with the enzyme, caused complete and irreversible inhibition. Had such been the case, inhibition should have been total or nearly so under those conditions where the ratio of inhibitor to enzyme was relatively high. Instead, it would appear that some sort of equilibrium was established between the inhibitor and enzyme.

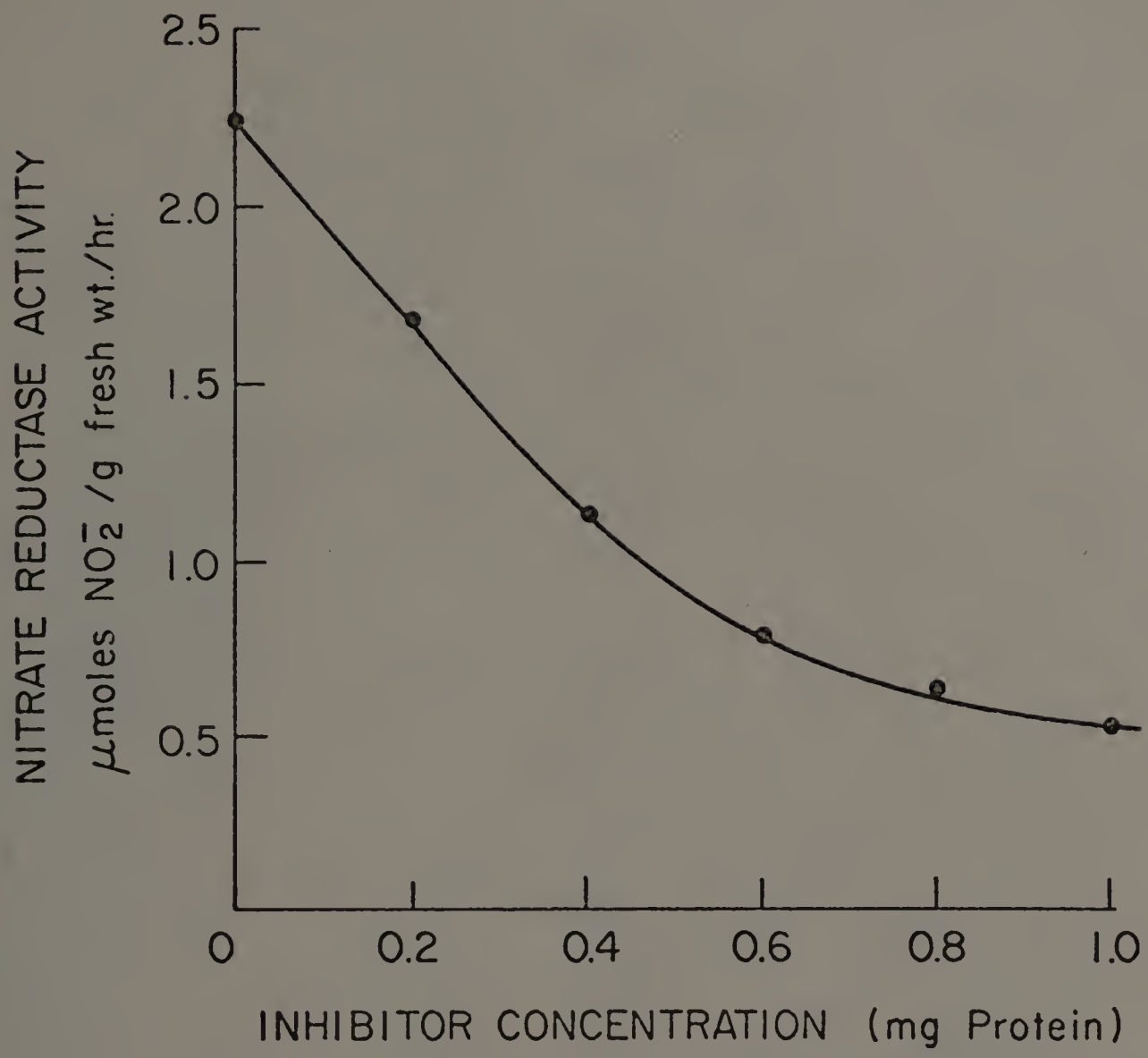

Figure 1. Effect of Increasing Inhibitor Concentration on the Activity of Nitrate Reductase

Inhibitor was directly added to the nitrate reductase reaction mixture as described in Materials and Methods.

Table I. Effect of the Inhibitor on Different Amounts of Nitrate Reductase Preparation

The inhibitor (0.4 mg protein) was directly added to the reaction mixture as described in Materials and Methods. The data are the average of two experiments each with 3 replicate determinations.

Amount of Enzyme	Activity			Inhibition
	Control	+ Inhibitor	Activity Lost	
ml	μmoles NO_2^-/g fresh wt/hr			%
0.2	0.48	0.27	0.21	44
0.4	1.30	0.70	0.60	46
0.6	2.20	1.20	1.00	45
0.8	2.94	1.74	1.20	41
1.0	3.42	2.02	1.40	41

One possibility which might explain the constant percentage of inhibition when the amount of enzyme was varied was that the inhibitor was affecting the availability of substrate or cofactor, which were kept constant in the preceding experiment, rather than the enzyme itself. It was found, however, that doubling the amount of both substrates (NO_3^- and NADH) had no effect on the inhibition.

