ABSTRACT

CONTROL OF NITRATE AND NITRITE REDUCTION
IN BARLEY ALEURONE LAYERS

By

Thomas Enrico Ferrari

Nitrate induces the formation of nitrate reductase activity in barley (Hordeum vulgare L. cv. Himalaya) aleurone layers. Previous work has demonstrated de novo synthesis of alpha-amylase by gibberellic acid in the same tissue. Aleurone layers therefore provide a convenient tissue for the study of both substrate- and hormone-induced enzyme formation. As measured in cell-free extracts, the nitrate-induced increase in nitrate reductase activity is inhibited by cycloheximide and 6-methylpurine, but not by actinomycin D. Nitrate does not induce alpha-amylase synthesis and it has no effect on the gibberellic acid-induced synthesis of alpha-amylase. Also, there is little or no direct effect of gibberellic acid (during the first 6 hours of induction) or of abscisic acid on the nitrate-induced formation of nitrate reductase. Gibberellic acid does interfere with nitrate reductase activity during long-term experiments (greater than 6 hours). However, the time

course of this inhibition suggests that the inhibition may be a secondary one.

Nitrate reductase activity in aleurone layers has also been determined in intact tissue, using two different methods. The first method measures the rate of appearance of $H_2{}^{18}O$ produced during the reduction of $KN^{18}O_3$. The second assay measures released nitrite resulting from nitrate reduction under anaerobic conditions. Nitrite production in this anaerobic, intact-tissue assay was dependent upon the presence of phosphate (pH 7.5) and was increased by ethanol and bisulfite.

After ten hours of nitrate induction, nitrate reductase activities measured by the $KN^{18}O_3$ assay are one-sixth, and those measured by the anaerobic intact-tissue assay are one-third, of those observed in cell-free extracts of aleurone layers. Addition of ethanol to the anaerobic intact-tissue medium increased the rate of nitrate reduction to a level greater than that found in the cell-free assay.

Oxygen inhibited nitrite release in the anaerobic intact-tissue assay. However, under aerobic conditions and in the presence of 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO) or antimycin A, nitrate reduction increased to rates comparable to those observed under anaerobiosis. Neither of these electron transport inhibitors affected anaerobic nitrate reduction.

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A method was devised for the detection and measurement of nitrite reductase in aleurone layers. Nitrite reductase activity of aleurone layers was determined by measuring nitrite disappearance with time. The method also allowed simultaneous determination of nitrite uptake by the tissue.

Enzyme activity was induced by nitrate, but measurable activity was present in noninduced tissue. Induced activity was inhibited by cycloheximide but not by actinomycin D. Activity in induced layers was inhibited by 2,4-dinitrophenol, antimycin A, 2-n-heptyl-4-hydroxyquinoline N-oxide, and anaerobiosis. Nitrite uptake was relatively insensitive to all inhibitors tested.

Nitrite uptake was rapid at pH 4.5 and negligible at pH 7.5. Nitrite accumulated anaerobically at pH 4.5, was rapidly released when transferred to medium at pH 7.5 --the pH of the anaerobic intact-tissue assay for nitrate reductase. Accumulated nitrite was released by the tissue whether held under anaerobic or aerobic conditions.

Nitrate-induced and noninduced aleurone layers were able to reduce low levels of nitrite anaerobically. But these rates (5 to 10 nmoles/layer/hour) were considerably lower than rates (25 nmoles/layer/hour) of nitrite production observed during the nitrate reductase anaerobic intact-tissue assay (with ethanol present).

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Thank you Sheila for maintaining the garden while I was away.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Guidance Committee:

This thesis is divided into sections I, II and III. Each was intended for publication, and was written in formats suited for publication in the Journal of Plant Physiology or the Proceedings of the National Academy of Science. The first two sections have already appeared in print.

INTRODUCTION

"'Nitrate assimilation' represents the biological conversion of nitrate to ammonia or to the amino acid or amide level for the ultimate synthesis of nitrogen containing cell constituents (48)." Approximately 10 billion tons of nitrogen are incorporated into plant life on a world wide basis per year (7). Although some of this fixed nitrogen comes from nitrogen fixing bacteria living symbiotically with plants, the bulk of fixed nitrogen arises from the reduction of nitrates by plants after being taken up from the soil.

The importance of nitrogen as a plant constituent is reflected by its occurrence in a wide variety of biologically prominent molecules e.g., proteins, nucleic acids, vitamins. Yet, unlike most other elements, nitrate nitrogen cannot be utilized directly by plant or animal cells; it must first undergo an eight electron reduction prior to its functioning in the cell's metabolism. Thus, considerable research has been devoted by investigators in an attempt to understand the reduction process and its regulation by environmental and cellular factors.

Detailed information on nitrate reductase--the first enzyme of the nitrate assimilating pathway--can be

found in several excellent reviews (7, 37, 46, 47, 48, 55). The following review will concentrate on how environmental and cellular factors control the activity of the nitrate reducing pathway.

LITERATURE REVIEW

Properties of Nitrate Reductase

In 1896, a nitrate reducing factor was detected in potato extracts by Bach (6). Eight years later, the enzymatic nature of the reduction of nitrate by crude extracts of plants was provided by Kastle and Elvove (33). These workers showed that potato and certain other plants contain a reducing substance or substances capable of effecting the reduction of nitrate to nitrite. Furthermore, they showed that reduction occurred most rapidly at 40 to 45°C, was augmented by an increase in the concentration of nitrate and reductant, was inhibited by certain metabolic poisons, was accelerated by specific substances, and that the reducing property of the extract was completely lost by boiling.

Except for an occasional observation (63), nitrate reductase has been found in all green and non-green plant tissues in which it has been sought. Though its presence in crude cell-free extracts was demonstrated as early as 1904 (33), Kessler (37) has credited Evans, Nason and Nicholas with identifying and characterizing nitrate reductase as the enzyme catalyzing the nitrate to nitrite reduction.

Nitrate reductase in both plants and microorganisms appears to be an adaptive enzyme. Its formation can be induced by nitrate, and molybdenum appears to be necessary for activity (7). Induction is preceded by a lag period as short as 20 minutes or as long as 25 hours, depending on plant material (11, 30). The enzyme appears to have a turnover rate (half-time) of 3 to 4 hours (2, 57). Evidence suggests that light either increases the rate of synthesis or prevents its breakdown (13, 25, 64).

Based on studies from a wide variety of tissues (7, 37), nitrate reductase has been found to be a metalloflavoprotein of molecular weight 500,000 to 600,000. The enzyme contains flavin adenine dinucleotide, molybdenum, iron and active sulfhydryl groups. Reduced nicotinamide adenine dinucleotide serves as hydrogen donor. The lability of the enzyme during extraction has hampered detailed characterization of the protein.

Effects of Metabolites on Nitrate Reduction

Feedback control at the molecular level is an important regulatory mechanism of enzyme activity in many organisms (5), and there is increasing evidence that nitrate reductase in plants is under such control. For example, the induction of nitrate reductase in tobacco cells grown in suspension culture may be repressed or derepressed by a variety of amino acids (20). Amino acids

with repressor-like activity included alanine, asparagine, glycine, methionine, proline, threonine, valine, aspartate, glutamate, histidine and leucine. In the presence of any of these repressors, arginine and lysine were found to act as derepressors. Except when in the presence of methionine or alanine, cysteine and isoleucine also derepressed. In addition, casein hydrolysate inhibited enzyme formation.

In corn seedlings, several secondary metabolites of inorganic nitrogen metabolism were evaluated as inhibitors of nitrate reductase induction (56). Of nine different phenylpropanoid compounds tested, only coumarin, trans-cinnamic acid and trans-o-hydroxycinnamic acid inhibited nitrate induction. In this same study, carbamyl phosphate and cyanate, were found to be competitive inhibitors (with nitrate) of nitrate reductase. The effects of carbamyl phosphate and cyanate are interesting in that they indicate regulation by directly interacting with the enzyme. Cell-free preparations of apple roots contain a potent inhibitor of nitrate reduction (24). The compound(s) was removed by dialysis against phosphate buffer or passage through Sephadex. The significance of this finding to the in vivo activity of nitrate reduction is uncertain.

Nitrate reductase activity extracted from sunflower leaves is increased two and three times by treatment of the seedlings with adenine and uracil, respectively (53). In cauliflower, serine is capable of stimulating production of nitrate reductase activity (1).

Regulation of Nitrate Reductase by Nitrate Uptake

Nitrate is necessary for the induction of nitrate reductase. The idea that nitrate has to be present in the cell to "turn on" the machinery necessary for enzyme synthesis, subjects this process to an important and often overlooked cellular control mechanism--regulation of nitrate uptake by the cell. There is considerable direct and indirect evidence in support of such an hypothesis, and this regulatory aspect may involve an active control mechanism.

Evidence for such a control mechanism is not uncommon in nature. For example, nitrate reductase in Escherichia coli is constitutive, but there exists an adaptive system for nitrate uptake (19). In the algae Ankistrodesmus braunii there appears to be at low nitrate concentrations a 2,4-dinitrophenol (DNP)-sensitive nitrate uptake mechanism (3). The authors propose that at low concentrations of nitrate, uptake is active. But, as concentrations are increased, passive uptake (DNP insensitive) accounts for a greater percentage of nitrate uptake.

There is evidence that nitrate uptake in higher plants also involves an active uptake mechanism. In excised wheat roots, nitrate uptake is increased by glucose and inhibited by galactose and salicylic acid (58). In rye seedlings, light was shown to promote nitrate uptake

and this was followed by increased enzyme activity (13). Whereas ammonium inhibited nitrate uptake by nitrogen starved wheat seedlings, it had no significant effect on the enzymatic reduction of accumulated nitrate (43). Experiments with tobacco cell suspensions indicate that nitrate was accumulated against a concentration gradient, and that this uptake was inhibited by cyanide, DNP and threonine (30). In addition, there appeared to be little or no passive uptake of nitrate into tobacco cells. Of particular interest was the finding that all nitrate in the cell is not available for enzyme induction. When fully induced tobacco cells were transferred to nitrateless media, nitrate reductase activity declined despite the presence of high nitrate concentrations within the cells.

Nitrate Reduction and Source of Reducing Equivalents

Several *in vitro* systems, often employing elaborate experimental conditions, have been devised which attempt to correlate an enzymatic reaction with the reduction of nitrate to nitrite (17, 18, 27, 39, 41). Without exception, each system studied utilizes two common components: nitrate reductase and an NADH-generating reaction(s). Nitrate reductase is an obligate component in all nitrate-reducing systems described. It appears that nitrate reduction *in vitro* may be coupled to any of a variety of light- or dark-mediated NADH-generating reactions *e.g.*, ethanol +

alcohol dehydrogenase (17, 27), 3-phosphoglyceraldehyde + glyceraldehyde-3-phosphate dehydrogenase (39), aldehyde + aldehyde oxidase (9), hydrogen + a bacterial hydrogenase (15), chloroplasts or grana + light (18, 41).

Thus, two nitrate reducing systems might operate in intact cells: one dependent on light-mediated generation of reducing equivalents and the other dependent on dark reactions.

Nitrate Reduction and the Generation of Reductant via Photosynthesis

Light has long been known to stimulate nitrate assimilation in green leaves and algae (8, 12, 35, 36, 42, 59). Since this discovery by Warburg and Neglein in 1920 (64), the explanation of this effect is still not yet agreed upon. Originally, Warburg and Neglein attributed the increase of nitrate assimilation by light to a permeability effect; that is, substrate becomes more readily available. They believed that in both the light and dark the reduction of nitrate to ammonia was coupled to respiration, and that extra carbon dioxide production observed during nitrate assimilation in the dark came from increased carbohydrate breakdown. Also, an indirect effect of light, due simply to the ample production of carbon compounds by photosynthesis was considered. The carbohydrate thus produced would--via respiration--serve as the electron donors required for the reduction of nitrate, just as they do in the dark.

A more direct consequence of light is possible. van Niel, Allen and Wright (60) proposed that light acted by providing increased amounts of reductant from photosynthetic electron transport. Extra oxygen production observed in the light during nitrate reduction was postulated to come from the water-splitting reaction of photosynthesis. And increased rates of electron flow occur as a result of the electrons being shuttled off to reduce nitrate after saturation of the carbon dioxide-fixing system.

In support of this latter hypothesis, Chlorella cells exposed to high light intensity and incubated in the presence of nitrate were found to evolve more oxygen compared to cells incubated in the absence of nitrate (60). This observation, coupled with the finding that the generation of reductants by isolated chloroplasts could be coupled to the reduction of nitrate by nitrate reductase (18, 32, 41), provides the major impetus for support of van Niel's hypothesis. In addition, light has been found to increase the amount of reductant in spinach chloroplasts in vivo (29).

The required stoichiometry of the photochemical reduction of nitrate or nitrite may be expressed by the following equations:

(a) $HNO_3 + H_2O \longrightarrow NH_3 + 2O_2$

(b) $2HNO_2 + 2H_2O \longrightarrow 2NH_3 + 3O_2$

The equations predict a ratio of oxygen evolved to nitrate or nitrite reduced (assimilated) of 2 and 1.5, respectively. There are several reports in the literature in which such ratios for nitrate (10, 14, 66) and nitrite reduction (10, 26, 61) were measured. The major obstacle for such an approach toward explaining the action of light on nitrate reduction is that the same ratios can be derived by expanding equations (a) and (b) to the following for nitrate (c and d) and nitrite reduction (e and f):

(a)

(c) $2CO_2 + 2H_2O \xrightarrow{\text{light}} 2(CH_2O) + 2O_2$

(d) $HNO_3 + 2(CH_2O) \xrightarrow{\text{dark}} NH_3 + H_2O + 2CO_2$

(b)

(e) $3CO_2 + 3H_2O \xrightarrow{\text{light}} 3(CH_2O) + 3O_2$

(f) $2HNO_2 + 3(CH_2O) \xrightarrow{\text{dark}} 2NH_3 + H_2O + 3CO_2$

In both cases, the ratio of nitrate or nitrite reduced to oxygen is the same as in equations (a) and (b). Since carbon dioxide production and utilization (via respiration and photosynthesis, respectively) are equal, only catalytic quantities of carbon dioxide would be required to permit reactions (c) through (f) to occur. And there is evidence that greater than 90% of carbon dioxide produced during anaerobiosis can be reutilized in photosynthesis (50, Table IV). Thus, one cannot unequivocably distinguish between the two sets of equations based on the reported ratios of oxygen evolved to nitrate or nitrite reduced.

Despite in vitro evidence in favor of light-mediated reduction of nitrate, several observations tend to detract from such an hypothesis. Perhaps the most important is the carbon dioxide requirement for light-stimulated nitrate reduction (10, 14, 22, 23). If nitrate were directly accepting electrons from photosynthetic light reactions, one would not expect a requirement for carbon dioxide. One cannot exclude at this time that electron transport is dependent in some way upon catalytic quantities of carbon dioxide, as appears to be the case in some algae (31). The observation that oxygen evolution is unaffected by the presence of nitrate, even at photosynthetically saturating light intensities (10) is in disagreement with van Niel's hypothesis which requires that at sufficiently high light intensities the rate of oxygen production would be enhanced by the addition of extra oxidant--namely nitrate (60). Furthermore, that only a loose "coupling" exists between light-generated reductants and nitrate reduction is indicated by (a) the findings that oxygen evolution, carbon fixation, the Hill reaction, and nitrate reduction show differential sensitivity to 3-(4-chlorophenyl)-1,1-dimethyl urea (CMU) (26), (b) the failure to obtain the theoretical ratios of oxygen evolved to nitrate assimilated by some investigators (22, 26, 64) (c) the observation that oxygen evolution remains the same in the presence or absence of nitrate at all light

intensities (10) and (d) the finding that nitrate reduction is saturated at relatively low light intensities (10, 22, 26).

Most arguments concerning the involvement of light in the reduction of nitrate have overlooked a significant study reported in 1955 by Good (21). In this study, mass spectroscopy was used to independently measure oxygen and carbon dioxide production by Chlorella cells incubated in the light with a helium atmosphere. Upon addition of ferricyanide to the reaction medium carbon dioxide evolution occurred; then, several minutes later, oxygen evolution began to occur. Ferricyanide also stimulated carbon dioxide evolution in the dark. By analogy to ferricyanide, these results suggest that nitrate might act as an electron acceptor of reductants generated via glycolysis, fermentation or the tricarboxylic acid cycle. The carbon dioxide thus produced by the operation of these pathways, could in turn act as a final electron acceptor in photosynthesis--thereby causing, indirectly, oxygen evolution to occur. Therefore it seems likely that there may be an indirect interaction between the processes of photosynthesis and respiration in nitrate assimilation.

Nitrate Reduction and the Generation of Reductant via Respiration and Photosynthesis--Use of Inhibitors

The use of metabolic inhibitors has indicated a relationship between the generation of reductants from

respiration or photosynthesis and nitrate reduction in both higher and lower plants. With suspensions of the marine chlorophyte Dunaliella tertiolecta, CMU reduced the rate of nitrate reduction in the light to rates comparable to those occurring in the dark in the absence of inhibitor (22). CMU had little or no effect on nitrate reduction in the dark. Alternatively, iodoacetate was without effect on nitrate reduction in the light, yet it abolished nitrate reduction in the dark.

Similar results for iodoacetate were obtained with tomato leaf disks (42). In this species, ^{15}N-nitrate assimilation was inhibited by malonate and iodoacetate in the dark but not in the light. Iodoacetate inhibition was overcome by succinate and citrate, indicating that nitrate reduction in the dark is dependent on some product(s) of glycolysis or the tricarboxylic acid cycle. The accumulation of nitrite in intact wheat root tips was also inhibited by iodoacetate (45). Thus, the differential effects of CMU and iodoacetate with respect to light regime indicates that nitrate reduction is influenced by two different electron generating systems.

