TURNOVER OF NITRATE REDUCTASE IN *NEUROSPORA CRASSA*

TURNOVER OF NITRATE REDUCTASE IN *NEUROSPORA CRASSA*

By

SHARON WALLS, B.Sc. (Hons.)

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AUTHOR: Sharon Walls, B.Sc. (Hons.) Queen's University, Kingston, Ontario

SUPERVISOR: Professor G. J. Sorger

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SCOPE AND CONTENTS:

Turnover of nitrate reductase in Neurospora crassa was examined in mycelia incubated in varying conditions. This study showed that the specific activity of the enzyme was very dependent on the age of the mycelia: it was highest in young mycelia which were in an exponential stage of growth. Both the rate of induction and the rate of decay in vivo and in cell-free extract were also highest in the mycelia in logarithmic growth phase. Comparison of the rate of induction and decay of nitrate reductase in exponentially growing mycelia on nitrate media and on media with no nitrogen suggested that the higher specific activity of the enzyme on nitrate media was perhaps a result of the protective effect of nitrate on the enzyme. The rate of decay of nitrate reductase in mycelia on ammonia media incubated at different ages and different temperatures was markedly different from the rate of decay of the enzyme in similarly treated mycelia on nitrate or no nitrogen media. This suggested that there might be two different mechanisms of decay.

An assay was developed to examine protease levels of the mycelia incubated in varying conditions. There was little evidence for the involvement of a protease in the decay of nitrate reductase, but the pattern of decay of the enzyme is consistent with the hypothesis that a protease may act as the mechanism of decay.

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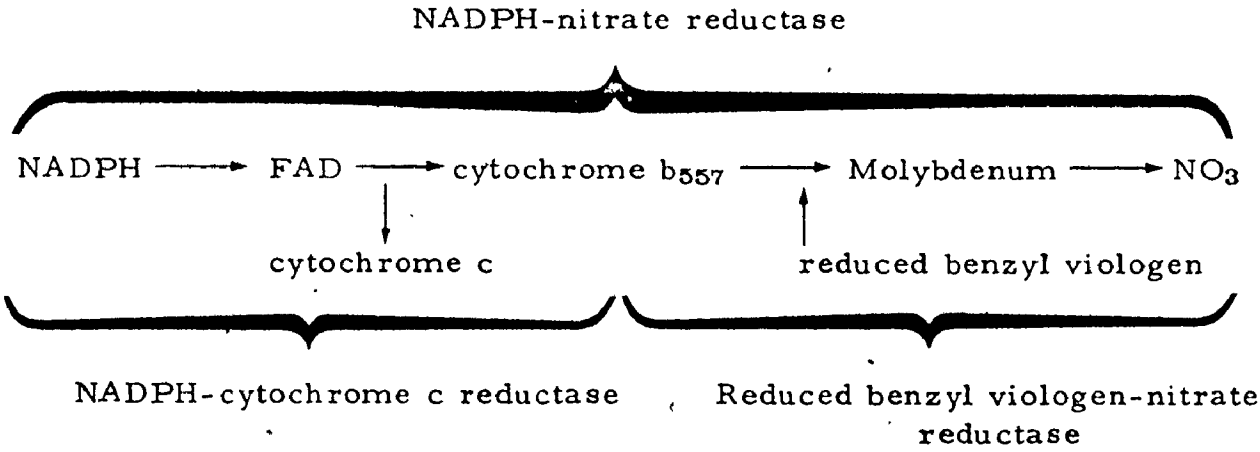
INTRODUCTION

General

This study was undertaken in order to clarify some of the factors which regulate the intracellular concentration of the enzyme nitrate reductase (NADPH-nitrate oxidoreductase, EC 1.6.6.3) in Neurospora crassa. This enzyme catalyzes the conversion of NO_3^- to NO_2^- during the assimilation of nitrogen in some bacteria, fungi, algae, and higher plants. Assimilatory nitrate reductase from some bacteria (Nicholas and Nason, 1955; Chiba and Ishimoto, 1973), and photosynthetic organisms (Syrett and Morris, 1963; Sanderson and Cocking, 1964; Stewart, 1968) require NADH as an electron donor, whereas the enzyme from fungi (Nason and Evans, 1953; Pateman et al., 1964; Sorger, 1965) requires NADPH as a reductant. The catalytic oxidation of NADPH by NO_3^- in the presence of nitrate reductase can be divided arbitrarily into two sequential steps as shown below (Sorger, 1966; Garrett and Nason, 1969). The two partial activities of nitrate reductase can be assayed separately: the FAD dependent activity can be assayed by measuring