We found in some instances that the amount of nitrate reductase activity varied from one batch of tissue to another for no obvious reasons (cf also 18). When this variation occurred, the total amount of inhibition accomplished by the inhibitor also varied: when activity was high,the total inhibition was great; when activity was low, there was little net inhibition (Table II). At first we thought that there might be some difference in the sensitivities of the different reductase preparations to the inhibitor. However, when the data were examined in terms of percent of inhibition, it turned out that this parameter was constant.

It was reported previously (Table II) (18) that preincubation of the enzyme with the inhibitor increased the percentage of inhibition, an observation which suggested that the inhibitor catalyzed the inactivation of the reductase such as a proteolytic enzyme could be expected to do. Inhibition of this nature could be predicted to give kinetics in which the nitrate reductase reaction in the presence of the inhibitor initially started at the same rate as the control but then rapidly fell off as the enzyme was inactivated. Such, however, was not entirely the case. An experiment was conducted in which inhibitor sufficient to cause about 45% inhibition in a standard 10 min assay (and 70% inhibition when enzyme and inhibitor were preincubated for 9 min and then assayed for 10 min) was added to

Table II. Effect of the Inhibitor on Two Nitrate Reductase Preparations with Different Activities

The inhibitor (0.6 mg protein) was directly added to the reaction mixture as described in Materials and Methods.

Enzyme Preparation	Activity			Inhibition
	Control	+Inhibition	Activity Lost	
	μmoles NO_2^-/g fresh wt/hr			%
I	6.12	2.94	3.18	52
II	2.16	0.96	1.20	56

a large reaction mixture. The reaction was immediately initiated by the addition of the substrates and the amount of nitrite formed was determined at short time intervals thereafter on small aliquots of the reaction mixture (Figure 2). The reaction rate of the control was linear for at least 25 min. In the presence of the inhibitor the reaction also appeared linear for about the first 9 min, but, in contrast to what would be expected from a proteolytic enzyme, the rate was already about 45% reduced at the initiation of the reaction. Furthermore, after the initial linear period the inhibition became progressively greater with time until after 20 min the reaction had essentially stopped. Thus, it appears that two types of inhibition are involved, one an initial rapid process such as could be attributed to a binding protein and a second which led to a progressive loss during preincubation in reductase activity.

The effect of temperature on the inhibition process was investigated with the rationale that if the inhibition which occurred during the preincubation period was enzyme catalyzed, it should be enhanced by an increased temperature. This indeed was the case (Figure 3). Initially, there was only a negligible difference in the amount of inhibition between the samples held at 4°C and those at 25°C. There was a significant loss of activity during the preincubation at 0°C for 60 min but there was much more loss of activity at 25°C, the rate of inactivation being almost 3-fold greater at this temperature than at 4°C.

The previous experiments indicated that the inhibitor preparation mediated two types of inhibition; one rapid and little influenced by temperature, the other temperature sensitive and relatively slow. This suggested that we were dealing