Nitrate Reduction and the Generation of Reductants via Respiration

Nitrate assimilation requires an extra reductive step compared to nitrite assimilation. Therefore, if respiration were involved in the generation of reductant

and energy in these processes, one would expect a greater amount of carbon dioxide evolution in the dark for nitrate assimilation compared to that for nitrite. This has been shown for intact cells of D. tertiolecta, where the ratio of carbon dioxide evolved to nitrogen assimilated was 2.0 for nitrate and 1.5 for nitrite (23). Similarly, respiratory quotients of barley roots incubated in the presence of nitrate and nitrite were 1.3 and 1.1, respectively (67).

Coupling reducing equivalents to nitrate reduction via respiration is also indicated by an increased carbon dioxide production in the dark when intact root tips of barley (67) or cells of D. tertiolecta (22) are incubated in the presence of nitrate. Intermediates of the tricarboxylic acid cycle also increased ^{15}N-nitrate assimilation in tomato leaf disks (42) and in cell-free preparations from a variety of plants (16). Finally, in tomato leaf disks, carbohydrate depleted tissue was found to assimilate less nitrate than non-depleted tissue (42). No difference could be detected between depleted and non-depleted tissue when placed in the light.

Nitrite Reduction

In addition to nitrate reduction, evidence indicates that nitrite reduction is also affected by light. With cells of D. tertiolecta (22), leaves of wheat (61), tobacco and broad bean (62), nitrite assimilation in the

light is approximately 2 to 20 times as great as rates observed in the dark. Light-stimulated nitrite assimilation was found to be dependent on carbon dioxide for Chlorella (14) and D. tertiolecta (22). In the absence of carbon dioxide, nitrite reduction in the light with D. tertiolecta occurred at rates similar to dark rates (22). Nitrite reduction in the light by Ankistrodesmus was accelerated by carbon dioxide (26). With Chlorella, glucose could overcome the requirement for carbon dioxide in the light (14). Light-stimulated reduction of nitrite by D. tertiolecta and Anabaena cylindrica was partially inhibited by CMU whereas dark reduction was unaffected (22, 26). However, nitrite assimilation by D. tertiolecta was inhibited by iodoacetate in both the light (35%) and dark (60%) (22).

For nonphotosynthetic tissues, one would expect an increase in the respiratory quotient during nitrite reduction if respiration was involved. This was found to be the case for barley roots growing in nitrite-containing solutions (67). The accumulation of nitrite by plants fed nitrate in the dark under anaerobic conditions also indicates a requirement for electron transport or oxidative phosphorylation in nitrite reduction (40, 44, 52). The ability to inhibit nitrite reduction by DNP in a variety of higher and lower plants suggests energy is the required factor (3, 26, 34, 54, 62). Because other uncouplers of

oxidative phosphorylation also inhibit nitrite reduction (4, 38), it seems unlikely that DNP is exerting its effect by alternatively accepting reducing equivalents as has been shown to be the case with isolates of Pseudomonas denitrificans (51) and reductants generated by spinach chloroplasts (65).

LITERATURE CITED

1. Afridi, M. M. R. K. and E. J. Hewitt. 1965. The inducible formation and stability of nitrate reductase in higher plants. II. Effects of environmental factors, antimetabolites, and amino acids on induction. J. Exp. Bot. 16:628-645.

2. Afridi, M. M. R. K. and E. J. Hewitt. 1964. The inducible formation and stability of nitrate reductase. J. Exp. Bot. 15:251-271.

3. Ahmad, J. and I. Morris. 1967. Inhibition of nitrate and nitrite reduction by 2,4-dinitrophenol in Ankistrodesmus. Archiv. Mikrobiol. 56:219-224.

4. Ahmad, J. and I. Morris. 1968. The effects of 2,4-dinitrophenol and other uncoupling agents on the assimilation of nitrite by Chlorella. Biochem. Biophys. Acta 162:32-38.

5. Atkinson, D. E. 1965. Biological feedback control at the molecular level. Science 150:851-857.

6. Bach, A. 1913. Zur kenntnis der reduktionsfermente. Pflanzliche perhydrase. IV. Mitteilung. Biochem. Zeit. 52:412-417.

7. Beevers, L. and R. H. Hageman. 1969. Nitrate reduction in higher plants. Annu. Rev. Plant Physiol. 20:495-522.

8. 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: 691-698.

9. Bernheim, F. 1928. The aldehyde oxidase of potato. Biochem. J. 22:344-352.

10. Bongers, L. S. H. 1958. Kinetic aspects of nitrate reduction. Neth. J. Agr. Sci. 6:79-88.

11. Brownell, P. F. and D. J. D. Nicholas. 1967. Some effects of sodium on nitrate assimilation in anabaena cylindrica. Plant Physiol. 42:915-921.

12. Burnstrom, H. 1943. Photosynthesis and assimilation of nitrate by wheat leaves. K. LantbrHogsk. Annlr. II, 1. [Cited by Grant (8)].

13. 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. 47:341-343.

14. Davis, E. A. 1953. Nitrate reduction by Chlorella. Plant Physiol. 28:539-544.

15. Del Campo, F. F., A. Paneque, J. M. Ramirez and M. Losada. 1965. Nitrate reduction with molecular hydrogen in a reconstituted enzymic system. Nature (Lond.) 205:387-388.

16. Delwiche, C. C. 1952. Reduction of nitrite and nitrate ions by preparations obtained from higher plants. Fed. Proc. 11:201-202.

17. Egami, F., K Omachi, K. Lida and S. Taniguchi. 1957. Nitrate reducing systems in cotyledons and seedlings of bean seed embryos of Vigna sesquipedalis during the germinating stage. Biokhim. 22:115-127.

18. Evans, H. J. and A. Nason. 1953. Pyridine nucleotide nitrate reductase from extracts of higher plants. Plant Physiol. 28:233-254.

19. Farkos-Hinsley, H. and M. Artman. 1957. Studies on nitrate reduction by Escherichia coli. J. Bacteriol. 74:690-692.

20. Filner, P. 1966. Regulation of nitrate reductase in cultured tobacco cells. Biochim. Biophys. Acta 118:299-310.

21. Good, N. 1955. Reduction of ferricyanide by algal suspensions. Plant Physiol. 30:483-484.

22. Grant, B. R. 1967. The action of light on nitrate and nitrite assimilation by the marine chlorophyte Dunaliella tertiolecta (Butcher). J. Gen. Microbiol. 48:379-389.

23. Grant, B. R. 1968. The effect of carbon dioxide concentration and buffer system on nitrate and nitrite assimilation by Dunaliella tertiolecta. J. Gen. Microbiol. 54:327-336.

24. Grassmanis, V. O. and D. J. D. Nicholas. 1967. A nitrate reductase from apple roots. Phytochemistry. 6:217-218.

25. Hageman, R. H. and D. Flesher. 1963. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35:700-708.

26. Hattori, A. 1962. Light induced reduction of nitrate, nitrite and hydroxylamine in a blue green algae, Anabaena cylindrica. Plant Cell Physiol. 3:355-369.

27. Hattori, A. and J. Myers. 1966. Reduction of nitrate and nitrite by subcellular preparations of Anabaena cylindrica. I. Reduction of nitrite to ammonia. Plant Physiol. 41:1031-1036.

28. Hattori, A. and J. Myers. 1967. Reduction of nitrate and nitrite by subcellular preparations of Anabaena cylindrica. II. Reduction of nitrate to nitrite. Plant Cell Physiol. 8:327-337.

29. Heber, V. W. and K. A. Santarius. 1965. Compartmentation and reduction of pyridine nucleotides in relation to photosynthesis. Biochim. Biophys. Acta 109:390-408.

30. Heimer, Y. 1970. Control of nitrate assimilation in cultured tobacco cells. Ph.D. Thesis. Michigan State University, East Lansing, Michigan.

31. Heise, J. J. and H. Gaffron. 1963. Catalytic effects of carbon dioxide in carbon dioxide assimilating cells. Plant and Cell Physiol. 4:1-11.

32. Huzisige, H., K. Satoh, K. Tanaka, and T. Hayasida. 1963. Photosynthetic nitrite reductase II. Further purification and biochemical properties of the enzyme. Plant and Cell Physiol. 4:307-322.

33. Kastle, J. H. and E. Elvove. 1904. On the reduction of nitrates by certain plant extracts and metals, and the accelerating effect of certain substances on the progress of the reduction. Amer. Chem. J. 31:606-641.

34. Kessler, E. 1955[a]. Über die Wirkung von 2,4-Dinitrophenol auf Nitratereducktion und Atmung von Grünalgen. Planta (Berl.) 45:94-105.

35. Kessler, E. 1955[b]. Role of photochemical processes in the reduction of nitrate by green algae. Nature 176:1069-1070.

36. Kessler, E. 1959. Reduction of nitrate by green algae. Soc. Exp. Biol. Symp. 13:87-105.

37. Kessler, E. 1964. Nitrate assimilation by plants. Annu. Rev. Plant Physiol. 15:57-72.

38. Kessler, E. and W. Bucher. 1960. Über die Wirking von Arsenat auf Nitratreduktion, Atmung und Photosynthese von Grünalgen. Planta (Berl.) 55:512-524.

39. Klepper, L. and R. H. Hageman. 1969. Dependence of nitrate reduction on generation of reduced nicotinamide adenine dinucleotide by glycolytic metabolism of leaf tissue. 11th Int. Bot. Congr. Abstr.

40. Kumada, H. 1953. The nitrate utilization in seed embryos of Vigna sesquipedalis. J. Biochem. 40: 439-450.

41. Losoda, M., J. M. Ramirez, A. Paneque and F. F. Del Campo. 1965. Light and dark reduction of nitrate in a reconstituted chloroplast system. Biochim. Biophys. Acta 109:86-96.

42. Mendel, J. L. and D. W. Visser. 1951. Studies on nitrate reduction in higher plants. I. Arch. Biochem. Biophys. 32:158-169.

43. Minotti, P. L., D. C. Williams and W. A. Jackson. 1969. The influence of ammonium on nitrate reduction in wheat seedlings. Planta 86:267-271.

44. Nance, J. F. 1948. The role of oxygen in nitrate assimilation by wheat roots. Amer. J. Bot. 35: 602-606.

45. Nance, J. F. 1950. Inhibition of nitrate assimilation in excised wheat roots by various respiratory poisons. Plant Physiol. 25:722-735.

46. Nason, A. 1956. Enzymatic steps in the assimilation of nitrate and nitrite in fungi and green plants, pp. 109-136. W. D. McElroy and B. Glass. Symposium Inorganic Nitrogen Metabolism. Johns Hopkins Press, Baltimore.

47. Nason, A. 1962. Symposium on metabolism of inorganic compounds II. Enzymatic pathways of nitrate, nitrite, and hydroxylamine metabolisms. Bacteriol. Rev. 26:16-41.

48. Nason, A. and McElroy. 1963. Modes of action of the essential mineral elements, Vol. III, p. 490. F. C. Steward. Plant Physiology, Academic Press, New York.

49. Nightingale, G. T. 1937. The nitrogen nutrition of green plants. Bot. Rev. V(III):85-174.

50. Nishida, K. 1962. Studies on the re-assimilation of respiratory CO_2 in illuminated leaves. Plant Cell Physiol. 3:111-124.

51. Radcliff, B. C. and D. J. D. Nicholas. 1968. Some properties of nitrite reductase from Pseudomonas denitrificans. Biochim. Biophys. Acta 153:545-554.

52. Randall, P. J. 1969. Changes in nitrate and nitrate reductase levels on restoration of molybdenum to molybdenum-deficient plants. Aust. J. Agr. Res. 20:635-642.

53. Ratner, E. I. and S. A. Samoilova. 1966. Influence of exogenous ribonucleic acid and some of its nitrogenous bases on the reduction of nitrates in plant tissue and the symbiotic fixation of atmospheric nitrogen in soya. [Transl. from Russian] Sov. Plant Physiol. 13:914-924.

54. Ritenour, G. L., K. W. Joy, J. Bunning and R. H. Hageman. 1967. Intracellular localization of nitrate reductase, nitrite reductase, and glutamic acid dehydrogenase. Plant Physiol. 42:233-237.

55. Robinson, M. E. 1929. The protein metabolism of the green plant. New Phytol. 28:117-149.

56. Schrader, L. E. and R. H. Hageman. 1967. Regulation of nitrate reductase activity in corn (Zea mays L.) seedlings by endogenous metabolites. Plant Physiol. 42:1750-1756.

57. Schrader, L. E., G. L. Ritenour, G. L. Eilrich, and R. H. Hageman. 1968. Some characteristics of nitrate reductase from higher plants. Plant Physiol. 43:930-940.

58. Stenlid, G. 1957. Some differences between the accumulation of chloride and nitrate ions in excised wheat roots. Physiol. Plant. 10:922-936.

59. Syrett, P. J. 1962. Nitrogen assimilation, pp. 171-188. In R. R. Lewis, Physiology and Biochemistry of Algae. Acad. Press, New York.

60. van Niel, C. B., M. B. Allen and B. S. Wright. 1953. On the photochemical reduction of nitrate by algae. Biochim. Biophys. Acta 12:67-74.

61. Vanecko, S. and J. E. Varner. 1955. Studies on nitrite metabolism in higher plants. Plant Physiol. 30:388-390.

62. Voskresenskaya, N. P. and G. S. Grishina. 1962. The significance of light for nitrite reduction in the green leaf. [Transl. from Russian] Sov. Plant Physiol. 1962. 9:4-10.

63. Wallace, W. and J. S. Pate. 1967. Nitrate assimilation in higher plants with special reference to the cockelbur (Xanthium pennsylvanicum). Ann. Bot. 31:213-228.

64. Warburg, O. and E. Negelein. 1920. Über die Reduktion der Saltpetersaüre in grünnen Zellen. Biochem. Zeit. 110:66-115.

65. Wessels, J. S. C. 1965. Mechanism of the reduction of organic nitro compounds by chloroplasts. Biochim. Biophys. Acta 109:357-371.

66. Warburg, O., G. Krippahl and C. Jetschmann. 1965. Widerlegung der Photolyse des H_2O und Beweis der von CO_2. Z. Naturforsch. 206:993-996.

67. Willis, A. J. and E. W. Yemm. 1955. The respiration of barley plants II. Nitrogen assimilation and the respiration of the root system. New Phytol. 54:163-181.

SECTION I

ABSTRACT

SUBSTRATE INDUCTION OF NITRATE REDUCTASE
IN BARLEY ALEURONE LAYERS

By

Thomas Enrico Ferrari

Nitrate induces the formation of nitrate reductase activity in barley (Hordeum vulgare L. cv. Himalaya) aleurone layers. Previous work has demonstrated de novo synthesis of alpha-amylase by gibberellic acid in the same tissue. The increase in nitrate reductase activity is inhibited by cycloheximide and 6-methylpurine, but not by actinomycin D. Nitrate does not induce alpha-amylase synthesis and it has no effect on the gibberellic acid-induced synthesis of alpha-amylase. Also, there is little or no direct effect of gibberellic acid (during the first 6 hours of induction) or of abscisic acid on the nitrate-induced formation of nitrate reductase. Gibberellic acid does interfere with nitrate reductase activity during long-term experiments (greater than 6 hours). However, the time course of this inhibition suggests that the inhibition may be a secondary one. Barley aleurone layers therefore provide a convenient tissue for the study of both substrate- and hormone-induced enzyme formation.

INTRODUCTION

Gibberellic acid induces increases of alpha-amylase and other hydrolases in barley half-seeds (8, 9). For amylase (5) and for protease (6) these increases have been shown to be the result of de novo synthesis which is dependent upon the continued presence of GA_3 (2) and continued synthesis of some unidentified kind (or kinds) of RNA (3). These increases in the hydrolases, in addition to being inhibited by protein synthesis and RNA synthesis inhibitors, are inhibited by abscisic acid (1), anaerobiosis and 1 to 2% ethyl alcohol. How many of these controls for gibberellin-induced hydrolase synthesis are characteristic of the de novo synthesis of any protein? And how many are specific for gibberellin-induced enzyme synthesis? To answer these questions we looked for the formation of a substrate-induced enzyme. More specifically, we looked for the nitrate-induced formation of nitrate reductase. There is no nitrate reductase in the dry seed and it does not appear during the incubation of the half-seed or isolated aleurone layer until nitrate is added.

MATERIALS AND METHODS

Enzyme Induction and Extraction

The procedure used to prepare aleurone layers was the same as described by Jones and Varner (7) and the extraction of nitrate reductase (NR) was similar to that of Filner (4). Briefly, the preparation of tissue and extraction of NR and alpha-amylase are as follows. Embryos were excised from barley (Hordeum vulgare L. cv. Himalaya) and the half-seeds sterilized in 1% sodium hypochlorite, rinsed in sterile deionized water, and imbibed on moist sand for 3 to 5 days prior to removal of the aleurone layer. Ten isolated aleurone layers were used per treatment. Unless stated otherwise, the basic incubation medium for all experiments with aleurone layers consisted of the following in a total of 2 ml: 2×10^{-3} M acetate buffer (pH 4.8), 2×10^{-2} M $CaCl_2$, 1 drop of 0.5 mg/ml chloramphenicol, and 5×10^{-2} M KNO_3. In experiments with half-seeds, the 2 ml incubation medium consisted of 1 drop of 0.5 mg/ml chloramphenicol and the concentration of KNO_3 as indicated in the text.

Aleurone layers were homogenized with sand, by morter and pestle in a total of 2 ml extraction medium consisting of 0.1 M tris-HCl buffer (pH 7.5) and 1 mM

cysteine. The homogenate was clarified by centrifugation at 5000g for 10 minutes, and the supernatant fraction used as the crude enzyme. All steps in the extraction procedure were carried out at 0 to 4°C.