the reduction of cytochrome c, and the catalytic activity of the second part of the enzyme complex can be assayed by using a reduced dye such as benzyl viologen as an artificial electron donor to reduce NO_3^- to NO_2^- (Sorger, 1966; Garrett and Nason, 1969). A similar situation has been described in algae and in higher plants, where the NADH nitrate reductase can be separated into an FAD-dependent NADH diaphorase, and a molybdenum-dependent $FMNH_2$ nitrate reductase (Beevers and Hageman, 1969; Wray and Filner, 1970; Relimpio et al., 1971).

Role of ammonium ions

An organism which is grown on media containing NH_4^+ has no nitrate reductase until the NH_4^+ has disappeared from the medium (Kessler and Oesterheld, 1970; Cardenas et al., 1971; Syrett and Hipkin, 1973). If the medium contains NH_4^+ plus NO_3^-, then there is a lag in the appearance of enzyme activity compared with the situation when NO_3^- alone is present, and the enzyme level after the usual period of induction is lower (Syrett and Morris, 1963; Subramanian et al., 1968; Ferguson, 1969; Subramanian and Sorger, 1972a). Orebamjo and Stewart (1974) showed that the rate of nitrate reductase formation during induction in Lemna could be increased by including an incubation period, on a medium containing no nitrogen source, between the transfer from the growth medium containing NH_4^+ to the induction medium containing NO_3^-.

Loss of intracellular nitrate reductase activity occurs when fungi, algae or plants which have been induced by NO_3^- are transferred to media lacking NO_3^- (Hewitt and Afridi, 1959; Morris and Syrett, 1965; Ferguson, 1969; Heimer and Filner, 1970; Subramanian and Sorger, 1972a; Orebamjo and Stewart, 1974). Addition of NH_4^+ to the culture medium results in the loss of enzyme activity in bacteria (Inderlied and

Delwiche, 1973), in fungi (Morton, 1956; Cove, 1966; Subramanian et al., 1968; Lewis and Fincham, 1970; Subramanian and Sorger, 1972a; Goldsmith et al., 1973), in algae (Syrett and Morris, 1963; Losada et al., 1970; Vega et al., 1971; Rigano and Violante, 1973); and in most higher plants (Ferguson, 1969; Smith and Thompson, 1971; Stewart, 1972; Radin, 1973; Orebamjo and Stewart, 1974). Some exceptions to the repressive effect of NH_4^+ in higher plants are found in early reports such as Ingle et al. (1966) and Filner (1966). They found that NH_4^+ did not repress nitrate reductase in radish cotyledons and cultured tobacco cells.

Morris and Syrett (1965) found that the rate of decay of enzyme activity in Chlorella transferred from inducing conditions to media containing either NH_4^+ or no nitrogen source, was about the same. Ferguson (1969) and Sorger et al. (1974) working with duckweed and Neurospora, respectively found that the disappearance of the enzyme activity was more rapid in an organism transferred to media containing no nitrogen source than in one transferred to media containing NH_4^+. This occurred in spite of the synthesis of some nitrate reductase on the media containing no nitrogen source.

It has been shown that some amino acids (or possibly their metabolites) repress nitrate reductase activity: alanine, asparagine, glycine, methionine, proline, threonine, valine, histidine, leucine, aspartate, and glutamate in cultured tobacco cells (Filner, 1966); arginine, histidine, asparagine, glutamine, isoleucine and cysteine in Neurospora (Subramanian et al., 1968); aspartic acid, glutamic acid, leucine, lysine, histidine, arginine, citrulline, and ornithine in Chlorella (Smith and Thompson, 1971); glutamine and asparagine in Penicillium (Goldsmith et al., 1973).