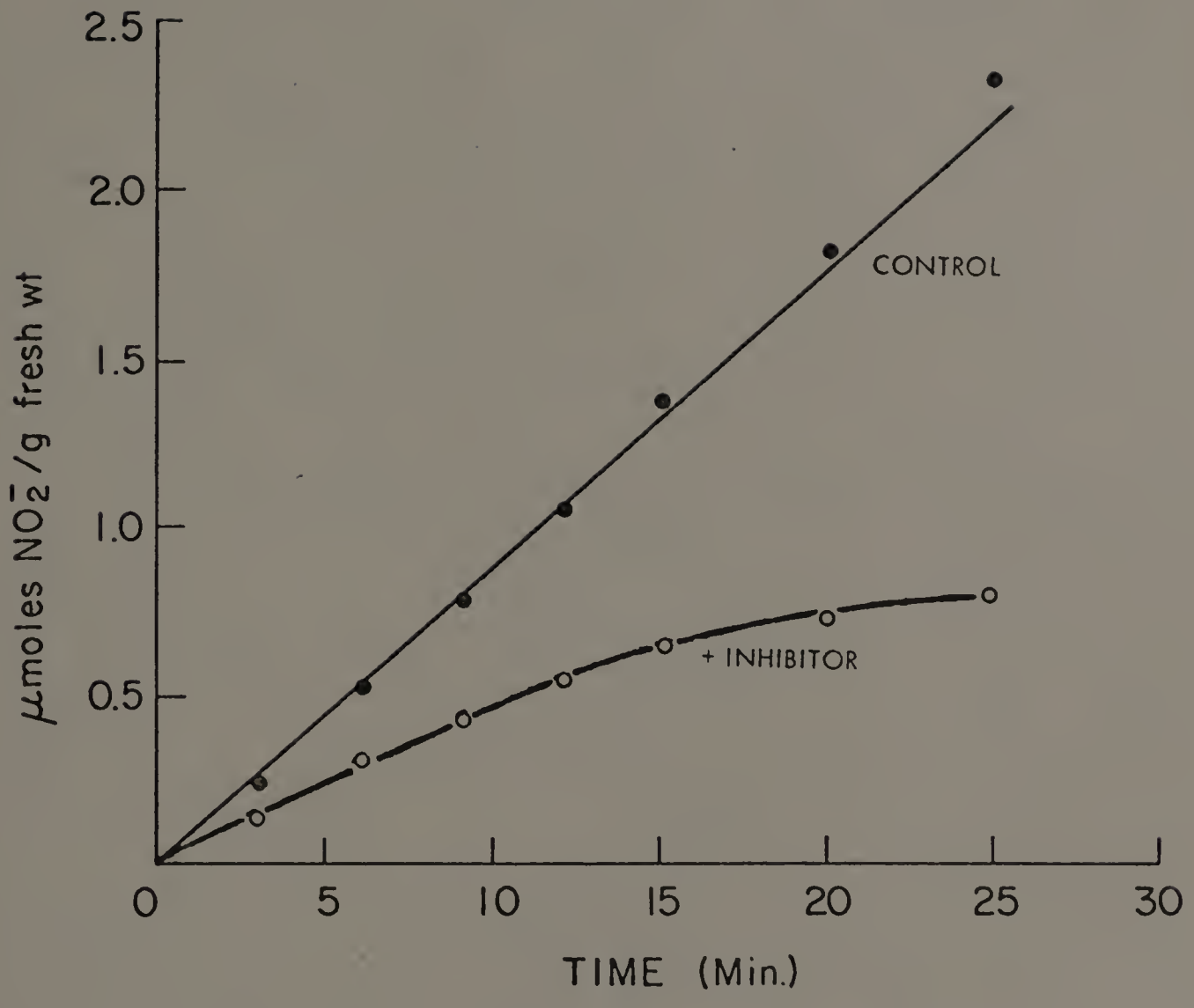

Figure 2. Time Course of Nitrite Production by Nitrate Reductase in the Presence and Absence of the Inhibitor

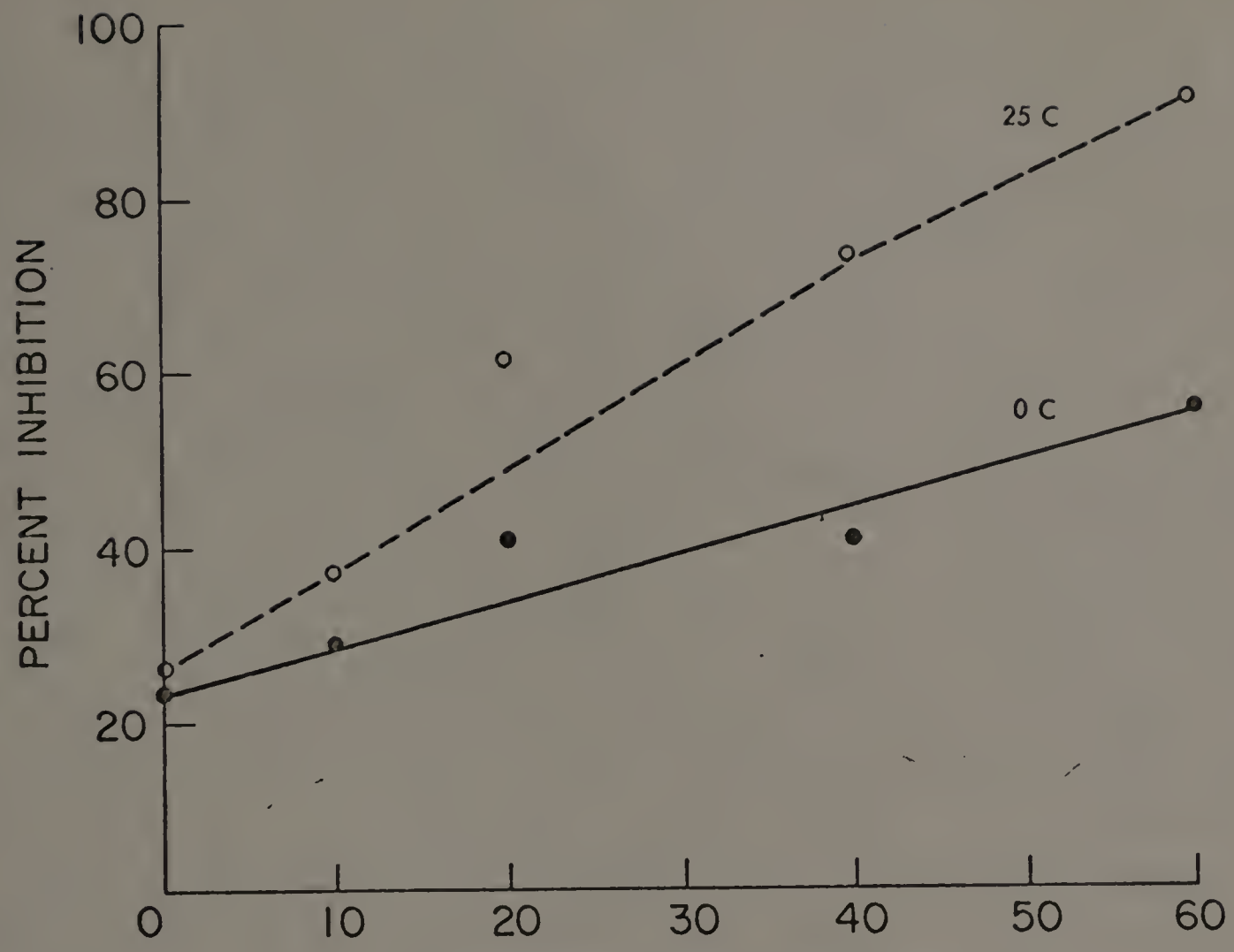

Figure 3. Effect of the Preincubation Temperature on the Degree of Nitrate Reductase Inhibition