Enzyme Assays

NR activity was determined in an assay medium consisting of the following components in a total of 0.4 ml: 0.12 ml, 0.167 M phosphate buffer (pH 7.5); 0.04 ml, 0.1 M KNO_3; 0.02 ml, 2 mM flavin mononucleotide; 0.02 ml $Na_2S_2O_4$, 8 mg/ml, in 0.095 M $NaHCO_3$; and 0.2 ml crude enzyme preparation. Up to this point, tubes were kept in an ice bath. Each tube was shaken gently to mix the reaction components and reduce the flavin mononucleotide (evidenced by a change in color from yellow to pale yellow). Just prior to placing the tubes in a water bath at 25°C to start the reaction, 1 drop of paraffin oil was run down the side of each tube. The paraffin formed a layer over the reaction medium, thereby preventing air oxidation of the reducing agent. After one-half hour, the reaction was stopped by shaking the reaction mixture vigorously until all the reducing agent was oxidized (evidenced by a return from the pale yellow to yellow color). Nitrite produced by enzymatic reduction of nitrate was determined by first adding 0.3 ml of 1% sulfanilamide in 3 N HCl, followed by 0.3 ml of 0.02% N-1-naphthylethylenediamine

dihydrochloride. Optical density at 540 mμ was measured after centrifuging at 5000g for 15 minutes.

alpha-amylase activity was determined from the induction medium or in the NR enzyme extract, as described by Jones and Varner (7).

RESULTS

Production of Nitrate Reductase by Half-seeds

From experiments with both dry and 3-day imbibed half-seeds, nitrate reductase (NR) could only be detected when incubated for 40 hours with nitrate. The optimum concentration of nitrate for induction was 5×10^{-2} M. No NR activity was detected in dry half-seeds or in half-seeds imbibed for 3 days on sand moistened with sterile deionized water.

Nitrate Reductase Production by Isolated Aleurone Layers

NR synthesized by isolated aleurone layers in response to nitrate occurs at a linear rate, after a lag period of about 30 minutes (Fig. 1), for at least 8 hours after the start of enzyme synthesis. The rate of NR formation is limited (Fig. 2) up to a concentration of approximately 10^{-2} M nitrate. Above 10^{-2} M nitrate the rate of enzyme synthesis decreased. No NR activity could be detected in the induction medium 24 hours after the start of the experiment, and no NR activity could be detected in aleurone layers incubated up to 24 hours without nitrate.

Figure 1.--Kinetics of nitrate reductase induction. Enzyme activity was measured in a 0.4 ml aliquot of the crude enzyme extract. Samples were removed at the indicated time intervals and enzyme activity determined.

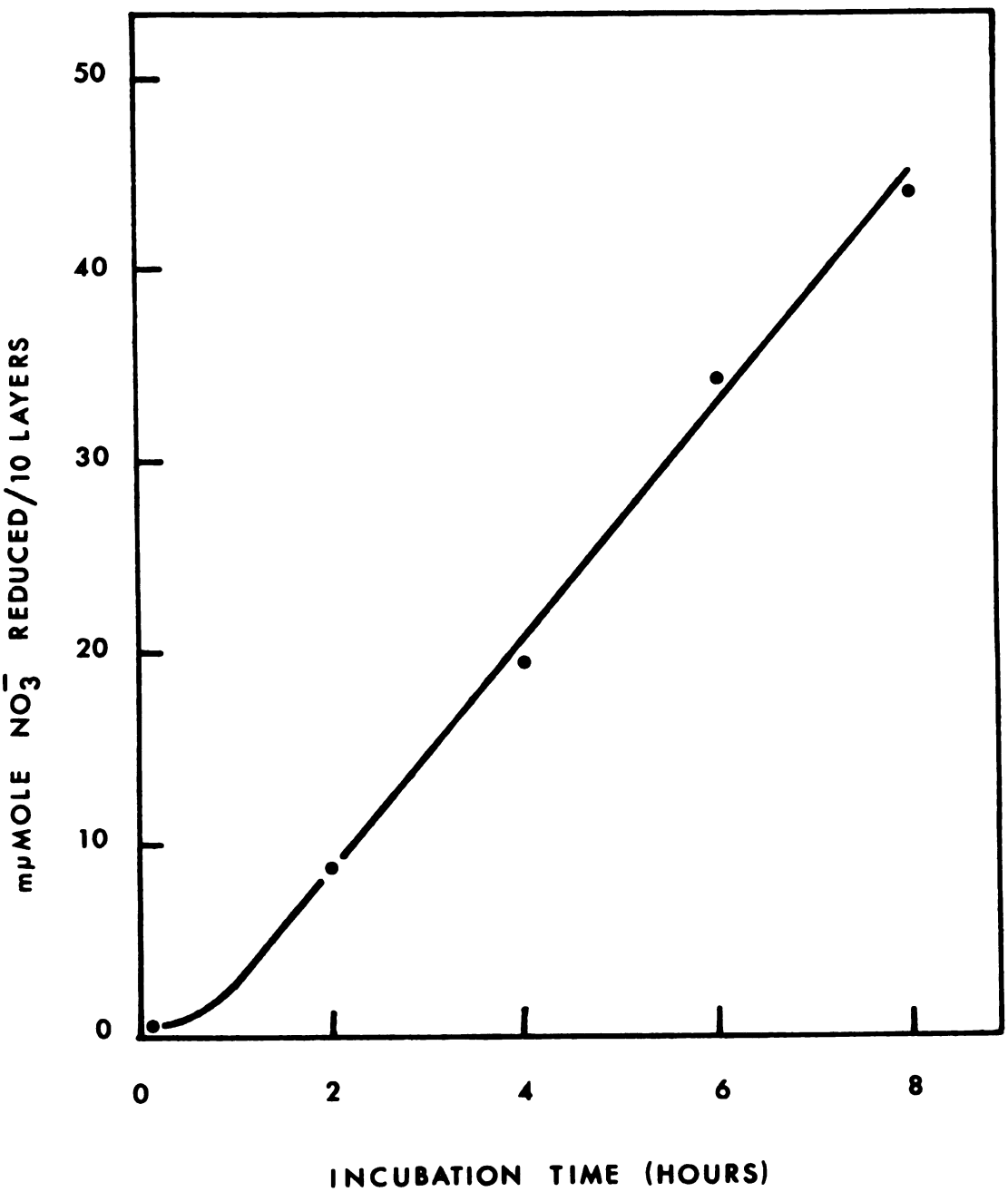
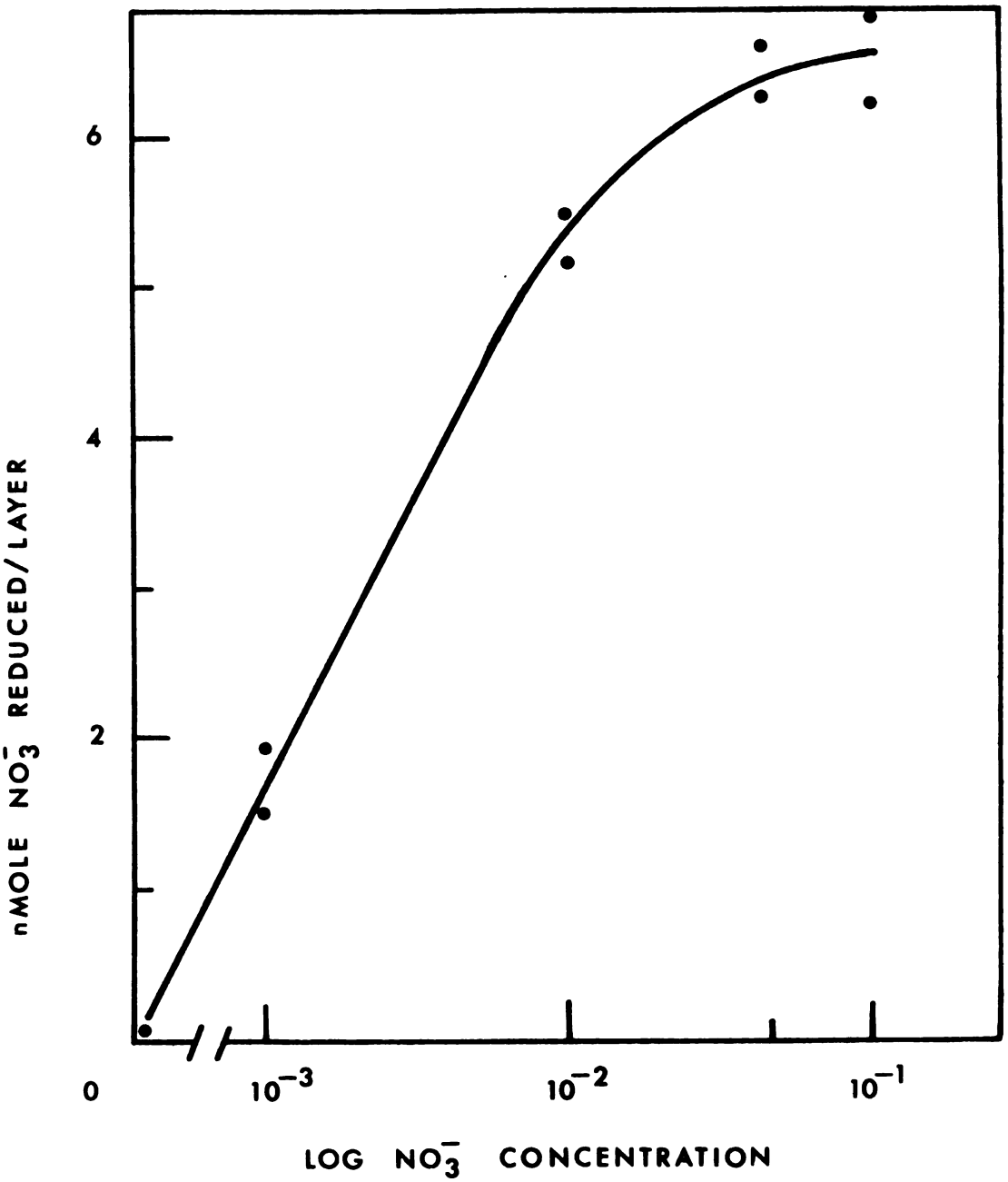

50
40
30
20
10
0
mμMOLE NO_3^- REDUCED/10 LAYERS
0
2
4
6
8
INCUBATION TIME (HOURS)

Figure 2.--Effect of substrate concentration on the induction of nitrate reductase. Nitrate reductase was measured after 16 hours incubation in 0.2 ml of crude enzyme extract.


6
4
2
0
nMOLE NO_3^- REDUCED/LAYER
10^{-3}
10^{-2}
10^{-1}
LOG NO_3^- CONCENTRATION

Effect of GA on Nitrate Reductase Synthesis

From the standpoint of induction specificity and control of enzyme synthesis, it seemed pertinent to determine if GA could substitute for nitrate in the induction of NR and conversely, whether or not nitrate has any effect on alpha-amylase induction. Experiments showed that (1) nitrate does not induce alpha-amylase; (2) GA does not induce NR; (3) nitrate does not prevent GA induction of alpha-amylase; and (4) GA does not directly prevent nitrate induction of NR (Table 1). GA does interfere with NR synthesis when incubated in the presence of nitrate for periods greater than 6 hours--the amount of inhibition being dependent on the concentration of GA used. With 10^{-6} M (Fig. 3) and 10^{-8} M GA (Table 1) the amount of nitrate reductase at 24 hours are 0% and 60% of control activities, respectively. The kinetics of GA interference of NR synthesis in a long-term (24 hours) experiment is shown in Figure 3. It can be seen that 10^{-6} M GA has little or no effect on NR activity for the first 6 hours; however, as noted above, enzyme activity is abolished at 24 hours. The time course of this interference suggests that the interference is a secondary one. For example, it might be due to the consequences of the appearance of the GA-induced protease.

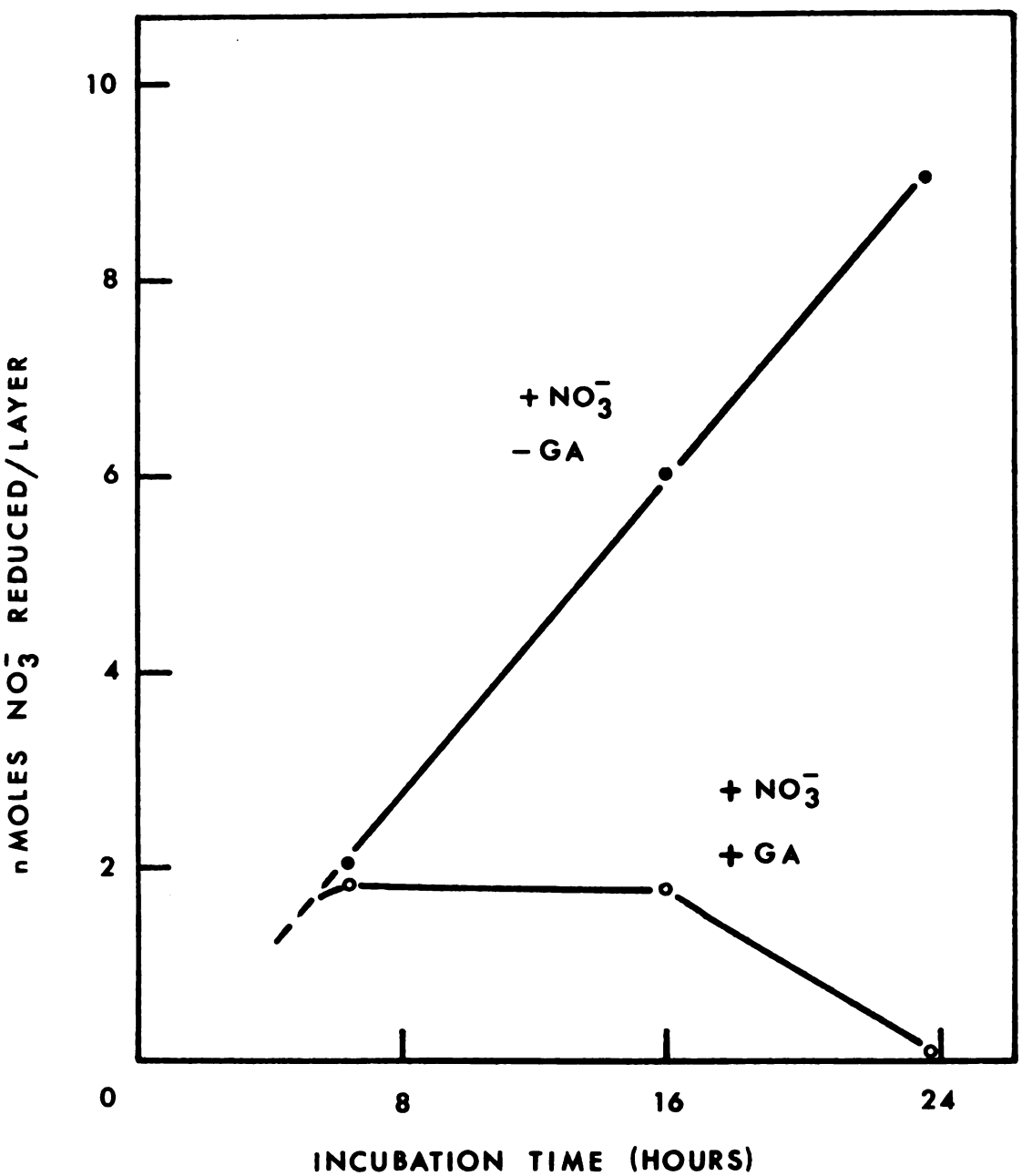
Table 1.--Effect of GA, NO_3^-, and GA + NO_3^- on induction of nitrate reductase and alpha-amylase.

Ten aleurone layers were incubated for 24 hours in 2 ml of 2 X 10^{-3} M acetate buffer (pH 4.8), 2 X 10^{-2} M $CaCl_2$, 1 drop of 0.5 mg/ml chloramphenicol and the concentration of KNO_3 and GA indicated.

Treatment	Activity[1]	
	NR of Extract	Amylase of Medium
	% of control	
Control ($-NO_3^-$, -GA)	0	100
+ KNO_3 (5 X 10^{-2} M)	100	90
+ GA (10^{-8} M)	0	500
+ KNO_3 + GA	60	500

[1]Each figure represents the average of 2 determinations. Control value for alpha-amylase in media represents activity by approximately 4 ug of alpha-amylase.

Figure 3.--Effect of GA on nitrate reductase synthesis. GA (10^{-6} M) was added at zero time and the amount of nitrate reductase in 0.4 ml of crude enzyme extract was assayed at indicated times. Potassium nitrate at 0.05 M was used during incubation.


10
8
6
4
2
0
8
16
24
nMOLES NO_3^- REDUCED/LAYER
INCUBATION TIME (HOURS)
+ NO_3^-
– GA
+ NO_3^-
+ GA

Effect of Abscisic Acid on Nitrate Reductase

NR synthesis is considerably less sensitive to abscisic acid (abscisin) than is alpha-amylase. With 10^{-6} M abscisic acid, NR was 80% of control activity, whereas alpha-amylase activity in the incubation medium and of the tissue extract were only 7% and 26%, respectively (Table 2). Inhibition of the order of magnitude observed with alpha-amylase was never observed with NR, even at the highest concentration of abscisic acid tested (10^{-5} M) (Table 2).

Sensitivity of Nitrate Reductase Synthesis to Several Metabolic Inhibitors

Cycloheximide was the most effective inhibitor of the chemicals tested (Table 3); actinomycin D (1 ug/ml) stimulated activity; 6-methylpurine at 5×10^{-4} M inhibited synthesis by 50%; and 8-azaguanine had little or no effect on production of the enzyme. The kinetics of mid-course inhibition of NR synthesis by 6-methylpurine are shown in Figure 4.

Table 2.--Inhibition of nitrate reductase and alpha-amylase induction by abscisic acid.

Ten aleurone layers were incubated in 2 ml of medium containing 2 X 10^{-3} M acetate buffer (pH 4.8), 2 X 10^{-2} M $CaCl_2$, 1 drop of 0.5 mg/ml chloramphenicol, 5 X 10^{-2} M KNO_3 or 10^{-8} M GA, and the concentration of abscisic acid as indicated.