Role of nitrate ions

There are numerous reports that nitrate reductase in algae is induced by NO_3^- (Morris and Syrett, 1965; Cardenas et al., 1971; Smith and Thompson, 1971; Rigano and Violante, 1973), and in fungi (Nason and Evans, 1953; Sorger, 1965; Cove, 1966; Lewis and Fincham, 1970; Goldsmith et al., 1973). In higher plants, the influence of NO_3^- on the level of activity of nitrate reductase, though still stimulatory, is often less marked than in fungi (Filner, 1966; Ingle et al., 1966; Schrader and Hageman, 1967; Smith and Thompson, 1971; Stevens and Oaks, 1973; Radin, 1973; Orebamjo and Stewart, 1974). Increasing the concentration of NO_3^- in the culture medium to more than 5mM had little effect on the rate of induction, or on the final induced level of nitrate reductase activity in the root tips of corn (Stevens and Oaks, 1973) or in Neurospora (Subramanian and Sorger, 1972a). In mature roots of corn, where the level of nitrate reductase activity was much lower than in root tips, there was a very gradual increase of activity with increasing NO_3^- concentration in the plant's environment up to 100mM.

Using cycloheximide and actinomycin D sequentially to try to separate transcription and translation of nitrate reductase, Sorger and coworkers (Subramanian and Sorger, 1972b; Sorger and Davies, 1973) showed that transcription occurred in the absence of NO_3^-, but that NO_3^- in the culture medium was necessary to stimulate the post-transcriptional events necessary for the induced appearance of the enzyme.

Several workers have shown that deprivation of amino acids in amino acid auxotrophs or addition of cycloheximide to the induction medium prevents the appearance of nitrate reductase activity (Sorger, 1965; Ingle et al., 1966; Stewart, 1968; Losada et al., 1970; Sawhney

and Naik, 1972; Syrett and Hipkin, 1973). This would suggest that de novo synthesis is involved in the induction of this enzyme. The best evidence for de novo synthesis of nitrate reductase is found in the work of Zielke and Filner (1971). They grew tobacco cells in a medium containing ^{15}N-labelled nitrate and ^{14}C-labelled arginine and the cells were then transferred to a medium containing ^{14}N-nitrate and ^{3}H-arginine. By following the buoyant density of nitrate reductase, ^{14}C-labelled proteins and ^{3}H-labelled proteins, they showed conclusively that the increase in nitrate reductase was from de novo synthesis and that synthesis continued during non-inducing conditions.

The induction of nitrate reductase does not appear to require NO_3^- in all systems. Transfer of algae (Morris and Syrett, 1965; Kessler et al., 1970; Cardenas et al., 1971; Vega et al., 1971; Rigano and Violante, 1973) and of fungi (Morton, 1956; Sorger and Davies, 1973) from growth media containing NH_4^+ to media with no nitrogen source, results in the appearance of some enzyme activity, though it is usually only a fraction of the activity induced by NO_3^-. Vega et al. (1971) and Rigano and Violante (1973) report very high levels of nitrate reductase in algae transferred to media with no added source of nitrogen. Kessler and Oesterheld (1970) suggested that the above induced activity may be in response to the production of NO_3^- by algal nitrification, but Syrett and Hipkin (1973) in comparable experiments and using a method sensitive to 5nM NO_3^-, were unable to detect any NO_3^- either in the cells or in the media.

There are reports of the induction of nitrate reductase on media containing amino acids as nitrogen sources. Herrera et al. (1972) observed that when Chlamydomonas was grown on glycine, alanine, or asparagine, nitrate reductase was induced to 30%, 40% or 40% respectively of the nitrate-induced levels. Orebamjo and Stewart (1974) found only 0.8 - 3.0% of the nitrate-induced level of activity in Lemna grown

on asparagine, allantoin, uric acid, or glutamine; Rigano and Violante (1973) reported 200% of the nitrate-induced level of activity in Cyanidium grown on glutamic acid.