A nitrate reductase preparation was preincubated with inhibitor (0.6 mg protein) or water in 0.1 M phosphate (K^+), pH 7.6 at 4°C and 25°C for different periods of time. The results are expressed as percent of the appropriate water controls. The activity of the control (without inhibitor at zero time) was 1.6 μmoles NO_2^-/g fresh wt/hr.

with two separate inhibitors. The alternative was that there was but one inhibitor which functioned initially as a binding protein causing a rapid partial loss of activity followed by a gradual loss of the remaining activity. Our previous observations (18) had not provided any positive evidence for more than one inhibitor. However, as the acetone-precipitated root preparation was relatively crude, it was entirely possible that it contained more than one inhibitor of nitrate reductase. The following experiment, discussed more fully later, provides some insight to this conundrum. A preincubation experiment was conducted as described in Materials and Methods. The initial inhibition was about 50% (Figure 4). As the preincubation proceeded there was a linear decline in activity until after 35 min no detectable nitrate reductase activity remained. At this point fresh enzyme equivalent to that initially used was added to the reaction mixtures which contained the inhibitor. (An entirely fresh control was set up at this time also.) Again, there was an immediate reduction (ca 50%) in enzyme activity followed by a linear loss of activity during the preincubation period. It is emphasized here that this loss of activity which was linear within the limits of experimental error has been observed in a number of similar experiments.

It was previously found (18) that the inhibitor had no detectable effect on a number of other enzymes which suggested a certain degree of specificity. It was of interest to determine the effect of the corn root inhibitor on the activities of nitrate reductase derived from several different species. We reasoned that if binding of the inhibitor to the reductase involved a site essential for activity, then nitrate reductases from diverse species should be similarly affected. On the other hand, if the interaction between enzyme and inhibitor involved some site

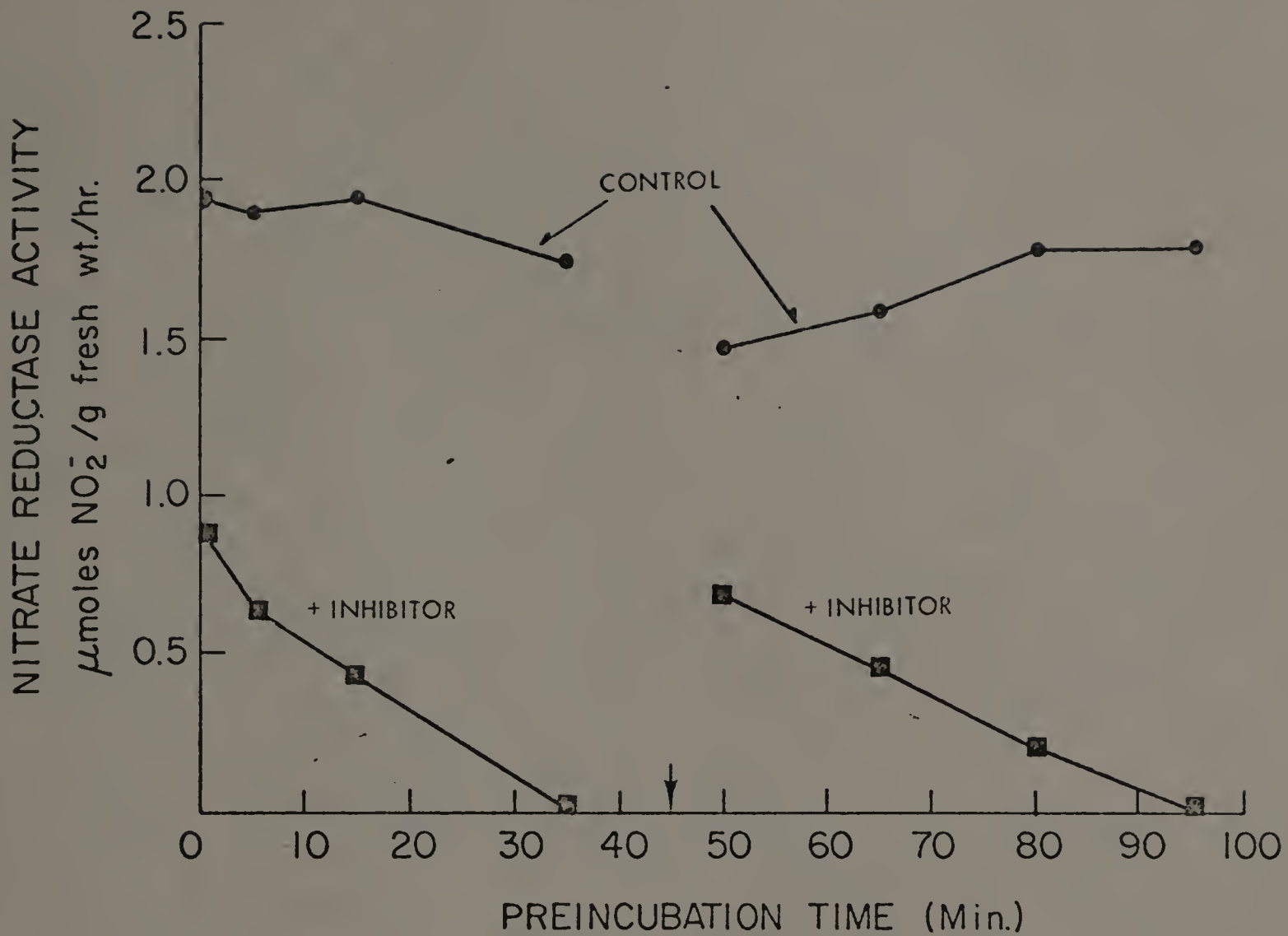

Figure 4. Effect of the Length of Preincubation on the Inhibition of Nitrate Reductase by its Inhibitor