Expt.	Time of Incubation	Concn. (M) of Abscisic Acid	Activity[1] NR	Activity[1] Alpha-amylase Medium	Activity[1] Alpha-amylase Extract
			% of control		
	hr.	control	100	100	100
I	24	10^{-6}	80	7	26
II	6	10^{-6}	78	---	---
III	6	10^{-5}	66	---	---

[1]Each number represents the average of 2 determinations. Percent control values for medium and extract represents activity of approximately 85 and 15 ug of alpha-amylase, respectively.

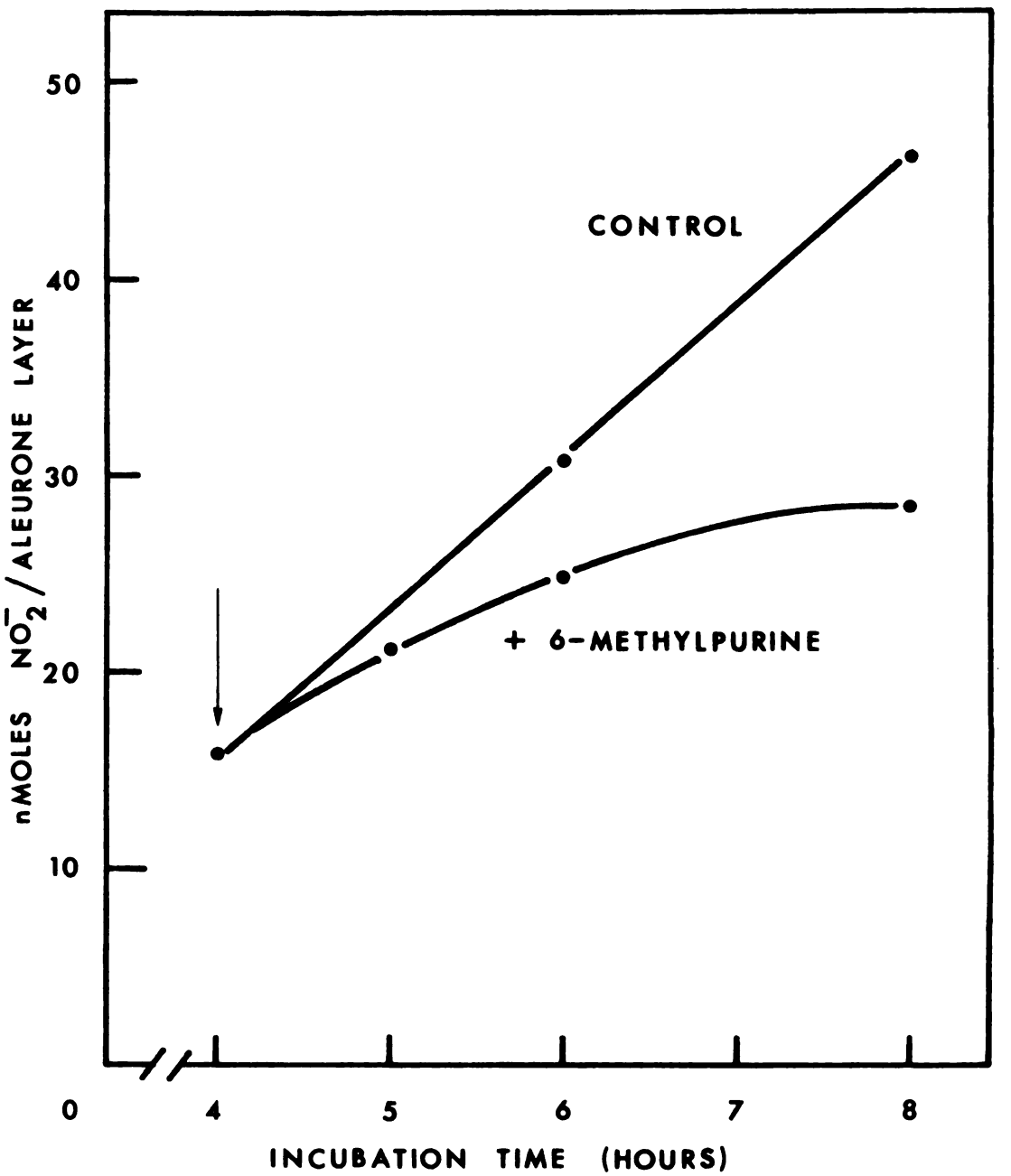
Table 3.--Effect of metabolic inhibitors on nitrate reductase synthesis.

Ten aleurone layers were incubated in 2 ml medium containing 2 X 10^{-3} M acetate (pH 4.8), 2 X 10^{-2} M $CaCl_2$, 5 X 10^{-2} M KNO_3, 1 drop of 0.5 mg/ml chloramphenicol and the concentration of the compound tested. Enzyme activity in a 0.4 ml aliquot of enzyme extract was determined 6 hours after the start of each experiment.

Expt.	Treatment	NR Activity[1] mumole NO_3^- reduced per layer per 30 min.
I	Control	22
I	Cycloheximide (1 ug/ml)	2
I	Cycloheximide (5 ug/ml)	3
I	Cycloheximide (10 ug/ml)	3
I	6-methylpurine (5 X 10^{-4} M)	11
I	6-methylpurine (10^{-4} M)	15
II	Control	15
II	Actinomycin D (1 ug/ml)	25
II	Actinomycin D (10 ug/ml)	20
II	Actinomycin D (40 ug/ml)	18
II	8-azaguanine (2.5 X 10^{-3} M)	16

[1]Each number represents the average of 4 determinations. Each experiment was repeated twice.

Figure 4.--Mid-course inhibition of nitrate reductase synthesis by 6-methylpurine (3 mM). Aleurone layers were incubated in a standard induction medium containing 0.05 M potassium nitrate for 4 hours. At this time, 0.2 ml of 0.02 M 6-methylpurine was added giving a final concentration of 3 mM. Nitrate reductase activity was measured at 1, 2, and 4 hours following addition of the chemical. A 0.4 ml aliquot of crude enzyme was used to determine enzyme activity.


50
40
30
20
10
0
4
5
6
7
8
CONTROL
+ 6-METHYLPURINE
nMOLES NO_2^- / ALEURONE LAYER
INCUBATION TIME (HOURS)

DISCUSSION

In barley aleurone layers, several differences exist between the substrate induction of nitrate reductase (NR) and the hormone induction of alpha-amylase. In contrast to what is found with alpha-amylase, there is no detectable NR in half-seeds or in isolated aleurone layers after imbibition on sand with distilled deionized water or incubation in the absence of inducer. Unlike alpha-amylase (2), NR is not secreted from the aleurone layer. In this study no NR was detected in the incubation medium as long as 24 hours after the start of the experiment. Also, NR synthesis is considerably less sensitive to inhibition by abscisic acid than is the synthesis of alpha-amylase. In contrast to what has been observed with alpha-amylase synthesis (3), 8-azaguanine has no effect on NR synthesis while actinomycin D enhances rather than inhibits NR activity. But as in alpha-amylase synthesis, 6-methylpurine and cycloheximide inhibit NR synthesis.

The independence of the NR and the alpha-amylase inductions within the same tissue is further evidenced by their specificity toward the inducers. That is, nitrate does not induce alpha-amylase nor does GA induce NR.

Furthermore, neither does GA prevent the induction of NR by nitrate, nor does nitrate prevent the induction of alpha-amylase by GA.

LITERATURE CITED

1. Chrispeels, M. J. and J. E. Varner. 1966. Inhibition of gibberellic acid induced formation of alpha-amylase by abscisin II. Nature 212:1066-67.

2. Chrispeels, M. J. and J. E. Varner. 1967[a]. Gibberellic acid-enhanced synthesis and release of alpha-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42:398-406.

3. Chrispeels, M. J. and J. E. Varner. 1967[b]. Hormonal control of enzyme synthesis: on the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant Physiol. 42:1008-16.

4. Filner, P. 1966. Regulation of nitrate reductase in cultured tobacco cells. Biochim. Biophys. Acta 118:299-310.

5. Filner, P. and J. E. Varner. 1967. A test for de novo synthesis of synthesis of enzymes: density labeling with $H_2{}^{18}O$ of barley alpha-amylase induced by gibberellic acid. Proc. Natl. Acad. Sci. 58: 1520-1526.

6. Jacobsen, J. V. and J. E. Varner. 1967. Gibberellic acid synthesis of protease by isolated aleurone layers of barley. Plant Physiol. 42:1596-1600.

7. Jones, R. L. and J. E. Varner. 1967. The bioassay of gibberellins. Planta (Berl.) 72:155-61.

8. Paleg, L. 1960. Physiological effects of gibberellic acid. 1. On carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol. 35: 293-99.

9. Yomo, H. 1960. Studies on the alpha-amylase activating action of gibberellin. Hakko Kyakaishi. 18:600-02. [Cited in Chemical Abstracts, 55:26145, 1961].

SECTION II

ABSTRACT

CONTROL OF NITRATE REDUCTASE ACTIVITY
IN BARLEY ALEURONE LAYERS

By

Thomas E. Ferrari

Nitrate reductase activity in barley (Hordeum vulgare L. cv. Himalaya) aleurone layers has been determined in the intact tissue, using two different methods. The first method measures the rate of appearance of $H_2{}^{18}O$ produced during the reduction of $KN^{18}O_3$. The second assay measures excreted nitrite resulting from nitrate reduction under anaeroboc conditions. Nitrite production in this anaerobic, intact-tissue assay was dependent upon the presence of phosphate (pH 7.5) and was increased by ethanol and bisulfite.

After ten hours of nitrate induction, nitrate reductase activities measured by the $KN^{18}O_3$ assay are one-sixth, and those measured by the anaerobic intact-tissue assay are one-third, of those observed in cell-free extracts of aleurone layers. Addition of ethanol to the anaerobic intact-tissue medium increased the rate of nitrate reduction to a level greater than that found in the cell-free assay.

Oxygen inhibited nitrite release in the anaerobic intact-tissue assay. However, under aerobic conditions and in the presence of 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO) or antimycin A, nitrate reduction increased to rates comparable to those observed under anaerobiosis. Neither of these electron transport inhibitors affected anaerobic nitrate reduction, though they were effective in inhibiting oxygen uptake in separate experiments.

INTRODUCTION

It is important to know if enzyme activities observed in cell-free extracts are representative of those which occur in the intact cell or tissue. For nitrate reductase, we have been able to measure its intracellular activity by two different methods, and to compare them with enzyme activity determined in cell-free extracts.

The first method is based on the use of $KN^{18}O_3$ as substrate for nitrate reductase, the rationale of which is as follows. The reduction of nitrate to ammonia is believed to occur via a pathway consisting of four reactions (1):

$$KN^{18}O_3 \longrightarrow \underset{+\ H_2{}^{18}O}{N^{18}O_2^-} \longrightarrow \underset{+\ H_2{}^{18}O}{(HN^{18}O)} \longrightarrow (NH_2{}^{18}OH) \longrightarrow \underset{+\ H_2{}^{18}O}{NH_4^+}$$

In the first and probably the rate limiting-step in the pathway, nitrate is reduced to nitrite by nitrate reductase. This reaction involves the release of one ^{18}O atom as $H_2{}^{18}O$. The products of nitrite reduction are not known exactly, but the second and third oxygens of nitrate are released during the final two or three steps of the pathway, or perhaps by nonenzymatic exchange of the $N^{18}O_2^-$, $(HN^{18}O)$ and $(NH_2{}^{18}OH)$ oxygens with oxygens of water. The oxygen atoms of $KN^{18}O_3$

have a very low exchange rate with water (half-life measured in years) at all pH values; however, those of nitrite exchange rapidly at low pH values. Thus, one-third of the ^{18}O released as water during the reduction of nitrate to ammonia is indicative of the *in vivo* activity of nitrate reductase. This is true whether the oxygens are enzymatically released from the nitrogen atom, as indicated in the above equation, or in the nonenzymatic exchange with cellular water at the nitrite, or latter, stages of reduction. Thus, the method measures the amount of enzyme involved in nitrate reduction--not necessarily total enzyme levels--in the presence of nitrate only, without addition of any other exogenous materials and under natural (aerobic) conditions.

The second, more rapid intact-tissue assay, is based on the observation (2) that under anaerobic conditions nitrite resulting from nitrate reduction is excreted from the tissue. The method differs from the $KN^{18}O_3$ assay in that assay conditions (anaerobiosis and high phosphate concentrations) are not normally what one might expect *in situ*. Nevertheless, it appears that this second assay technique will be especially useful as a rapid method for studying total levels of nitrate reductase in different biological materials and studying the factors which control the cellular activity of this enzyme.

Barley aleurone layers develop nitrate reductase activity upon exposure to nitrate (3). We have used isolated

aleurone layers to compare nitrate reductase activity of cell-free extracts to enzyme activity observed in the two intact-tissue assays.

MATERIALS AND METHODS

The procedure used to prepare aleurone layers was similar to that described by Chrispeels and Varner (4). Barley seeds (Hordeum vulgare L. cv. Himalaya) were cut in half, the embryoless halves were imbibed on moist sand, and after 4 to 5 days, the aleurone layers were removed.

Nitrate reductase was induced at 23 C under aseptic conditions in either of two ways:

(1) For the $KN^{18}O_3$ assay, a 5-ul drop of $KN^{18}O_3$ (0.05 M) was added to each of 20 aleurone layers. Chloramphenicol (10 ug/ml) was added to the sterilized $KN^{18}O_3$ stock solution to insure against microbial growth during the subsequent induction period. The layers were placed in 5-cm, sterilized, foil-covered Petri dishes. A moistened filter paper (4.25 cm diameter) placed against the lid of the Petri dish prevented dehydration of the tissue. After a 10-hr induction period, water was sublimed (at 80 to 100 μ pressure) from the tissue and collected in a trap cooled with liquid nitrogen. The sublimed water was weighed and placed in 12-ml conical Pyrex tubes containing 46 mg of $NaHCO_3$. The tubes were stoppered with serum vial caps, frozen, and evacuated to approximately 100 μ. The solution was made acidic by injection of 0.2 ml lactic acid, thereby

releasing CO_2. After about 24 hr equilibration at 40 C, the tubes were placed in dry ice and the atom percent excess of $C^{18}O_2$ determined with a MAT GD 150 mass spectrometer. From the weight of extracted water and the isotopic enrichment of CO_2, the number of moles of nitrate reduced were computed.

(2) For the anaerobic intact-tissue assay, nitrate reductase was induced by placing 40-50 aleurone layers and two drops of chloramphenicol (0.5 mg/ml) in a 50-ml Erlenmeyer flask containing 5 ml of sterilized 0.05 M KNO_3. The flasks were stoppered with cotton plugs and placed in a metabolic shaker set at 200 rpm. After 2 to 3 hr of induction the tissue was rinsed with approximately 20 ml of 0.05 M KNO_3, and the effect of various conditions on enzyme activity during the assay was determined. The intact-tissue assay media contained in 2 ml: 10 aleurone layers, 0.1 M phosphate buffer (pH 7.5), 0.02 M KNO_3, and the treatment solution as indicated in the text. To start the assay, the reaction mixture, contained in a 25-ml Erlenmeyer flask, was de-aerated by bubbling nitrogen gas through the medium for one minute, and then stoppered. Nitrite in the media was determined at zero time and after 20 or 30 minutes of incubation, by adding aliquots to 0.3 ml each of 1 per cent sulfanilamide in a 3 N HCl and 0.02 per cent N-1-naphthylethylenediamine dihydrochloride. Optical density at 540 nm was measured after centrifuging at 2000 X g for 10 minutes.

Cell-free activity of nitrate reductase was determined as described previously (3).

Oxygen uptake was measured polarographically with a Clark oxygen electrode. The 2.7-ml reaction media contained 0.02 M KNO_3, 0.1 M phosphate buffer (pH 7.5), 10 aleurone layers, and the test substance dissolved in ethanol. Pure ethanol was added to controls.

RESULTS

Comparison of Nitrate Reduction Rates in the Intact Tissue and Cell-Free Assays

Enzyme activities as determined by the standard cell-free assay, the anaerobic intact-tissue assay, and the $KN^{18}O_3$ *in vivo* assay are shown in Table 1. Nitrate reductase induced under exactly the same conditions shows 6 and 2.5 times more activity in the cell-free and anaerobic intact-tissue methods, respectively, than in the $KN^{18}O_3$ *in vivo* assay.

In an attempt to determine what factor was limiting the rate of nitrate reduction in the intact-tissue, nitrate-induced aleurone layers were assayed in the same medium as used in the cell-free assay. For this, the layers were placed in 2 ml of media containing flavin mononucleotide (FMN), $Na_2S_2O_4$, KNO_3, and phosphate buffer. The reaction was run in the presence of nitrogen to prevent oxidation of the $Na_2S_2O_4$ by air. Under these conditions nitrite was released from the tissue, and the amount of nitrate reductase activity estimated by removing aliquots of the reaction medium after a suitable time period and analyzing for nitrite. Enzyme activity in the complete system, and its dependence on each of the reaction components is shown in Table 2.

Table 1.--Nitrate reductase activity as determined by the cell-free and intact-tissue assay methods.

For each assay data were obtained from tissue induced 10 hours under identical conditions by method 1 as described in Materials and Methods. Numbers represent averages of three experiments.

Assay Method	Nitrate Reductase Activity (nmoles NO_3^- reduced/layer.hr)
Cell-free	8.4
Anaerobic intact-tissue	3.2
$KN^{18}O_3$ intact-tissue	1.4

Table 2.--Dependence of nitrate reductase activity upon components of the cell-free assay media.

Data from tissue induced 22 hr by method 1 as described in Materials and Methods. Numbers represent averages of three experiments.