The decay of nitrate reductase in vivo in media containing NO_3^- has been examined by preventing further synthesis of the active enzyme using either cycloheximide, which is a general protein synthesis inhibitor, or tungstate, which appears to replace molybdate in nitrate reductase and renders the enzyme inactive (Wray and Filner, 1970; Notton and Hewitt, 1971; Vega et al., 1971; Subramanian and Sorger, 1972c). Stevens (1972) found that the decay of nitrate reductase in corn roots exposed to cycloheximide was the same in the presence and in the absence of NO_3^-; therefore, nitrate seemed not to protect the enzyme from decay. There is evidence suggesting that cycloheximide affects the rate of decay of nitrate reductase in some organisms (Ingle et al., 1966; Travis et al., 1969; Lewis and Fincham, 1970; Subramanian and Sorger, 1972a), consequently the use of tungstate ($WO_4^=$) which inhibits the synthesis of the active form of the enzyme without interfering with the decay process, is recommended in these cases (Sorger et al., 1974). Using this method Sorger et al. (1974) found that nitrate reductase in Neurospora was partially protected from decay by NO_3^-, the decay of the enzyme in vivo on a medium containing NO_3^- being about one-third of that on a medium containing no nitrogen source.

The presence of NO_3^- in the culture medium then, enhances the level of nitrate reductase activity in most organisms, but its mechanism of action is not known.

Decay of nitrate reductase activity

The mechanism of the above loss of nitrate reductase activity is not known in detail. The following possibilities can be considered for Neurospora:

1) inhibition of enzyme activity by NH_4^+ or other metabolites which accumulate in the presence of NH_4^+, and/or in the absence of NO_3^-;

2) prevention or decrease in enzyme synthesis because of NH_4^+ or lack of NO_3^- and loss of activity due to destruction by proteases;

3) destruction of nitrate reductase by a specific protease activity under non-inducing conditions; or

4) some combination of the above.

"Repressive" amino acids or ammonium ions added to cell free preparations of nitrate reductase, had no effect on the enzyme activity (Cove, 1966; Schrader et al., 1967; Subramanian et al., 1968; Garrett and Nason, 1969; Lewis and Fincham, 1970). Subramanian and Sorger (1972a) have shown that the activity of nitrate reductase in mixtures of extracts from fully induced and uninduced Neurospora mycelia was identical to that in the extracts of fully induced controls.

NH_4^+ could have an indirect effect on nitrate reductase activity in vivo. The ions might act as a trigger that would alter either the pH, or the redox state inside the cell or the permeability of a membrane separating nitrate reductase and a protease. Ammonium repression of nitrogenase has been investigated in Azobacter and Klebsiella using methionine sulfone and methionine sulfoxamine; both glutamate analogues that inhibit glutamate synthetase and glutamine synthetase (Gordon

and Brill, 1974). In the presence of these analogues, NH_4^+ is excreted and no repression of nitrogenase occurs. Therefore the NH_4^+ by itself appears not to be the repressor of nitrogenase in vivo.

There is some evidence that changes in the redox state of nitrate reductase affect its activity in algae (Vennesland and Jetschmann, 1971; Herrera et al., 1972; Moreno et al., 1972; Losada et al., 1973; Solomonson, 1974). The inactivation of nitrate reductase by NH_4^+ in Chlamydomonas and Chlorella in vivo can be reversed in vitro, by oxidation of the extract with ferricyanide or nitrate (Vennesland and Jetschmann, 1971; Herrera et al., 1972). Such a reactivation of the enzyme was not found in Neurospora crassa (Subramanian and Sorger, 1972a). Addition of NADH to an extract of Chlamydomonas reinhardii, Chlorella fusca, or Chlorella vulgaris produces a rapid inactivation of nitrate reductase activity (Herrera et al., 1972; Moreno et al., 1972; Losada et al., 1973; Solomonson, 1974). The presence of NADH plus cyanide results in an inactivation of the enzyme in extracts of spinach (Relimpio et al., 1971). The enzyme can be protected from this inactivation by NO_3^- (Relimpio et al., 1971; Herrera et al., 1972; Moreno et al., 1972), and NO_3^- can also reactivate the NADH-inactivated enzyme (Moreno et al., 1972). Garrett and Greenbaum (1973) found that the sensitivity of Neurospora nitrate reductase to metal binding agents is increased by incubation of the enzyme with NADPH. The molybdenum in the enzyme was thought to have a greater affinity for the metal binding agents when the latter is reduced than when it is oxidized. This finding is postulated to have regulatory significance.