The inhibitor (0.5 mg protein) was preincubated with nitrate reductase (1.3 mg protein) in 1 ml 0.1 M phosphate (K^+), pH 7.6 at 25°C. At selected time intervals the standard nitrate reductase assay was initiated by the addition of NO_3^- and NADH. At the time indicated by the arrow, fresh nitrate reductase (1.3 mg protein) was added to the samples preincubated with inhibitor since zero time.

Table III. Effect of the Inhibitor from Corn Roots on Nitrate Reductase Extracted from Various Plant Tissues

The various nitrate reductases were either assayed directly or preincubated with the inhibitor for 15 minutes at 25°C.

Plant Tissues	Treatments	Activity		
		Control	+Inhibitor	Inhibition
		μmoles NO_2^-/g fresh wt/hr		%
Experiment I				
Corn	None	2.7	1.56	42
	Preincubation	2.4	0.33	86
Spinach	None	5.0	2.3	54
	Preincubation	4.9	1.08	78
Experiment II				
Corn	None	6.3	3.5	45
	Preincubation	3.8	0.45	88
Radish	None	0.77	0.48	39
	Preincubation	0.48	0.12	75
Tomato	None	1.9	1.0	47
	Preincubation	1.0	0	100
Pepper	None	1.12	0.6	47
	Preincubation	0.42	0	100
Cucumber	None	7.13	4.0	56
	Preincubation	5.8	1.57	74

other than a site required for maintaining active enzyme, then nitrate reductase from species other than corn might not be affected by the inhibitor. The data (Table III) indicated that the inhibitor was approximately equally effective, at least for the initial inhibition, for the enzyme from six different species. It appears that there may be some differences between the different enzymes in the amount of activity lost during the preincubation but this does not necessarily indicate a difference in the initial interaction between enzyme and inhibitor but rather may be due to differences in the stabilities of the postulated enzyme-inhibitor complexes.

DISCUSSION

A protein inhibitor of a specific enzyme could be expected to be either a protease (9) which degrades the enzyme or a binding protein which forms an inactive complex with the enzyme (1, 3, 4, 5, 10, 25). Proteolytic enzymes have been found in plants but the physiological function of this class of enzymes, beyond the hydrolysis of reserve proteins in germinating seeds and the general degradation of proteins in dying cells, is uncertain (9). The relatively random nature of the cleavage of peptide bonds by proteolytic enzymes would have to be accounted for in order to make this type of potential inhibitor specific for an unique enzyme species. Theoretically, protein molecules can exist in several different thermodynamic states (21). It is possible that a protein becomes subject to degradation only when it is in one of a number of possible configurations. Such configurations could be altered by interactions with small molecules, similar or dissimilar peptides

and subunits, or by association with various intracellular organelles (21). Presumably, the configuration of the enzyme-substrate complex is sufficiently different from that of the free enzyme that the enzyme is resistant to proteolysis (13, 14, 15, 27). The stabilizing effect of the substrate thus could provide the basis for the selective degradation of specific enzymes, that is, below some critical level of substrate an enzyme might be especially prone to proteolysis. Several examples of this type of specific inactivation have been demonstrated in animals and microorganisms (2, 15, 23), but, to date, none in plants, just as in the present case the substrates conferred no protection against inactivation (Figure 2).

Many studies have been made on the inhibition of enzymes, especially proteases, from animals and microorganisms by specific binding proteins (1, 3, 4, 5, 7, 12). In contrast, there are few detailed reports of binding proteins which inhibit or inactivate specific enzymes in plants. Two apparent exceptions, however, both involve invertase. A low molecular weight protein which non-competitively inhibited invertase from many plants was found by Pressey in potato tubers (19, 20). Reduction of invertase activity by increasing amounts of inhibitor occurred linearly which led Pressey to suggest that the effect of the inhibitor was on the free enzyme. Jaynes and Nelson (10) reported that invertase I from maize endosperm was noncompetitively inhibited by an inactivator protein from the same tissue. In contrast to the potato tuber inhibitor, the inhibitor from maize endosperm caused a curvilinear decrease in activity with an increase in inhibitor concentration (10). It was not established in either case, however, how the invertases were inhibited.

The experiments reported in this paper present evidence that the maize root inhibitor of nitrate reductase caused two types of inhibition. One was rapid and quickly inhibited the reductase activity when added directly to the reaction mixture. The other was temperature dependent, slow acting and became apparent only after preincubation with the enzyme. Previous observations (18) suggested that only one inhibitor occurred in the root extract and that it was a protein. Since the inhibitor has not been purified, obviously the possibility cannot be excluded that the inhibition is due to two factors. Also, in view of the fact that the nitrate reductase preparations were not purified it is possible that the seemingly different forms of inhibition were rather a reflection of one inhibitor affecting two forms of nitrate reductase, as Pressey (20) suggested for the potato tuber inhibitor of diverse invertases. There is no evidence for two forms of nitrate reductase from any species, and, therefore, our assumption, for lack of convincing evidence otherwise, was that there was but one inhibitor affecting but one nitrate reductase.