	Nitrate Reductase Activity (nmole NO_2^- formed/layer.hr)	
	Anaerobic-intact Tissue Assay*	Cell-free Assay
Complete	3.6	9.2
Complete-noninduced	0.0	0.0
- FMN	3.7	0.0
- $Na_2S_2O_4$	3.2	0.0
- Phosphate buffer	0.9	2.3
- KNO_3	0.3	0.0

*Actually represents the anaerobic, intact-tissue assay supplemented with components of the cell-free assay.

Nitrate reduction in the intact layers in the presence of $Na_2S_2O_4$, FMN, and inorganic phosphate was greater than in the $KN^{18}O_3$ *in vivo* assay, but only approximately one-third the rates observed in the cell-free assay.

Effect of Arsenate, Ethanol, Antimycin A and HOQNO on Nitrate Reduction

From the results presented in Table 2 it seemed that phosphate in some way limits nitrate reduction in the tissue under anaerobic conditions. Though it is less effective, arsenate can substitute for phosphate in enhancing nitrate reduction (Table 3). In later experiments, Tris buffer was also found to be effective; therefore, it is not possible to decide whether the enzyme's response to these compounds is a non-specific ion or pH effect.

Ethanol also enhanced nitrate reduction in the anaerobic intact-tissue assay (Figure 1). Ethanol increased nitrite release under both anaerobic and aerobic conditions. This increased sensitivity for measuring rates of nitrate reduction allowed the detection of low levels of nitrate reductase in noninduced tissues (Table 4). The ethanol-enhanced nitrate reduction was strongly dependent on phosphate buffer (Table 4).

Because bisulfite inhibits the reduction of acetaldehyde during glycolysis, its effect on the anaerobic reduction of nitrate was determined. Results qualitatively similar to those obtained with ethanol were obtained with

Table 3.--Dependency of nitrate reductase activity upon phosphate or arsenate in the anaerobic intact-tissue assay.

Tissue induced for 3 hours by method 1 as described in Materials and Methods. Numbers represent an average of two experiments. The arsenate containing media were adjusted to pH 7.5 with NaOH.

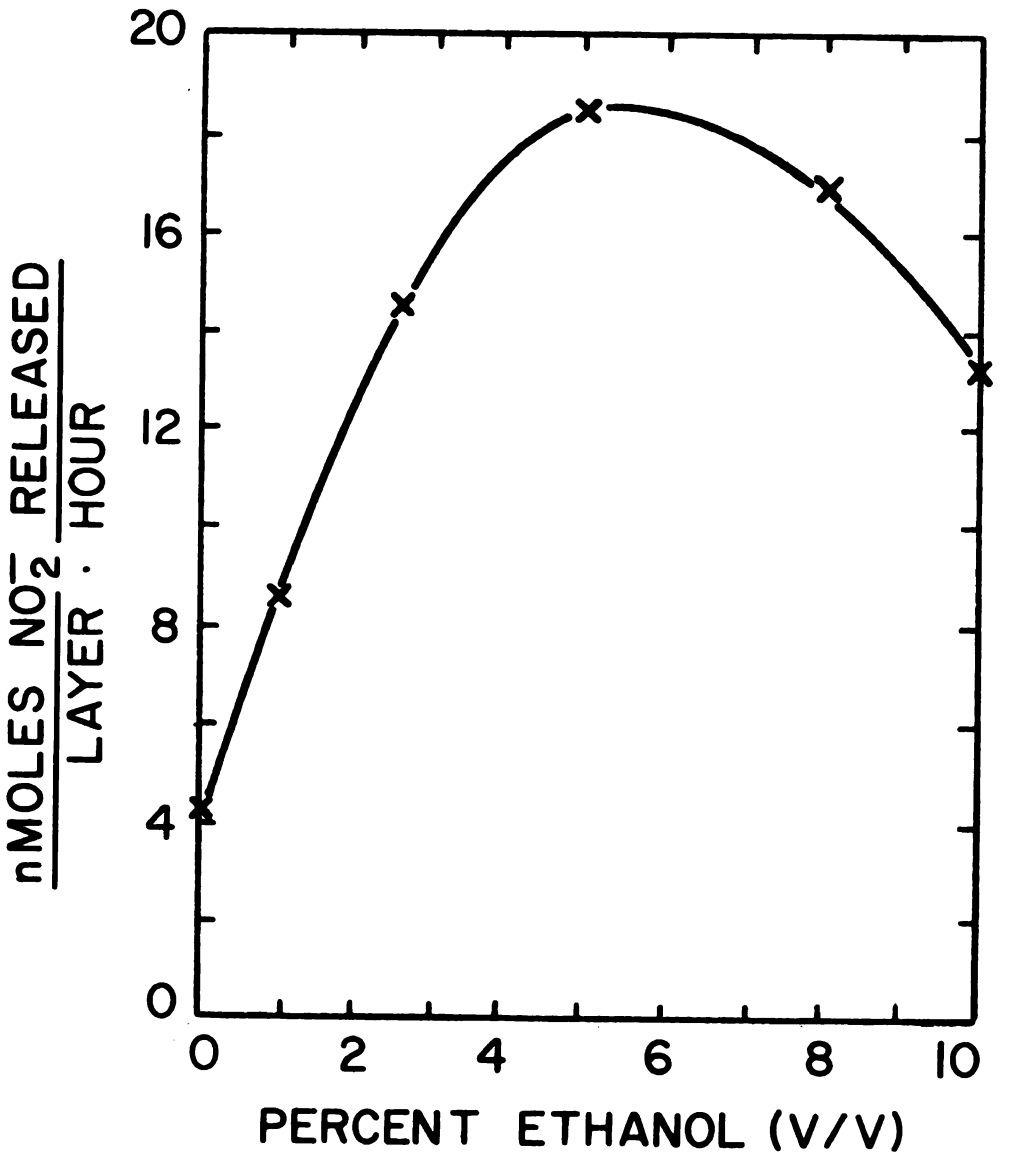
Treatment	Nitrate Reductase Activity (nmoles NO_2^- formed/layer.hr)
KNO_3 - HPO_4^{--}	0.3
KNO_3 + HPO_4^{--}	2.7
KNO_3 + AsO_4^{--}	1.9

Table 4.--Effect of ethanol on nitrate reductase in the anaerobic intact-tissue assay.

Tissue induced 3.5 hours by method 2 as described in Materials and Methods. Numbers represent an average of two experiments.

Assay Condition	Nitrate Reductase Activity (nmoles NO_2^- formed/layer.hr)
N_2 - EtOH	3.6
N_2 + EtOH	16.6
N_2 + EtOH - tissue	0.0
Air + EtOH	2.0
Air - EtOH	0.1
N_2 + EtOH - phosphate	1.0
N_2 + EtOH noninduced	2.5
N_2 - EtOH noninduced	0.3

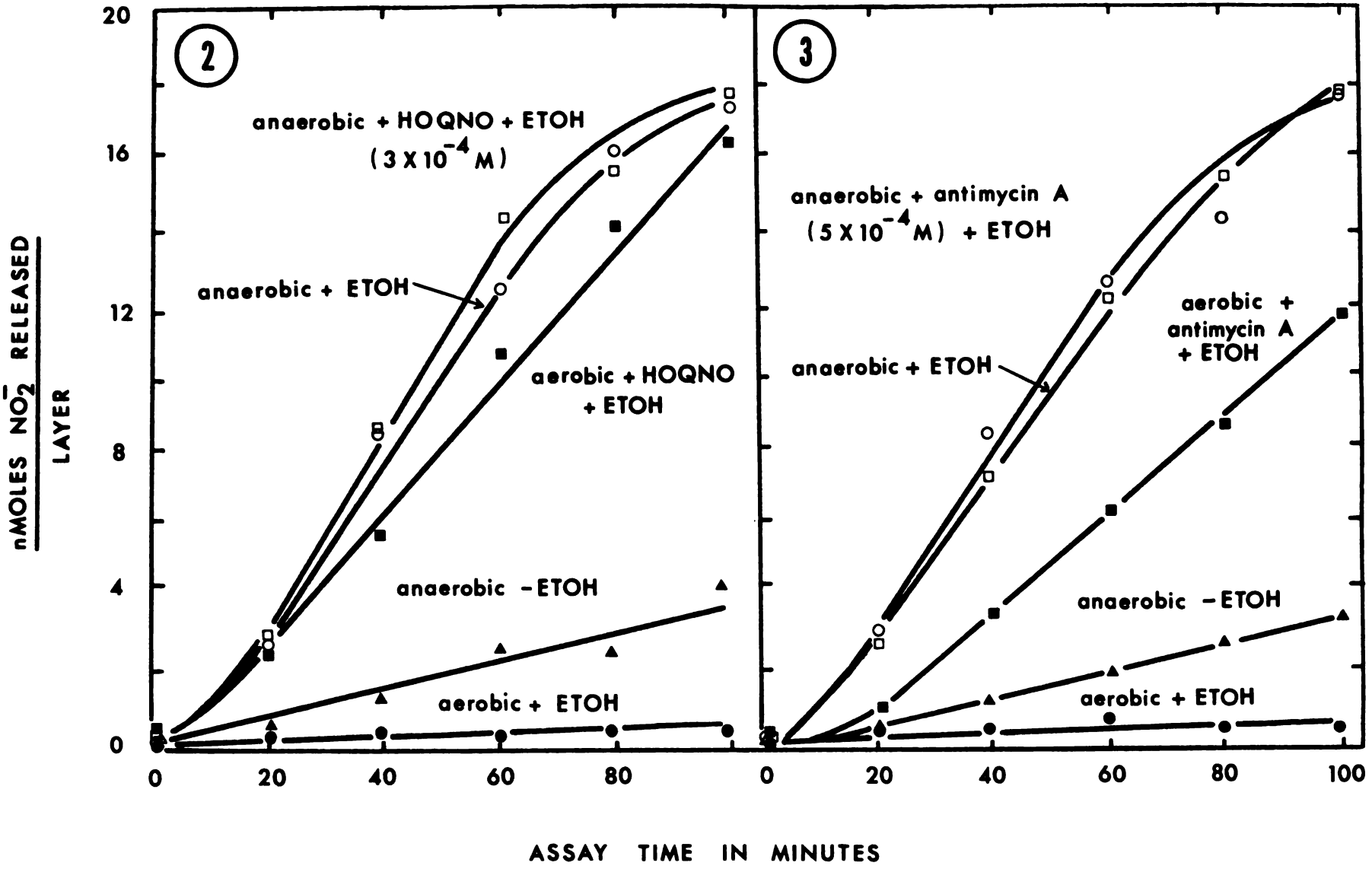
Figure 1.--Concentration curve of ethanol-enhanced nitrite released in the anaerobic intact-tissue assay. Tissue was induced 3 hours by method 2 as described in Materials and Methods.


20
16
12
8
4
0
0
2
4
6
8
10
PERCENT ETHANOL (V/V)
nMOLES NO2- RELEASED / LAYER · HOUR

10^{-3} M. bisulfite ($Na_2S_2O_4$). The chloramphenicol normally present as antiseptic in the assay media possesses a potentially sulfite-reactive carboxyl group; therefore, penicillin was added in its place. Also, the pH of the reaction media was reduced from 7.5 to 5.5 to ensure the presence of the reactive HSO_3^- ion species. Neither ethanol nor bisulfite exerted their effect by inhibiting respiration (see next paragraph) because neither chemical inhibited oxygen uptake (Table 5).

The reduction of nitrate under anaerobic conditions and the excretion of the reduced product, nitrite, resembles "nitrite respiration" as described for bacteria (5) and reported to occur in Vigna sesquipedalis cotyledons (2). For this reason the effect of some respiratory inhibitors on nitrate reduction was determined. Both antimycin A and HOQNO, at concentrations which strongly inhibit oxygen uptake (Table 5), had negligible effects on the anaerobic reduction of nitrate (Figures 2 and 3). However, under aerobic conditions, both these chemicals caused nitrite to be excreted from the tissue (Figures 2 and 3). The HOQNO and antimycin A effect was not observed with noninduced tissue, and was phosphate dependent. The rate of the aerobic HOQNO-induced nitrite release is nearly identical to that observed under anaerobic conditions (Figure 2). In the presence of antimycin A, the rate of nitrite release begins to approach rates observed under anaerobic conditions

Figures 2 and 3.--Antimycin and HOQNO-enhanced nitrite release under aerobic conditions. Tissue was induced 2-3 hours by method 2 as described in Materials and Methods.


2
3
anaerobic + HOQNO + ETOH
(3 X 10⁻⁴ M)
anaerobic + ETOH
aerobic + HOQNO
+ ETOH
anaerobic - ETOH
aerobic + ETOH
anaerobic + antimycin A
(5 X 10⁻⁴ M) + ETOH
anaerobic + ETOH
aerobic +
antimycin A
+ ETOH
anaerobic - ETOH
aerobic + ETOH
nMOLES NO2⁻ RELEASED
LAYER
ASSAY TIME IN MINUTES
0
4
8
12
16
20
0
20
40
60
80
100

only after a lag of approximately 10 to 15 minutes (Figure 3). This is consistent with the effect of antimycin A on oxygen uptake: a 15-minute preincubation period is required before oxygen uptake is inhibited 70 per cent (Table 5).

Table 5.--Effect of HOQNO and antimycin A on oxygen uptake.

The rate of oxygen uptake of ten layers was determined over a period of approximately 5 minutes, 2 to 4 minutes after addition of the test chemical to the reaction media. Chemicals were added in ethanol solutions (final ethanol = 5 per cent). Tissue was induced 2-3 hours by method 1 as described in Materials and Methods. Numbers represent an average of two experiments. Phosphate buffer at pH 7.5, except for the bisulfite treatment which was pH 5.5.

Treatment	Oxygen uptake (% of Control)
Control	100
5% ethanol	135
$NaHSO_3$ (10^{-2} M)	110
Antimycin A (5×10^{-4} M)	65
Antimycin A (5×10^{-4} M) 15-min. preincubation	30
HOQNO (3×10^{-4} M)	40
HOQNO (3×10^{-4} M) 15-min. preincubation	10

Induction Kinetics of Nitrate Reductase

Figure 4. compares the kinetics of nitrate reductase induction as determined by the intact-tissue and the cell-free assay methods. Included in this figure, for purposes of comparison, is a plot of enzyme activity determined by the anaerobic intact-tissue method in the presence of ethanol. Nitrate reductase activity determined by the $KN^{18}O_3$ assay yields the lowest values of nitrate reduction and is thought to be a correct measure of the rate of nitrate reduction in unmolested tissue. In the anaerobic intact-tissue assay, greater rates of nitrate reduction are observed, but enzyme activity still falls short of that observed in cell-free extracts. However, addition of ethanol to the anaerobic assay medium causes the rate of nitrate release to exceed nitrite production in cell-free extracts, and is believed to most closely represent total enzyme levels.

Decay Kinetics of Nitrate Reductase

Induction and decay of nitrate reductase activity has been determined using the anaerobic intact-tissue assay to measure enzyme activity (Figure 5). The assay was performed in the presence of 5 per cent ethanol. Only a brief exposure to 0.05 M KNO_3 is required to induce enzyme activity. The rate of nitrate release begins to decrease 3 hours after nitrate treatment, but there is still measurable nitrate reductase activity after 22 hours. The enzyme activity

Figure 4.--Nitrate reductase induction kinetics as determined by three different assay methods. Tissue was induced by method 1 as described in Materials and Methods for the indicated times. $KN^{16}O_3$ was substituted for $KN^{18}O_3$ in all but the isotope assay.