Experiments with cycloheximide in fungi and higher plants suggest that not only is protein synthesis necessary for the induced appearance of nitrate reductase, but that it is also necessary for the loss of activity (Ingle et al., 1966; Travis et al., 1969; Lewis and Fincham, 1970;

Subramanian and Sorger, 1972a; Goldsmith et al., 1973). Zielke and Filner (1971) showed that there is both synthesis and degradation of nitrate reductase during inducing and non-inducing conditions in cultured tobacco cells. The above experiments suggest that the loss of enzyme activity under non-inducing conditions in fungi and higher plants involves more than an inhibition of the synthesis of nitrate reductase. The rate of decay of nitrate reductase in Neurospora cultured on media containing NO_3^-, NH_4^+, or no nitrogen source varies widely, even in the presence of $WO_4^=$, which should prevent the appearance of the active form of the enzyme (Sorger et al., 1974). If loss of activity occurred solely because of the absence of nitrate reductase synthesis, then the rate of decay of the enzyme in mycelia incubated in media containing NO_3^- and $WO_4^=$ and in no nitrogen source should be the same, since the growth rate of the mold on these two media is the same. Therefore it is possible that nitrate reductase activity is actively destroyed in non-inducing conditions.

The destruction of nitrate reductase by a protease could be regulated in several ways:

a) activation of an inactive protease or removal of an inhibitor from a protease under non-inducing conditions for nitrate reductase;

b) compartmentation of nitrate reductase and protease with a release of protease under non-inducing conditions for nitrate reductase; or

c) increased sensitivity of nitrate reductase enzyme to protease under non-inducing conditions for nitrate reductase.

There is immunological evidence that the loss of nitrate reductase activity in non-inducing conditions is a result of the disappearance of the enzyme protein (Sorger et al., 1974). Wallace (1973, 1974) has isolated an inactivating enzyme in corn roots which seems specific for

nitrate reductase. There are several examples of proteases apparently specific for certain enzymes, such as those acting on pyridoxal enzymes in rat tissue (Afting et al., 1972; Katunuma et al., 1972), on fructose 1-6 bisphosphatase in yeast (Molano et al., 1974), on pyruvate decarboxylase in yeast (Juni and Heym, 1968) and on tryptophan synthase in both yeast and Neurospora (Tsai et al., 1973). Evidence exists in the literature suggesting that such proteases can be regulated in one or more of each of the ways listed above:

a) There are several examples of inhibitors of specific proteases isolated from crude extracts of yeast (Juni and Heym, 1968; Lenney, 1973; Molano and Gancedo, 1974) and of Neurospora (Yu et al., 1973). These inhibitors did not react either with other proteases in cell free extracts, or with trypsin. It is possible that a similar inhibitor is involved in regulating the decay of nitrate reductase by protease digestion.

b) Compartmentation may be involved in the regulation of protease A and protease B in yeast (Matile and Wiemken, 1966; Lenney, 1973). The two proteases are found in the cell vacuoles, while inhibitors of these proteases are found in the cytosol outside the vacuole. Another example of regulation by compartmentation of proteases appears in the work of Cabib et al. (1973). They found that zymogen, the inactive form of yeast chitin synthetase, is activated by a proteolytic activating factor found in yeast vacuoles. Since chitin synthetase is needed at the site of primary septum synthesis, release of the activating factor from vacuoles can result in localized activation of the zymogen.

c) The correlation of the conformation of the enzyme and its sensitivity to protease is well documented. The vulnerability of rat liver enzymes to protease digestion in vitro correlated with their

decay *in vivo* (Bond, 1971). Two mutant proteins in *E. coli*, B-galactosidase synthesized in ochre and ambre mutants (Goldsmith, 1970), and lac repressor synthesized in a deletion mutant (Platt *et al.*, 1970), both exhibited higher rates of degradation *in vivo* than the corresponding wild type proteins. When proteins were synthesized with analogues of amino acids, or when their synthesis was prematurely terminated with puromycin, the abnormal products were much more unstable *in vivo* than the normal control proteins (Goldberg, 1972a; Pine, 1967). The increased susceptibility of these abnormal proteins to endoproteases *in vitro* was well correlated with their increased susceptibility to degradation *in vivo*, when compared with normal control proteins (Goldberg, 1972b). A mutant form of pyruvate decarboxylase in yeast showed greater lability *in vitro* than the wild type enzyme (Juni and Heym, 1968). Strains *nit 1* and *nit 3* of *Neurospora*, which produce mutant forms of nitrate reductase both have a nitrate reductase partial activity that is more stable in non-inducing conditions than their wild type counterparts (Subramanian and Sorger, 1972a; Sorger *et al.*, 1974).