The mechanism by which the inhibitor caused a loss of nitrate reductase activity is not clear. However, certain deductions can be made. The action of a binding protein on an enzyme is supposedly rapid, analogous to a heavy metal or other small inhibitors. The apparently instantaneous inhibition observed here (Figure 2) indicates that the inhibitor is first of all a protein which forms a complex with the reductase (binding protein) and thus causes inhibition. The reduction of nitrate reductase activity by increasing amounts of inhibitor approached linearity in the low concentration range of the inhibitor, a characteristic to be expected of a binding protein also. A seemingly puzzling aspect of the problem, however, was

the failure to obtain complete inhibition when the inhibitor theoretically should have been present in considerable excess of the reductase. One might presuppose that this failure to obtain complete inhibition represented the few remaining uncombined nitrate reductase molecules with which the inhibitor had not come in contact. On the other hand, the relatively constant degree of inhibition which resulted from a constant amount of inhibitor and varying amounts of reductase suggested alternative explanations. The reversible formation of an enzyme-inhibitor complex can probably be eliminated because it would not lead to a constant percentage of inhibition. But the assumption of the formation of an irreversible complex which retained partial nitrate reductase activity would explain the constant percentage inhibition and would also provide a basis for the explanation of the subsequent loss of reductase activity with time.

Studies of allosteric enzymes (16) indicate that a proper configuration is required for enzyme activity and that any change in configuration could influence activity. We postulate that the mechanism of inhibition in the present case involves the formation of a complex between the inhibitor and nitrate reductase with a consequent alteration of the reductase configuration which causes an immediate partial loss of activity. Attendant upon this change in configuration is a loss of stability and a gradual loss of activity.

In the experiment described in Figure 4 the inhibitor caused a 50% reduction in activity without any preincubation. This was so even though sufficient inhibitor was used to have caused a 50% inhibition of at least three times as much nitrate reductase as was used in this experiment (cf Table II). The relatively constant percentage of inhibition (40 to 50%) observed with one level of inhibitor independent

of the amount of enzyme is consistent with the concept that the inhibitor-enzyme complex is partially active. (This assumes, of course, still an excess of inhibitor at the highest enzyme level.) The critical point to this argument, however, is the linear loss of the remaining reductase activity upon preincubation.

If free, fully active nitrate reductase were being inactivated by a proteolytic enzyme, the kinetics of the inactivation should be first order, that is, the rate of inactivation should have decreased as the amount of enzyme decreased. However, the observed linear decrease in activity means that the inactivation process followed zero order kinetics, that is, the loss of activity was independent of the amount of enzyme remaining in the solution. The conclusion, then, is that the observed loss of activity during the preincubation period is due to the further and complete loss of activity from the partially active enzyme-inhibitor complex. This further loss of activity could be accelerated by elevated temperatures as observed (Figure 3) and still not be an enzyme-catalyzed reaction.

One observation apparently in conflict with the above argument is the greater degree of inhibition when the level of inhibitor was significantly increased. A possible explanation consistent with our model is that at the higher inhibitor concentrations secondary binding of the inhibitor to the enzyme occurs which leads to further, but still not complete, inhibition.

LITERATURE CITED

1. Bechet, J. and J. M. Wiame. 1965. Indication of a specific regulatory binding protein for ornithine transcarbamylase in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 21: 226-34.

2. Berger, L., M. W. Slein, S. P. Colowick, and C. F. Cori. 1946. Isolation of hexokinase from baker's yeats. J. Gen. Physiol. 29: 379-91.

3. Blobel, G. and V. R. Potter. 1966. Relation of ribonuclease and ribonuclease inhibitor to the isolation of polysomes from rat liver. Proc. Natl. Acad. Sci. 55: 1283-88.

4. Bowman, D. E. 1945. Amylase inhibitor of navy beans. Science 102: 358-359.

5. Dvorak, H. F., Y. Anrabu, and L. A. Heppel. 1966. The occurrence of a protein inhibitor for 5-nucleotidase in extracts of E. coli. Biochem. Biophys. Res. Commun. 24: 628-32.

6. Engelsma, G. 1967. Effect of cycloheximide on the inactivation of phenylalanine deaminase in gherkin seedlings. Naturwissenschaften 54: 319-20.

7. Ferguson, J. J.,Jr., M. Ball, and H. Holzer. 1967. Yeast malate dehydrogenase: Enzyme inactivation in catabolite repression. European J. Biochem. 1: 21-5.

8. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-62.

9. Greenberg, D. M. 1955. Plant proteolytic enzymes. In: S. P. Colowick and N. O. Kaplan (Eds.), Methods in Enzymology. Vol. II, Academic Press, New York, pp 54-64.

10. Jaynes, T. A. and O. E. Nelson. 1971. An invertase inactivator in maize endosperm and factors affecting inactivation. Plant Physiol. 47: 629-34.

11. Kenney, F. T. 1967. Turnover of rat liver tyrosine transaminase: stabilization after inhibition of protein synthesis. Science 156: 525-28.