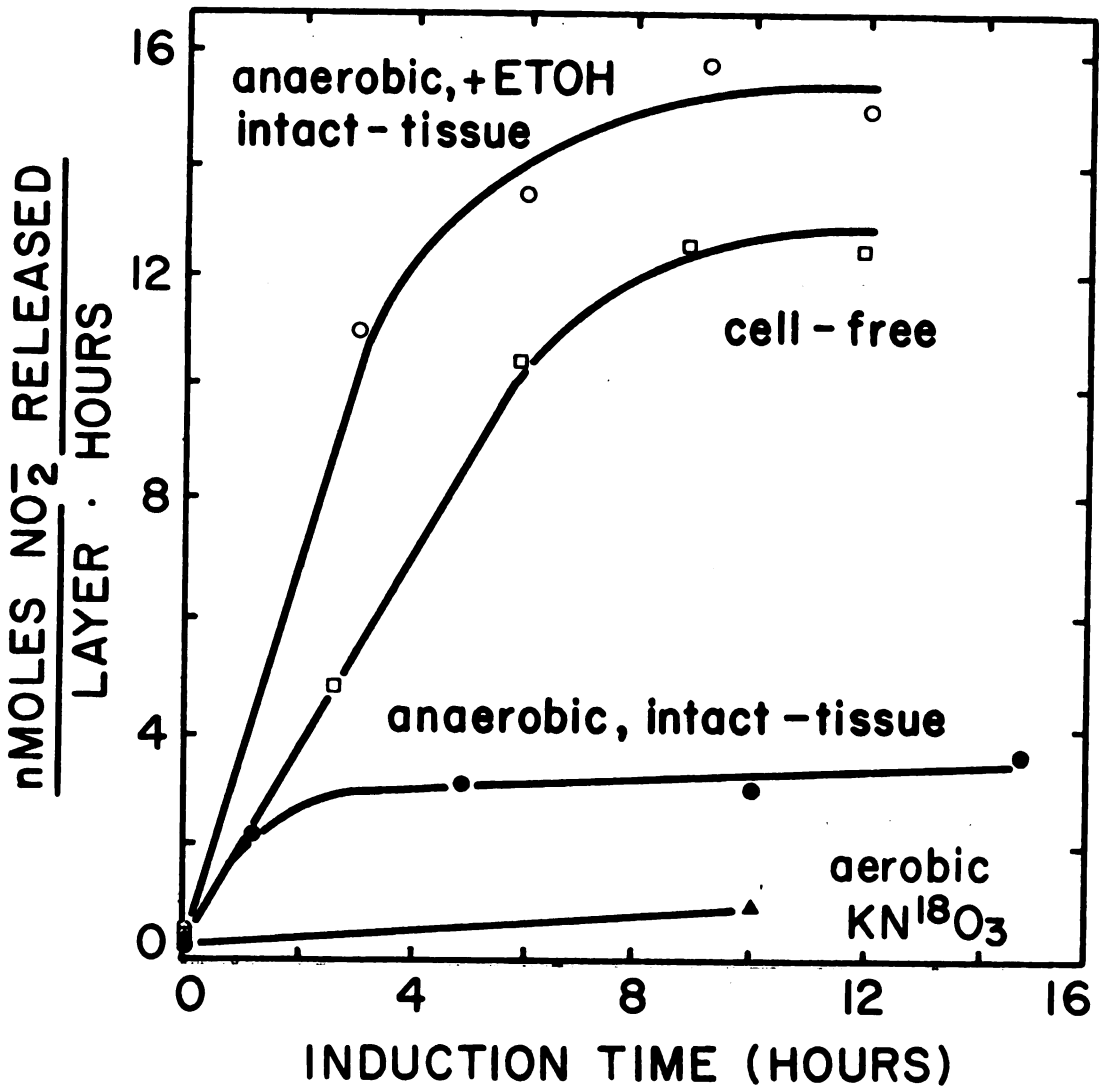
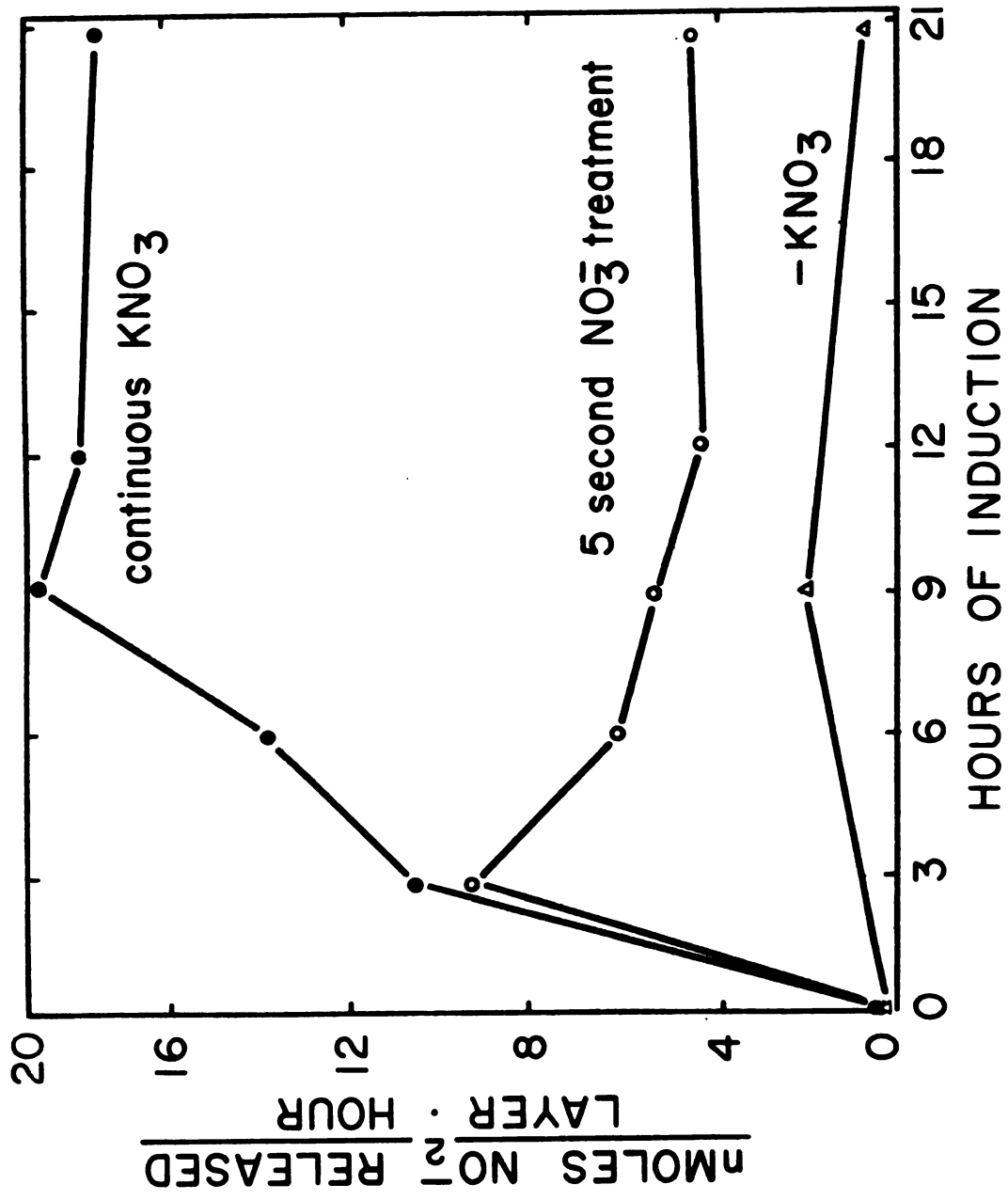

anaerobic, + ETOH
intact - tissue
cell - free
anaerobic, intact - tissue
aerobic
KN18O3
nMOLES NO2 RELEASED / LAYER · HOURS
INDUCTION TIME (HOURS)
0
4
8
12
16

Figure 5.--Decay kinetics of nitrate reductase. In the 5-second nitrate treatment, 5 ml of 0.05 M KNO_3 was poured over aleurone layers as they were held in a forceps. This treatment was followed immediately by a wash with 40-50 ml of sterile distilled water. The tissue was placed in an additional 40-51 ml water and rinsed once again. The tissue was finally added to 5 ml of sterile distilled water containing two drops of chloramphenicol (0.5 mg/ml). Control tissue was incubated in 5 ml of 0.05 M KNO_3. Throughout the experiment, tissue samples were removed at various time intervals for the assay of nitrate reductase activity.


continuous KNO3
5 second NO3 treatment
-KNO3
HOURS OF INDUCTION
nMOLES NO2 RELEASED / LAYER · HOUR
0
4
8
12
16
20
0
3
6
9
12
15
18
21

induced as described in Figure 5 was inhibited 90 per cent by cycloheximide (10 ug/ml) and 20 per cent by 6-methylpurine (2×10^{-3} M) after three-hours induction (both chemicals were present throughout the three-hour incubation period). These kinetic and inhibitor data provide evidence that the ethanol affect is physiological and not a nonenzymatic reduction of nitrate. This experiment also demonstrates the usefullness of the anaerobic intact-tissue assay as a convenient and rapid measure of nitrate reductase activity in plant tissue.

DISCUSSION

A comparison of nitrate reduction rates in the cell-free and in the $KN^{18}O_3$ *in vivo* assay methods indicates that only a small fraction of the total enzyme activity induced in response to nitrate is functioning in the tissue. The low *in vivo* activity is not due to an isotope effect since nitrate reductase induced and assayed in either $KN^{18}O_3$ or $KN^{16}O_3$ (Table 6) showed similar rates of nitrate reduction in the anaerobic intact-tissue assay.

The dependence of the anaerobic intact-tissue assay upon phosphate, and the effect of ethanol, suggests that these compounds may, under certain conditions, be involved in the *in vivo* controls of nitrate reduction. For example, phosphate might act indirectly by overcoming the limitation of some phosphate-dependent step in the generation of NADH (e.g., in the oxidation of 3-phosphoglyceraldehyde), or it might act directly with the enzyme. Phosphate has been reported to double the activity of FADH-dependent nitrate reductase isolated from *Neurospora* (6).

Ethanol may act by indirectly or directly providing reductant for nitrate reduction. Ethanol and several other oxidizable substances have been reported to stimulate nitrate reduction in crude extracts of potato (7). Reductants

Table 6.--Effect of $KN^{18}O_3$ and $KN^{16}O_3$ on nitrate reductase.

Tissue was induced in $KN^{18}O_3$ or $KN^{16}O_3$ and was assayed in $KN^{18}O_3$ or $KN^{16}O_3$, respectively. Enzyme activity was measured with the anaerobic intact-tissue assay. Nitrate reductase was induced by method 1 as described in Materials and Methods. Numbers represent an average of two experiments.

Treatment	Nitrate Reductase Activity (nmoles NO_2^- formed/layer.hr)
Induced and assayed in:	
$KN^{18}O_3$	2.4
$KN^{16}O_3$	2.6

generated from aldehydes by isolated potato aldehyde oxidase are capable of reducing nitrate to nitrite (8). Klepper and Hageman (9) reported stimulation of nitrate reduction by 3-phosphoglyceraldehyde in leaf disks in an *in vivo* assay similar to the anaerobic intact-tissue assay described in this paper.

The enhancement of nitrate reduction by HOQNO and by antimycin A under aerobic condition suggests that the low activity of nitrate reductase observed in the $KN^{18}O_3$ assay, may be the result of oxygen (via electron transport) competing with the enzyme for reducing equivalents. The fact that antimycin A and HOQNO inhibit oxygen uptake at a site between cytochromes b and c (10), taken together with their failure to affect the anaerobic reduction of nitrate, argues against the participation of cytochrome c in anaerobic nitrate reduction in this system.

LITERATURE CITED

1. Nason, A. 1956. Enzymatic steps in the assimilation of nitrate and nitrite in fungi and green plants. In A Symposium on Inorganic Nitrogen Metabolism, ed. W. D. McElroy and B. Glass, (Baltimore: The Johns Hopkins Press) pp. 109-136.

2. Kumada, H. 1953. The nitrate utilization in seed embryos of Vigna sesquipedalis. J. Biochem. 40:439-450.

3. Ferrari, T. E. and J. E. Varner. 1969. Substrate induction of nitrate reductase in barley aleurone layers. Plant Physiol. 444:85-88.

4. Chrispeels, M. J. and J. E. Varner. 1967. Gibberellic acid-enhanced synthesis and release of alpha-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42:398-406.

5. Taniguchi, S., R. Sato and F. Egami. 1956. The enzymatic mechanisms of nitrate and nitrite metabolism. In A Symposium on Inorganic Nitrogen Metabolism, ed. W. D. McElory and B. Glass, (Baltimore: The Johns Hopkins Press) pp. 87-108.

6. Kinsky, S. C. and W. D. McElroy. 1958. Neurospora nitrate reductase: the role of phosphate, flavin and cytochrome c reductase. Arch. Biochem. Biophys. 73:466-483.

7. Kastle, J. H. and E. Eluove. 1904. On the reduction of nitrates by certain plant extracts and metals, and the accelerating effect of certain substances on the progress of the reduction. Amer. Chem. J. 31:606-641.

8. Bernheim, F. 1928. The aldehyde oxidase of the potato. Biochem. J. 22:344-352.

9. Klepper, L. and R. H. Hageman. 1969. Dependence of nitrate reduction on generation of reduced nicotinamide adenine dinucleotide by glycolytic metabolism of leaf tissue. XI International Botanical Congress, Abstracts. p. 112.

10. Hackett, D. P. 1964. Enzymes of terminal respiration. In Modern Methods of Plant Analysis, ed. H. F. Linskens, B. D. Sanwal, M. V. Tracy, (Berlin: Springer-Verlag) vol. 7. pp. 654-655.

SECTION III

ABSTRACT

NITRITE REDUCTION IN BARLEY ALEURONE LAYERS

By

Thomas E. Ferrari

A method has been devised for the detection and measurement of nitrite reductase in intact barley (Hordeum vulgare L. cv. Himalaya) aleurone layers. The technique involves feeding aleurone layers nitrite and measuring nitrite disappearance after a given time period. The method also allows simultaneous determination of nitrite uptake by the tissue. Quantitative recovery of nitrite is obtained by rapid heating of tissue in the presence of dimethyl sulfoxide.

Using the procedure described, nitrite reductase activity in intact barley aleurone layers was determined. Enzyme activity was increased by incubation with nitrate, but considerable activity was present in noninduced tissue. Nitrate-induced activity was inhibited by cycloheximide but not by actinomycin D. Enzyme activity in induced layers was inhibited by 2,4-dinitrophenol, antimycin A, 2-n-heptyl-4-hydroxy-quinoline N-oxide, and anaerobiosis. Activity in

noninduced tissue appeared to be less sensitive to the above respiratory inhibitors; however, both activities were inhibited greater than 90 per cent by anaerobiosis. Nitrite uptake was relatively insensitive to the inhibitors tested.

Nitrite uptake was rapid at pH 4.5 and negligible at pH 7.5. Nitrite accumulated at pH 4.5 anaerobically, was rapidly released when transferred to media at pH 7.5--the pH of the anaerobic intact-tissue assay for nitrate reductase. Accumulated nitrite was released by the tissue whether held under anaerobic or aerobic conditions.

Nitrate-induced and noninduced aleurone layers were able to reduce low levels of nitrite (25 uM) anaerobically. But these rates (5 to 10 nmoles/layer/hour) were considerably lower than rates (25 nmoles/layer/hour) of nitrite production observed during the nitrate reductase anaerobic intact-tissue assay (with ethanol present).

CONTROL OF NITRITE REDUCTION IN BARLEY ALEURONE LAYERS

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The reduction of nitrate by intact plant tissues and the release of the reduced product nitrite, occurs either under anaerobic conditions (4, 7, 10), or aerobically in the presence of respiratory inhibitors (4). Why nitrite leaks from the tissue is not known. Either the rate of nitrate reduction may exceed nitrite reduction, or nitrite reduction may not proceed under conditions in which nitrite release occurs. In either case nitrite might accumulate and leak from the tissue, thereby causing the observed release phenomenon.

To test these possibilities requires that nitrite reductase activity be measured in the intact tissue and under conditions similar to those in which nitrite release occurs. This report describes a method for the detection and measurement of nitrite reductase activity in intact tissue, and the effects of conditions favoring nitrite release on the

enzyme's activity. The technique involves administering nitrite to aleurone layers and measuring nitrite disappearance. The method also allows simultaneous determination of nitrite uptake by the tissue.

MATERIALS AND METHODS

Nitrite reductase activity was measured after incubation of 50 isolated aleurone layers at 23° with chloramphenicol (50 ug) and 5 ml of sterilized 0.05 M KNO_3. Erlenmeyer flasks (50 ml) containing the tissue and medium were stoppered with cotton plugs and placed in a metabolic water-bath shaker at 200 rpm. After 6 to 12 hours of incubation the tissue was rinsed with approximately 50 ml of distilled water, and the effect of various conditions on enzyme activity determined.

Unless otherwise indicated, nitrite reductase activity was assayed by measuring nitrite disappearance after placing 10 aleurone layers in 2 ml of media containing 0.1 M KH_2PO_4, chloromycetin (20 ug), nitrite and the treatment solution as indicated in the text. Nitrite (final $NaNO_2$, 2.5×10^{-4} M) was added to start the assay. Two 0.1 ml aliquots were immediately removed to provide a measure of the initial nitrite concentration of the media. After 40 minutes, two 0.1 ml aliquots were removed for nitrite determination. The difference between the final and initial nitrite concentration of the medium indicates the amount of nitrite taken up by the tissue. Dimethyl sulfoxide (DMSO) was then added to the medium (DMSO final

concentration = 50 per cent) and the 25 ml Erlenmeyer flask and its contents placed on a hot plate until the medium boiled (about 20 to 30 seconds). Under these conditions the nitrite of the tissue rapidly leaks back into the medium. After cooling, two 0.2 ml aliquots were then removed for nitrite determination. The difference between this nitrite concentration and the initial concentration measures the amount of nitrite reduced.

Nitrite was determined colorimetrically by the Griess-Ilosvay method (11) after adding 0.3 ml each of 1 per cent sulfanilamide in 3 N HCL and 0.02 per cent N-1-naphthylethylenediamine dihydrochloride. Optical density at 540 nm was determined after centrifugation at 2000 x g for 10 minutes.

Results

Properties of Nitrite Uptake and Disappearance

Figure 1 shows the effect of pH on nitrite uptake by aleurone layers. Uptake decreased with increasing pH up to pH 7.5, after which little or no depletion of nitrite from the media occurred. Nitrite uptake was rapid, and not affected by prior incubation of tissue in nitrate (Figure 2). Within 20 minutes, approximately 50 per cent of the added nitrite had been taken up by the tissue at either of two nitrite concentrations.

Figure 1.--Effect of pH on nitrite uptake. Nitrite uptake by 10 aleurone layers was determined over a 90 minute period in 4 ml of medium containing 1.25 mM sodium nitrite, 0.1 M phosphate buffer at the indicated pH and 20 µg chloromycetin.