In this study the possible role of protease in the disappearance of nitrate reductase was examined. The rate of decay of nitrate reductase was measured under different conditions and compared with the levels of protease activity. Measurement of the rates of induction of nitrate reductase under similar conditions allowed speculation as to the relative importance of synthesis and decay in determining the level of activity of the enzyme.

METHODS AND MATERIALS

Fungal strain

Neurospora crassa wild type strain ORa, was used.

Media

The basic medium was that described by Sorger and Giles (1965), to which the following nitrogen sources were added: ammonium tartrate, 20mM (NH_4^+ medium), $NaNo_3$, 20mM (NO_3^- medium), or no nitrogen source (-N medium). Sodium tungstate (6mM) was added to the medium when the rate of decay of nitrate reductase was measured.

Culture conditions

Conidia from a mature culture in a small culture tube were introduced into 200ml of NH_4^+ medium. Aliquots of 10ml of this inoculated medium were incubated in standing culture at 27 $\pm$ 1°C in 50ml Erlenmeyer flasks until a mycelial mat was formed. Since the number of conidia in the original inoculum varied, growth curves of the dry weight of mycelial pads were done for each experiment. Figure 1 shows a sample of these curves, it is apparent that varying the inoculum size had little effect on the duration of the lag phase.

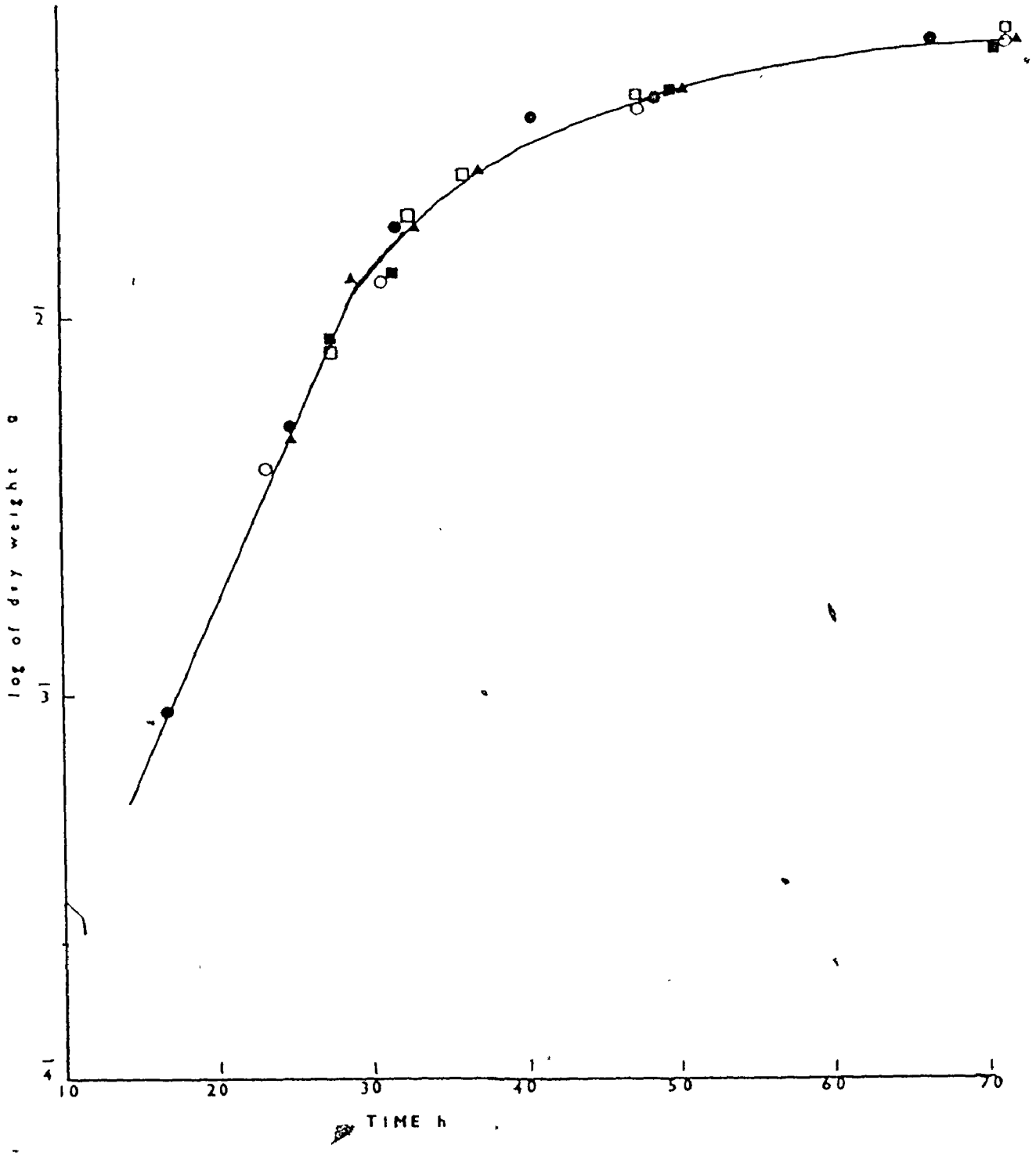
Induction and decay

At specified intervals mycelial pads were harvested from the NH_4^+ medium, washed in distilled water, and placed in NO_3^- medium for induction, 1 pad per 10ml media. The pads were induced for four hours

FIGURE 1

Growth curves for Neurospora crassa

Mycelial pads from 5 different experiments (● ○ ▲ ■ and □) were harvested at the indicated times and dried at 65°C for at least 12 hours before weighing on a micro balance.


log of dry weight g
2̄
1̄
4̄
10
20
30
40
50
60
70
TIME h