12. Kern, M. and R. Natale. 1958. A diphosphopyridine nucleotidase and its protein inhibitor from Mycobacterium butyricum. J. Biol. Chem. 231: 41-51

13. Markus, G. 1965. Protein substrate conformation and proteolysis. Proc. Natl. Acad. Sci. 54: 253-58.

14. Markus, G., D. K. McClintock, and B. A. Castellani. 1967. Ligand-stabilized conformations in serum albumin. J. Biol. Chem. 242: 4402-08.

15. McClintock, D. and G. Markus. 1968. Conformational changes in aspartate transcarbamylase. I. Proteolysis of the intact enzyme. J. Biol. Chem. 243: 2855-62.

16. Monod, J., J. P. Changuex, and F. Jacob. 1963. Allosteric proteins and cellular control system. J. Mol. Biol. 6: 306-29.

17. Onwuene, I. O., H. M. Laude, and R. C. Huffaker. 1970. Nitrate reductase activity in relation to heat stress in barley seedlings. Crop Sci. 11: 195-200.

18. Pan, Y. T. 1972. An investigation of a nitrate reductase inhibitor in the roots of Zea mays. Part I, Ph.D. Thesis, University of Massachusetts, Amherst.

19. Pressey, R. 1966. Separation and properties of potato invertase and invertase inhibitor. Arch. Biochem. Biophys. 113: 667-74.

20. Pressey, R. 1967. Invertase inhibitor from potato: purification, characterization and reactivity with plant invertase. Plant Physiol. 42: 1780-86.

21. Schimke, R. T. 1970. Regulation of protein degradation in mammalian tissues. In: H. N. Munro (Ed.), Mammalian Protein Metabolism. Vol. IV, Academic Press, New York and London, pp 177-228.

22. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1965. The role of synthesis and degradation in the control of rat liver trypotphan pyrrolase. J. Biol. Chem. 240: 322-31.

23. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1965. Studies of the stability in vivo and in vitro of rat liver tryptophan pyrrolase.

24. Schrader, L. E., G. L. Ritenour, G. L. Eilrich, and R. H. Hageman. 1968. Some characteristics of nitrate reductase from higher plants. Plant Physiol. 43: 930-40.

25. Schwimmer, S., R. U. Makower, and E. S. Rorem. 1961. Invertase and invertase inhibitor in potato. Plant Physiol. 36: 313-16.

26. Travis, R. L., W. R. Jordan, and R. C. Huffaker. 1969. Evidence for an inactivating system of nitrate reductase in Hordeum vulgare L. during darkness that requires protein synthesis. Plant Physiol. 44:1150-56.

27. Zito, R., E. Antonini, and J. Wyman. 1964. The effect of oxygenation on the rate of digestion of human hemoglobins by carboxypeptidases. J. Biol. Chem. 239: 1804-08.

SUMMARY

The activity of nitrate reductase in root extracts prepared from young corn (Zea mays) seedlings decreased with age until 10 days after germination when it was no longer detectable. A factor which inhibited nitrate reductase developed in the extracts coincident with the disappearance of the reductase activity. The inhibitor, which could be precipitated by acetone or ammonium sulfate and redissolved in buffer, was nondialyzable and was not affected by Polyclar-AT, indicating that it was not a phenolic compound or other small molecule. Heating the inhibitor at 60°C for 10 minutes led to a partial loss of its action and complete loss of activity at 80°C. The inhibitor was also completely inactivated when held at pH 2 at 0°C for 30 minutes but was relatively stable at higher pH's. Passage of the inhibitor preparation through a Bio-Gel P-100 column indicated that all of the inhibitor was associated with one large molecular weight component. All of these observations considered together indicated that the corn root inhibitor of nitrate reductase is probably a protein.

The inhibitor exhibited two types of inhibition towards nitrate reductase: one was little affected by temperature and was apparently instantaneous; the other was temperature sensitive, slow acting and caused a linear decrease in nitrate reductase activity when enzyme and inhibitor were incubated together in the absence of NO_3^- and NADH. The substrates did not afford any protection against inactivation. A simple stoichiometric relationship did not exist between the inhibitor and enzyme. Instead, it appeared that some sort of equilibrium was established between the two. The possibility was discussed that the inhibitor was a binding protein which caused an immediate partial loss of nitrate reductase activity and at the same time makes the enzyme unstable. The latter would account for the linear loss of the balance of activity observed in preincubation experiments.

The inhibitor appears to be relatively specific for nitrate reductase. It had no effect on phenylalanine ammonia-lyase, invertase, glutamate dehydrogenase, malate dehydrogenase or glucose oxidase. The action of the inhibitor did not appear to be through an effect on the flavin nor molybdenum components of the enzyme nor on the NADH electron donor.

Nitrate reductase activity could be detected in the roots by an in vivo assay but the level was less than 15% of that found in the leaves by the same method. It was concluded that the inhibitor may play a physiological role in corn by causing a shift in the site of nitrate reduction from the roots to the leaf.

APPENDIX

Table 1. Changes with Age in Nitrate Reductase Activity in Corn Seedlings. Experiment 1.

Seedlings were grown in a growth chamber (14 hr white light at 30°C and 10 hr dark at 24°C).