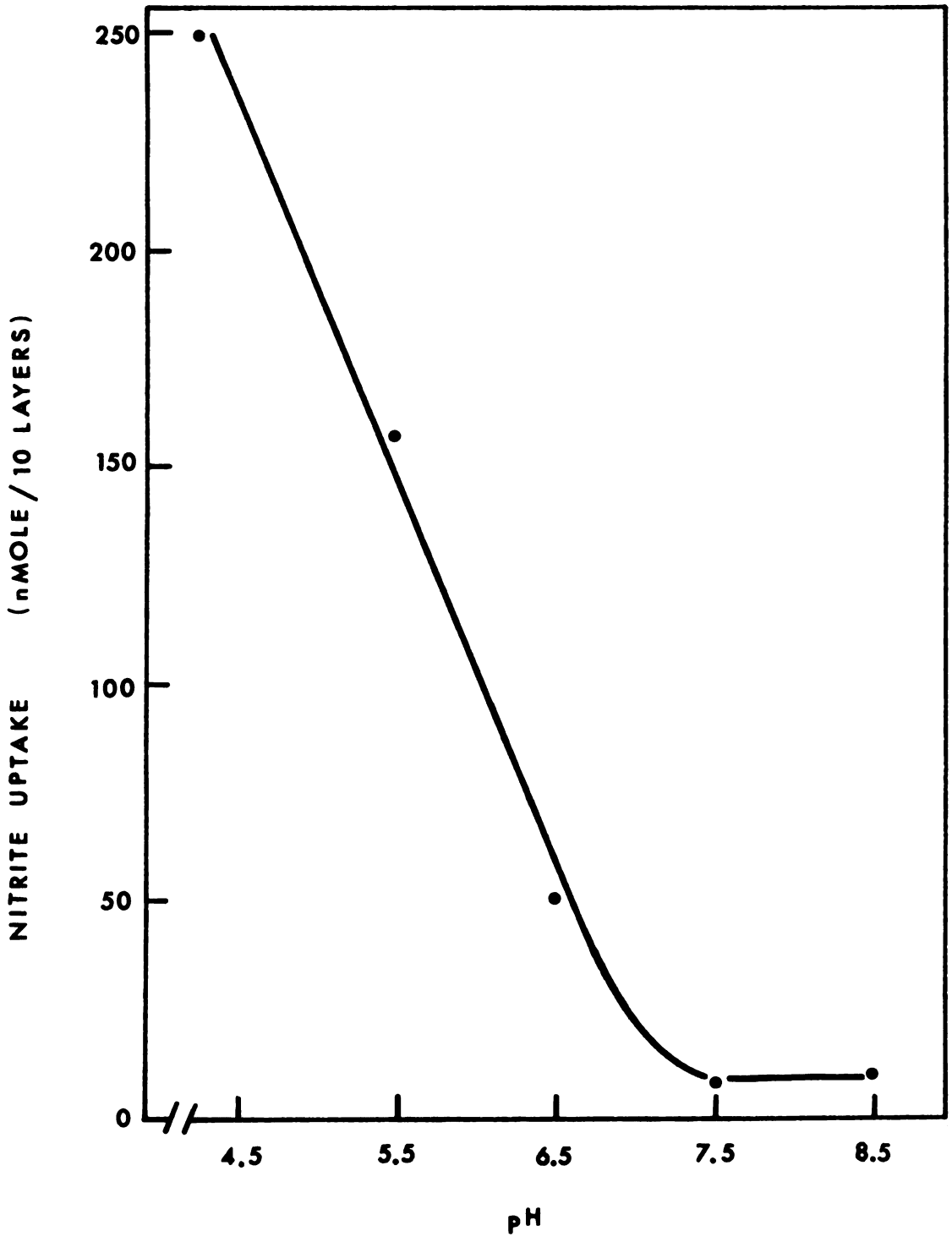
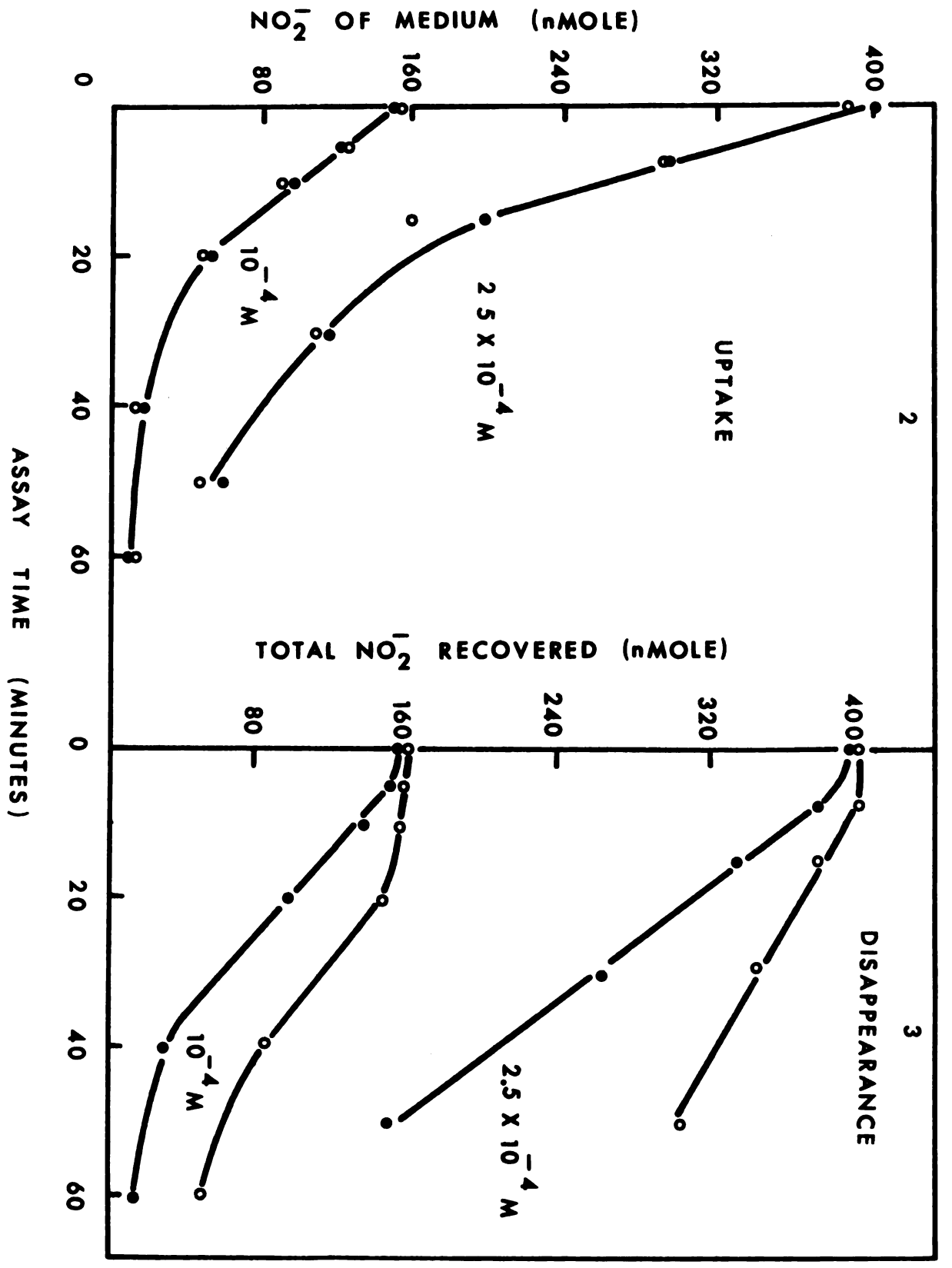

250
200
150
100
50
0
NITRITE UPTAKE (nMOLE / 10 LAYERS)
4.5
5.5
6.5
7.5
8.5
pH

Figure 2.--Kinetics of nitrite uptake. Prior to determination of nitrite uptake, tissue was incubated 12 hours in the presence or absence of 0.05 M potassium nitrate. Uptake was measured with 10 aleurone layers incubated in 2 ml of medium containing 0.25 mM sodium nitrite and 0.1 M phosphate buffer, pH 4.5. Open and closed circles represent noninduced and nitrate-induced tissues, respectively.

Figure 3.--Kinetics of nitrite disappearance. Assay conditions were as described in Figure 2. Nitrite disappearance was measured as described in Materials and Methods.



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Assay
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As measured by nitrite disappearance, nitrite reductase activity was increased by prior incubation with nitrate, but considerable activity was present in noninduced tissue (Figure 3). The temperature coefficient (Q_{10}) of nitrite uptake was approximately 1.4 compared to 2.6 and 2.4 for nitrite disappearance in induced and noninduced tissue, respectively (Table 1).

The increase in nitrite reductase activity by incubation with nitrate, was completely inhibited by cycloheximide and enhanced slightly by actinomycin D (Table 2). Nitrite disappearance by noninduced tissue was not affected by either inhibitor (Table 2).

Enzyme activity in induced layers was inhibited by 2,4-dinitrophenol (DNP) (Figure 4), antimycin A, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) and anaerobiosis (Table 3). Activity in uninduced tissue was insensitive to antimycin A and HOQNO (Table 3), and sensitive to anaerobiosis. Nitrite uptake was relatively unaffected by the inhibitors (Table 4, Figure 5).

Nitrite Release by Aleurone Layers

The following experiments were performed to determine if aleurone layers are able to retain nitrite at pH 7.5 under anaerobiosis--conditions under which nitrite release occurs during anaerobic nitrate reduction (4). Since no nitrite is taken up by the tissue at pH 7.5 (Figure 1)--the

Figure 4 and 5.--Effect of 2,4-dinitrophenol on nitrite uptake and disappearance. Tissue was incubated 13 hours in the presence or absence of 0.05 M potassium nitrate and 10 μg/ml chloromycetin. Uptake and disappearance of nitrite was determined for 10 aleurone layers incubated 40 minutes in 2 ml of media containing 2,4-dinitrophenol at the concentration indicated, 0.25 mM sodium nitrite, 0.1 M phosphate buffer (pH 4.5), and 20 μg chloromycetin.

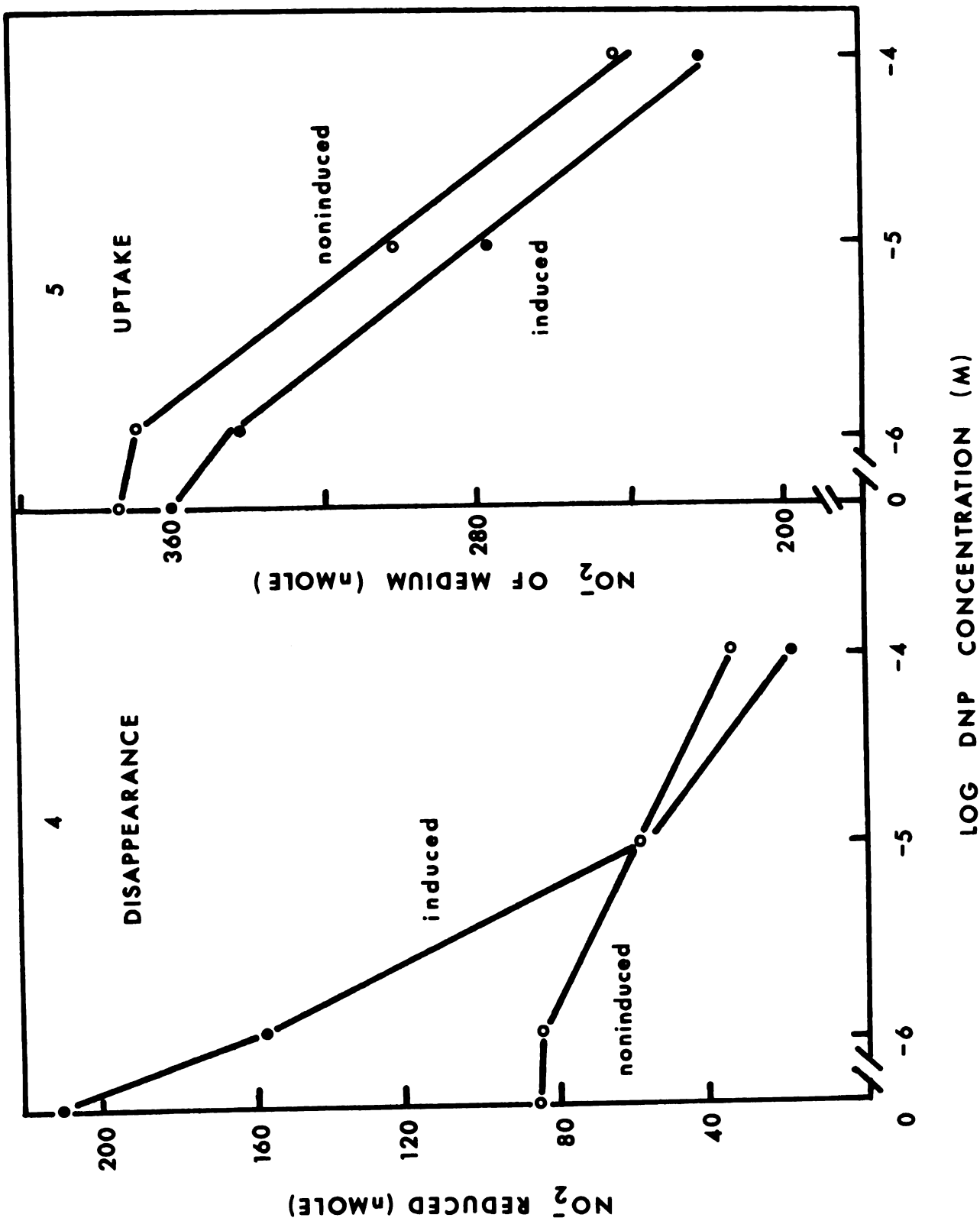
4
DISAPPEARANCE
induced
noninduced
200
160
120
80
40
0
-6
-5
-4
5
UPTAKE
noninduced
induced
360
280
200
0
-6
-5
-4
LOG DNP CONCENTRATION (M)
NO2- REDUCED (nMOLE)
NO2- OF MEDIUM (nMOLE)

Table 1.--Temperature coefficients for uptake and disappearance of nitrite by nitrate-induced and noninduced tissue.

After 6 hours induction with nitrate, nitrite uptake and disappearance was measured over a 20 and 40 minute period, respectively. Temperature coefficients were determined over a 15 to 35° range.

± nitrate induction	Q_{10} for uptake	Q_{10} for disappearance
+	1.4	2.6
-	1.5	2.4

Table 2.--Effect of protein synthesis inhibitors on the uptake and disappearance of nitrite in noninduced and nitrate-induced aleurone layers.

Tissue incubated 7 hours in the presence or absence of 0.05 M potassium nitrate, cycloheximide (10 ug/ml) and actinomycin D (10 ug/ml). All treatments contained 20 ug chloromycetin.

	Disappearance		Uptake	
$\pm$ NO_3^- Induction	+	-	+	-
	(nmoles/40 min./10 layers)			
Control	170	68	344	388
Cycloheximide (10 ug/ml)	66	66	322	311
Actinomycin D (10 ug/ml)	207	65	336	312

Table 3.--Effect of respiratory inhibitors on the disappearance of nitrite by aleurone layers.

Tissue was induced in the presence or absence of 0.05 M potassium nitrate. After 12 hours of incubation, tissue was placed in media containing phosphate buffer, 0.1 M (pH 7.5), and the inhibitor being tested. Ethanol (final concentration = 5 per cent) was used as solvent for the inhibitors. After 30 minutes incubation at 23°, the media containing the inhibitors was discarded and the tissue rinsed 2 times with 4 ml of 0.1 M potassium phosphate (monobasic). Nitrite uptake by the tissue over a 20 minute period was then determined as described in Materials and Methods.

Treatment	Nitrite disappearance			
	Total noninduced	% of control	Induced Activity (Total Induced - noninduced)	% of control
	(nmoles/10 layers/40 min.)			
Control	56	100	80	100
HOQNO (0.25 mM)	54	97	38	48
Antimycin A (0.5 mM)	49	88	46	58
Nitrogen Atm.	6	11	0	0

Table 4.--Effect of respiratory inhibitirs on the uptake of nitrite by aleurone layers.

Experimental procedure as described in Table 3.

Treatment	Nitrite Uptake			
	Induced	% of control	Noninduced	% of control
	(nmoles/10 aleurone layers/20 min)			
Control	157	100	194	100
HOQNO (0.25 mM)	125	80	129	67
Antimycin A (0.5 mM)	117	81	148	76
N_2 Atm.	147	94	145	75

pH of the anaerobic nitrate reduction medium--tissue was first preloaded with nitrite at pH 4.5 and under anaerobic conditions to prevent its reduction. Nitrite of the media at the start of the preloading period, after 40 minutes, and after DMSO extraction, are shown in Table 5. No nitrite was lost during the 40-minute uptake period. When tissue was subsequently washed and placed in nitrite-less media at pH 7.5, nitrite leaked from the tissue whether incubated an additional 40 minutes under aerobic or anaerobic conditions (Table 6). The ability of tissue to reduce some nitrite during the pH 7.5 incubation, is indicated by the failure to recover at 40 minutes all of the nitrite present at the start of the pH 7.5 incubation. The kinetics of nitrite release and disappearance under aerobic conditions are indicated in Figure 6. Approximately 50 per cent of the nitrite accumulated in the tissue during incubation with nitrite at pH 4.5 leaked into the medium during the first 5 minutes of incubation at pH 7.5.

Anaerobic Nitrite Dissppearance at Low Nitrite Concentrations

Nitrite was found to disappear under anaerobic conditions when noninduced tissue was incubated in 25 μM nitrite (Table 7)--a ten fold lower concentration than what was used in previous experiments. DNP partially inhibited this disappearance (Table 7). On the other hand, nitrate-induced tissue showed no apparent ability to reduce nitrite when

Table 5.--Nitrite accumulation by aleurone layers at pH 4.5.

Tissue was incubated in the presence or absence of 0.05 M potassium nitrate for 12 hours. Tissue was then incubated in a nitrogen atmosphere for 40 minutes in 2 ml of medium containing 0.1 M phosphate buffer (pH 4.5), 0.25 mM sodium nitrite, and 20 ug chloromycetin. Nitrite of the medium and tissue was determined as described in Materials and Methods.

	Nitrite of media			Nitrite
			after DMSO extraction	Of tissue*
Assay time (min.)	0	40	40	40
± KNO_3 induction	(nmole/2 ml)			(nmole/ 10 layers)
+	429	139	452	313
-	436	135	427	292

* Nitrite of tissue = nitrite of media after DMSO extraction minus nitrite of media prior to DMSO extraction.

Table 6.--Release of accumulated nitrite under aerobic and anaerobic conditions at pH 7.5.

Nitrite of tissue at 40 minutes was taken from table 4. Experimental procedure was as described in Figure 6.

	Nitrite of tissue (nmoles/10 layers)			Nitrite of media (nmoles/2 ml)			Nitrite disappearance* (nmoles/10 layers)	
Assay time (min.)	40	80		40	80		80	
		air	nitrogen		air	nitrogen	air	nitrogen
induced	313	24	60	5	204	220	90	38
noninduced	292	17	65	6	207	215	74	18

*Nitrite disappearance = total (media and tissue) nitrite at time 40 minus total nitrite at time 80.