	Activity	
Age	Root	Leaf
days	μmoles NO_2^-/g fresh wt/hr	
5	0.12	4.98
6	0.08	6.9
7	0.00	6.12
8	0.00	6.26

Table II. Effect of Temperature on the Distribution of Nitrate Reductase Activity in Corn Leaves

	Activity	
Leaf Parts	Low Temperature (°F) 62°(night) - 68°(day)	High Temperature (°F) 75°(night) - 85°(day)
	μmoles NO_2^-/g fresh wt/hr	
Tip	0	1.06
Part I*	0.33	1.72
Part II	0.88	1.74
Part III	0.83	0.86
Part IV*	0.53	0.24

Table III. Nitrate Reductase Activity in Corn Leaves Grown in Different Temperatures

	Activity			
	Temperature °F			
Leaf	40°(N) - 50°(D)	50°(N) - 60°(D)	65°(N) - 75°(D)	75°(N) - 85°(D)
	μmoles NO_2^-/g fresh wt/hr			
Part II	3.64	4.24	2.16	
			1.72	2.72

Table IV. Effect of Temperature on Nitrate Reductase Activity in Corn Leaves

Corn seedlings were grown in flats at the indicated temperature regimes. At zero time, after sampling, flat A_1 was transferred from the "low" to the "high" temperature and flat B_2 was transferred from the "high" to the "low" temperature. Each number is the means of three replicate determinations.

	Temperature (Low)		Temperature (High)	
Tissue	62°(N) - 68°(D)		75°(N) - 85°(D)	
	μmoles NO_2^-/g fresh wt/hr			
	Zero Time			
	A_1	A_2	B_1	B_2
Tip	0.0	0.0	0.1	0.5
Leaf	0.9	1.4	2.5	1.5
Sheath	0.8	0.7	0.1	0.2
	Day 2			
	B_2	A_2	B_1	A_1
Tip	0.3	0.0	0.9	0.0
Leaf	4.8	2.5	6.5	0.7
Sheath	0.3	0.5	0.4	0.7
	Day 4			
	B_2	A_2	B_1	A_1
Tip	0.0	0.0	0.5	0.6
Leaf	2.2	3.2	3.2	2.2
Sheath	0.5	0.35	0.7	0.1

Table V. Effect of Temperature on the Appearance of Nitrate Reductase Inhibitors in the Tip Portion of Corn Leaf

Extracts prepared from the tips of Day-4 seedlings (Appendix, Table IV) were added to a leaf extract.

Enzyme Source	Activity
	μmoles/g hr
Leaf extract alone	2.9
Leaf extract + Tip (A_1)	3.1
Leaf extract + Tip (A_2)	0.8
Leaf extract + Tip (B_1)	2.8
Leaf extract + Tip (B_2)	1.6

Table VI. The Effect of Different Concentrations of Tip Extract on Nitrate Reductase Activity from Corn Leaves

Enzyme Source	Activity
	μmoles/g fresh wt/hr
Leaf (0.5 ml)	0.98
Leaf + Tip (0.1 ml)	0.58
Leaf + Tip (0.2 ml)	0.46
Leaf + Tip (0.5 ml)	0.16
Leaf + Tip (1.0 ml)	0.08

Table VII. Effect of Polyclar-AT on Nitrate Reductase in Corn Leaves

Enzyme Source	Activity
	μmoles NO_2^-/g fresh wt/hr
Leaf	4.86
Leaf (Polyclar-AT)	6.05
Tip	0
Tip (Polyclar-AT)	0
Leaf + Tip	1.92
Leaf + Tip (Polyclar-AT)	1.86
Leaf (Polyclar-AT) + Tip	2.58

Table VIII. The Effect of Two Fractions of the Tip Extract on Nitrate Reductase Activity in Corn Leaves

The tip extract was treated with 1.5 volumes of cold (-10°C) acetone and stored at -10°C for 20 min, after which the insoluble material was collected by centrifugation at 10,000 g for 10 min. The precipitated material was resuspended in 0.1 M phosphate (K^+) buffer, pH 7.5 equivalent to the initial volume of the tip extract. This fraction is referred to as the "acetone-precipitated fraction". The supernatant fluid obtained from the above centrifugation was taken to dryness in vacuo and the dried residue dissolved in 0.1 M phosphate (K^+) buffer equivalent to the initial volume of the tip extract. This fraction is referred to as the "supernatant fraction".

Treatment	Activity	% of Control
	μmoles NO_2^-/g fresh wt/hr	
Leaf	6.66	100
Leaf + Tip	0	0
Leaf + Tip (supernatant fraction)	3.42	51
Leaf + Tip (acetone ppt fraction)	0.31	5
Leaf + Tip (boiled supernatant fraction)	3.7	55
Leaf + Tip (boiled acetone fraction)	9.3	140
Leaf + Tip (dialyzed supernatant fraction)	6.12	92
Leaf + Tip (dialyzed acetone ppt fraction)	0.70	10

Table IX. The Effect of the Acetone-Precipitated Tip Extract on Various Enzyme Activities

Enzyme	Nitrate Reductase	Invertase	β -Amylase
		U	μU
Control	3.8	1.1	1.37
Control + Tip extract	2.54	1.3	1.5

Table X. The Effect of NO_3^- on Nitrate Reductase Activity Measured by Intact Tissue Assay

	Activity			
	Corn Grown under Low		Corn Grown under High	
Additives	Temperature 62°(N) - 68°(D)		Temperature 75°(N) - 85°(D)	
	μmoles NO_2^-/g fresh wt/hr			
	Tip	Leaf	Tip	Leaf
--	1.10	0.94	0	0
NO_3^-	1.14	...	1.10	0.52
NADH	1.40	...	0	0
NO_3^-, NADH	1.34	1.14	0.90	0.50