Figure 6.--Release of accumulated nitrite by aleurone layers. Tissue was induced in the presence or absence of 0.05 M potassium nitrate for 12 hours. Tissue was then preloaded with nitrite by incubation in a nitrogen atmosphere for 40 minutes and in 2 ml of medium containing 0.1 M potassium phosphate (monobasic), 0.25 mM sodium nitrite, and 20 µg chloromycetin. Tissue was next rinsed 3 times with 10 ml cold phosphate buffer (pH 7.5), 0.1 M, and placed in 2 ml of nitrite-less media containing 0.1 M phosphate buffer (pH 7.5), and 20 µg chloromycetin. Nitrite of the medium and tissue was determined at the times indicated, as described in Materials and Methods. o = noninduced tissue. ● = nitrate induced tissue.

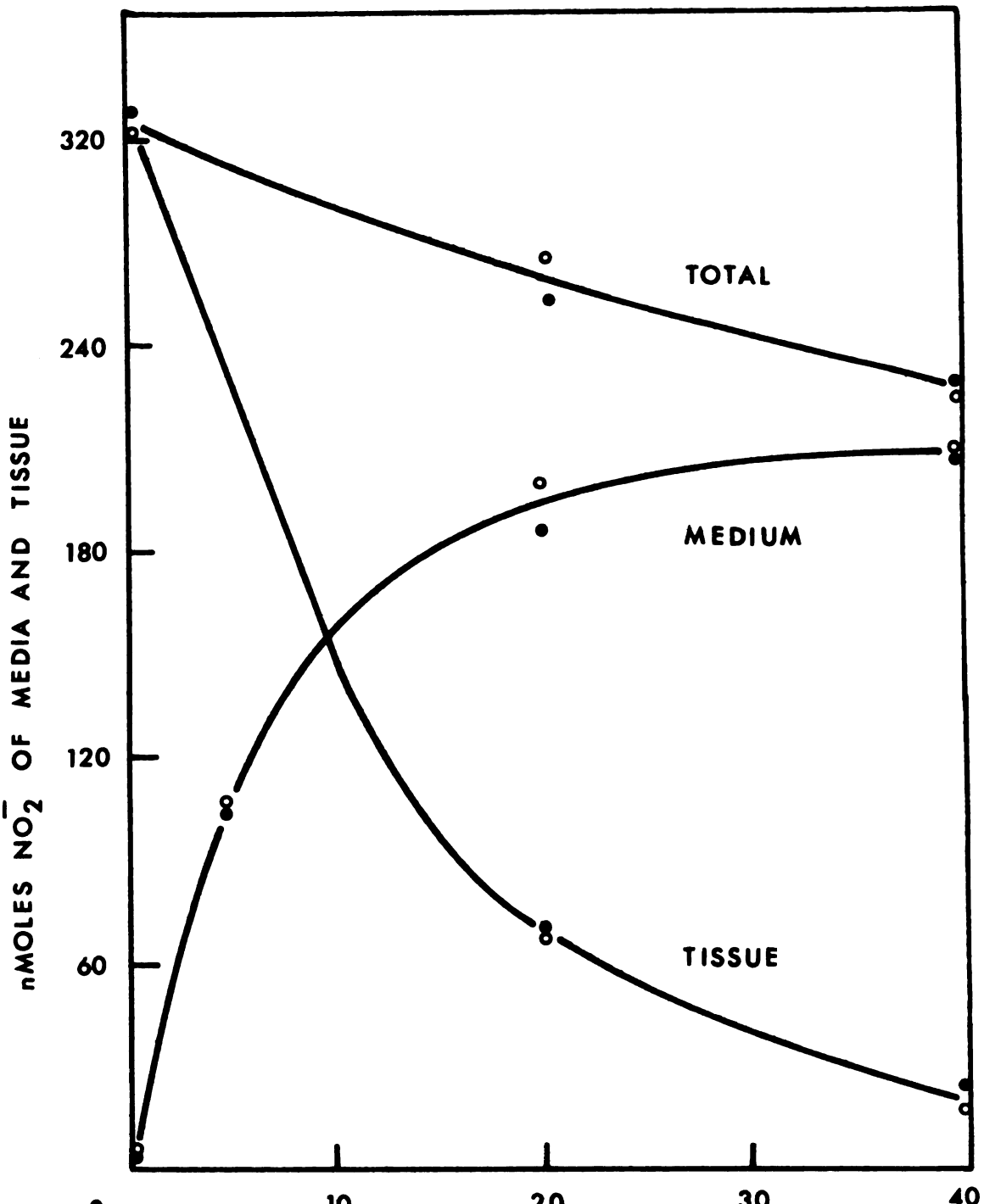
320
240
180
120
60
0
10
20
30
40
TOTAL
MEDIUM
TISSUE
nMOLES NO_2^- OF MEDIA AND TISSUE
MINUTES INCUBATION AT pH 7.5

Table 7.--Nitrite production and disappearance by nitrate-induced and noninduced tissue assayed in the presence or absence of low nitrite concentration.

After incubation in the presence of potassium nitrate for 9 hours, tissue was rinsed 3 times with approximately 50 ml of distilled water. Ten aleurone layers were then incubated in 2 ml of media containing ± sodium nitrite (25 uM), phosphate buffer (0.1 M, monobasic) and 20 umoles of chloromycetin. Tissue was added to the incubation media just prior to deairation for 1 minute with nitrogen. DNP = 0.1 mM.

	Nitrite of medium			Nitrite of medium		
			after DMSO extraction			after DMSO extraction
Assay time (min.)	0	40	40	0	40	40
	(nmole/2 ml)					
Assay Condition	noninduced tissue			induced tissue		
+ nitrite	44	6	10	42	13	45
+ nitrite + DNP	41	11	21	41	28	112
- nitrite	0	2	2	1	5	21
- nitrite + DNP	0	2	3	2	18	74

incubated with low levels of nitrite (Table 7). When DNP was included in the assay media, nearly 2.5 times more nitrite was found at the end of the assay than was present at the start (Table 7). When tissue was assayed in the absence of nitrite, 20 nmoles of nitrite were produced by nitrate-induced tissue (Table 7). Again, DNP increased considerably (3.5 fold) the amount of nitrite accumulated by nitrate-induced tissue. Little or no nitrite is present in induced tissue at the start of the assay. Thus, it appeared that nitrite was being produced from the anaerobic reduction of nitrate which had accumulated during the nitrate incubation phase of the experiment. Therefore, it appears that nitrite disappearance occurred in induced tissue as well as noninduced tissue, and reduction was inhibited by DNP.

Effect of PCP and Ioxynil on the Uptake and Disappearance of Nitrite

Two other uncouplers of oxidative phosphorylation, pentachlorophenol (PCP) (8) and 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) (3), were tested for their ability to inhibit nitrite disappearance in aleurone layers. While causing only relatively slight inhibitions of uptake, both PCP (10^{-3} M) and ioxynil (10^{-4} M) strongly inhibited nitrite disappearance in induced and noninduced tissue (Table 8).

Table 8.--Effect of pentachlorophenol and ioxynil on nitrite uptake and disappearance by nitrate-induced and noninduced aleurone layers.

Tissue was incubated 9 hours in the presence or absence of 0.05 M potassium nitrate and 10 ug/ml chloromycetin. Tissue was then rinsed 3 times with approximately 50 ml of distilled water and placed in 2 ml of media containing 0.25 mM sodium nitrate, 0.1 M potassium phosphate (monobasic) and the inhibitor being tested.*

	Nitrite uptake		Nitrite disappearance	
	induced	noninduced	induced	noninduced
	(per cent of control)			
Control	100	100	100	100
Pentachlorophenol 10^{-3} M	89	83	23	55
Pentachlorophenol 10^{-4} M	88	85	61	65
Ioxynil 10^{-4} M	74	75	15	34
Ioxynil 10^{-5} M	84	87	73	69

* All treatments contained inhibitor dissolved in acetone (Final concentration = 1 per cent). Acetone added to control.

Effect of DNP on Nitrate Reduction

The effect of DNP on the anaerobic intact-tissue assay (4) for nitrate reductase is shown in Table 9. When the assay was done in the absence of ethanol, DNP increased nitrite recovery by 65 per cent; however, DNP had little or no effect on ethanol-enhanced nitrite production.

Table 9.--Effect of DNP on nitrate reduction.

After 4 hours induction with nitrate, tissue was placed in 2 ml of media containing 0.1 M phosphate buffer (pH 7.5), 0.1 M potassium nitrate and nitrogen atmosphere. Nitrite of media at 30 minutes (after DMSO extraction) was determined as described in Materials and Methods. DNP = 0.1 mM. Ethanol = 5 per cent.

Assay condition	nmoles nitrite produced/layer.hour
- ethanol - DNP	4.0
- ethanol + DNP	6.6
+ ethanol - DNP	25.6
+ ethanol + DNP	27.0

Discussion

When nitrate-induced tissue is incubated anaerobically in the presence of nitrate, nitrite leaks from the tissue (4, 7, 10). Nitrite production and release was enhanced considerably by ethanol (4). The failure of 2,4-dinitrophenol (DNP)--an inhibitor of nitrite reduction in higher and lower plants (1, 6, 12)--to further increase nitrite production in the presence of ethanol (Table 9), indicated that the inhibition of nitrite reduction by anaerobiosis was complete. However, DNP did increase nitrite accumulation in tissue assayed in the absence of ethanol (Table 9). This additional nitrite accumulated may represent inhibition of the tissue's ability to reduce low levels of nitrite (Table 7) under anaerobic conditions.

The failure to observe nitrite disappearance at high levels of nitrite (250 uM) might be due to the accumulation of toxic levels of nitrite within the cell. Assuming 0.01 ml of water and 30 umoles of nitrite accumulated (Table 5) per layer, the concentration of nitrite within an aleurone layer would be approximately 3 mM. Such concentrations are toxic to Anabaena cylindrica (2) and cultured tobacco cells (5). Although induced and noninduced aleurone layers were able to reduce low levels of nitrite (25 uM) anaerobically, these rates (5 to 10 nmoles/layer/hour) are considerably lower than rates of nitrite production from

nitrate under anaerobic conditions in the presence of ethanol (25 nmoles/layer/hour). Thus, it appears nitrite cannot be reduced fast enough, and because nitrite cannot be retained by the tissue (Table 6) it leaks into the medium.

The DNP-inhibited reduction of nitrite under aerobic conditions in aleurone layers, and in intact cells of Chlorella (6) and Ankistrodesmus (1), suggests that nitrite reduction is dependent on oxidative phosphorylation. The ability to inhibit nitrite disappearance by other uncouplers of oxidative phosphorylation (Table 8) (1, 6)--with and without $-NO_2$ functional groups--argues against the possibility that DNP is alternatively accepting reducing equivalents, as has been shown to be the case with isolates of Pseudomonas denitrificans (9) and reductants generated by spinach chloroplasts (13).

Evidence that nitrite disappearance in both induced and noninduced tissue is enzymatically mediated, is indicated by their temperature coefficients, and sensitivity to anaerobiosis and uncouplers of oxidative phosphorylation. The ability to inhibit nitrate-induced nitrite reductase activity with cycloheximide argues for the requirement for protein synthesis in the induced system.

The differential sensitivity of induced and non-induced nitrite disappearance to all inhibitors tested,

suggests that there might be two enzymes responsible for nitrite reduction, or that one is present in different cellular compartments.

LITERATURE CITED

1. Ahmad, J. and I. Morris. 1968. The effects of 2,4-dinitrophenol and other uncoupling agents on the assimilation of nitrite by chlorella. Biochim. Biophys. Acta 162:32-38.

2. Brownell, P. F. and D. J. D. Nicholas. 1967. Some effects of sodium on nitrate assimilation and N_2 fixation in Anabaena cylindrica. Plant Physiol. 42:915-921.

3. Ferrari, T. E. and D. E. Moreland. 1969. Effects of 3,5-dihalogenated-4-hydroxybenzonitriles on the activity of mitochondria from white potato tubers. Plant Physiol. 44:429-434.

4. Ferrari, T. E. and J. E. Varner. 1970. Control of nitrate reductase activity in barley aleurone layers. PNAS. 65:729-736.

5. Kelker, H. Ph.D research. MSU/AEC Plant Research Lab, Michigan State University. Personal Communication.

6. Kessler, E. and W. Bucher. 1960. Über die Wirking von Arsenate auf Nitratreduktion, Atmung und Photosynthese von Grünalgen. Planta (Berl.) 55:512-524.

7. Kumada, H. 1953. The nitrate utilization in seed embroys of Vigna sesquipedalis. J. Biochem. 40: 439-450.

8. Moreland, D. E. 1967. Mechanisms of action of herbicides. Ann. Rev. Plant Phys. 18:365-386.

9. Radcliff, B. C. and D. J. D. Nicholas. 1968. Some properties of nitrite reductase from Pseudomonas denitrificans. Biochim. Biophys. Acta 153:545-554.

10. Randall, P. J. 1969. Changes in nitrate and nitrite reductase levels on restoration of molybdenum to molybdenum-deficient plants. Aust. J. Agr. Res. 20:635-642.

11. Snell, F. D. and C. T. Snell. Colorimetric Methods of Analysis. Vol. II. Van Nostrand, Princeton, third edition. 1949. p. 804.

12. Voskresenskaya, N. P. and G. S. Grishina. 1962. The significance of light for nitrite resuction in the green leaf. Fiziol. Rast. 9:7-15. (Translated in Soviet Plant Physiol. 1962. 9:4-10.)

13. Wessels, J. S. C. 1965. Mechanism of the reduction of organic nitro compounds by chloroplasts. Biochim. Biophys. Acta 109:357-371.

DISSERTATION SUMMARY

Nitrate induced the formation of nitrate reductase activity as measured in cell-free extracts of barley. (Hordeum vulgare L. cv. Himilaya) aleurone layers. No nitrate reductase activity was detected in dry half-seeds or in aleurone layers after imbibition or incubation in the absence of inducer. Induction of nitrate reductase activity was not affected by abscisic acid or 8-azaguanine. Actinomycin D enhanced rather than inhibited enzyme activity; however, 6-methylpurine and cycloheximide were inhibitory.

In addition to determining activity in cell-free extracts, nitrate reductase activity was also measured in the intact tissue using two different methods. The first method measured the rate of appearance of $H_2{}^{18}O$ produced during the reduction of $KN^{18}O_3$. The second assay measured released nitrite resulting from nitrate reduction under anaerobic conditions. After 10 hours of nitrate induction, nitrate reductase activities measured by the $KN^{18}O_3$ assay were only one-sixth, and those measured by the anaerobic intact-tissue assay were one-third, of those observed in cell-free extracts of aleurone layers.

Nitrite production in the anaerobic intact-tissue assay system was inhibited by oxygen and increased by

ethanol and bisulfite. Oxygen-inhibited nitrite production was reversed by 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO) or antimycin A--two inhibitors of mitochondrial electron transport. Neither of these inhibitors affected the anaerobic reduction of nitrate.

A method was devised for the detection and measurement of nitrite reductase activity in aleurone layers. The technique involved administering nitrite to aleurone layers and measuring nitrite disappearance after a given time period. The method also allowed simultaneous measurement of nitrite uptake. Recovery of nitrite from tissue was obtained by rapid heating of the medium plus tissue in the presence of dimethyl sulfoxide.

Using the above procedure, nitrite reductase activity was found to be increased by prior incubation of tissue with nitrate, but considerable activity was present in noninduced tissue. The temperature coefficient (Q_{10}) of nitrite uptake was approximately 1.4, compared to 2.6 and 2.4 for nitrite disappearance in induced and noninduced layers, respectively.

Nitrite-induced activity was inhibited by cycloheximide but not by actinomycin D. Enzyme activity in induced layers was inhibited by 2,4-dinitrophenol (DNP), antimycin A, HOQNO and anaerobiosis. Activity in noninduced tissue was insensitive to antimycin A and HOQNO, and sensitive to anaerobiosis and DNP.

Nitrite uptake was rapid at pH 4.5 and negligible at pH 7.5. Nitrite accumulated at pH 4.5 anaerobically, was rapidly released when transferred to media at pH 7.5--the pH of the anaerobic intact-tissue assay for nitrate reductase. Accumulated nitrite was released by the tissue whether held under anaerobic or aerobic conditions.

Nitrate-induced and noninduced aleurone layers were able to reduce low levels of nitrite (25 uM) anaerobically. But, these rates (5 to 10 nmoles/layer/hour) were considerably lower than rates (25 nmoles/layer/hour) of nitrite production observed during the nitrate reductase anaerobic intact tissue assay (with ethanol present). DNP increased nitrite accumulation during the nitrate reductase anaerobic intact-tissue assay in the absence, but not in the presence of ethanol. This additional nitrite recovery in the absence of ethanol may represent an inhibition of the tissue's ability to reduce low levels of nitrite under anaerobic conditions.

Thus, it appears nitrite cannot be reduced fast enough during the nitrate reductase anaerobic intact-tissue assay; and, because nitrite cannot be retained by the tissue at neutral pH, it leaks into the medium.

APPENDIX

Reproducibility[1] of the nitrate reductase anaerobic intact tissue assay, and the effect of different amounts of tissue[2] on enzyme activity.

Experiment	Number of layers ($nmoles\ NO_2^-$ produced/hour)		
	5	10	15
1	41.3	95.6	100.0
2	53.2	64.3	113.0
3	47.5	101.3	127.0
mean	47.3±3.5	87.0±13.1	113.3±7.8

[1]Standard deviation from the mean calculated from the formula:

$$S^2 = \frac{(d)^2}{n(n-1)^2}$$

where S = the standard deviation from the mean, d = deviation from the mean, the n = number of samples.

[2]Barley seeds (1965 harvest) used in this and all previous studies were obtained from Dr. R. A. Nilan, Department of Agronomy, Washington State University, Pullman, Washington.