in shaking culture at 27 ± 1°C on a rotary shaker at medium speed (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). When the decay of nitrate reductase was to be measured, the induced pads were washed in distilled water, transferred to an appropriate decay medium and shaken at 27°C or 37°C for specified intervals.

Preparation of cell free extracts

Mycelial pads were harvested, washed in distilled water and blotted between paper towels until no moisture could be extruded. The mycelia were then frozen in liquid nitrogen and stored at -60°C until used. The frozen fungus was ground in an ice-cold mortar with approximately the same volume of silica, and the resulting paste suspended in 2.0ml of cold potassium phosphate buffer (0.1M, pH 7.0) per pad. This brei was centrifuged at 12,000 g for 10 minutes. (Sorvall Model RC2B Norwalk, Conn.) and the resulting supernatant was kept on ice, and usually assayed within one hour.

Enzyme Assays

Nitrate reductase

Nitrate reductase was assayed as described by Subramanian et al. (1968). Extract and potassium phosphate buffer (0.1M, pH 7.0) to a combined volume of 0.1ml were incubated at 30 ± 1°C, with 0.4ml of substrate (10ml of 0.1M $NaNO_3$, 20ml of 0.2 M sodium pyrophosphate buffer, pH 7.0, and 5ml of 10^{-5}M FAD in the same sodium pyrophosphate buffer). The reaction was started by adding 0.05ml of 0.2% NADPH and was usually terminated after 10 minutes with 4.0ml of a color reagent (3 parts water: 1 part 1.0% sulfanilamide in 0.3N HCl: 1 part 0.01% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water). The colour

was allowed to develop for 20 minutes and then read at 540nm in a 1cm path length cuvette in a Beckman DB spectrophotometer. The amount of NO_2^- was determined from a standard curve. One unit of activity is defined as the production of 1 nmol of NO_2^- / min. at 30 $\pm$ 1°C. Specific activity is expressed as units/mg of protein. Figure 2 shows the amount of NO_2^- released per minute with increasing amounts of extract in the assay mixture.

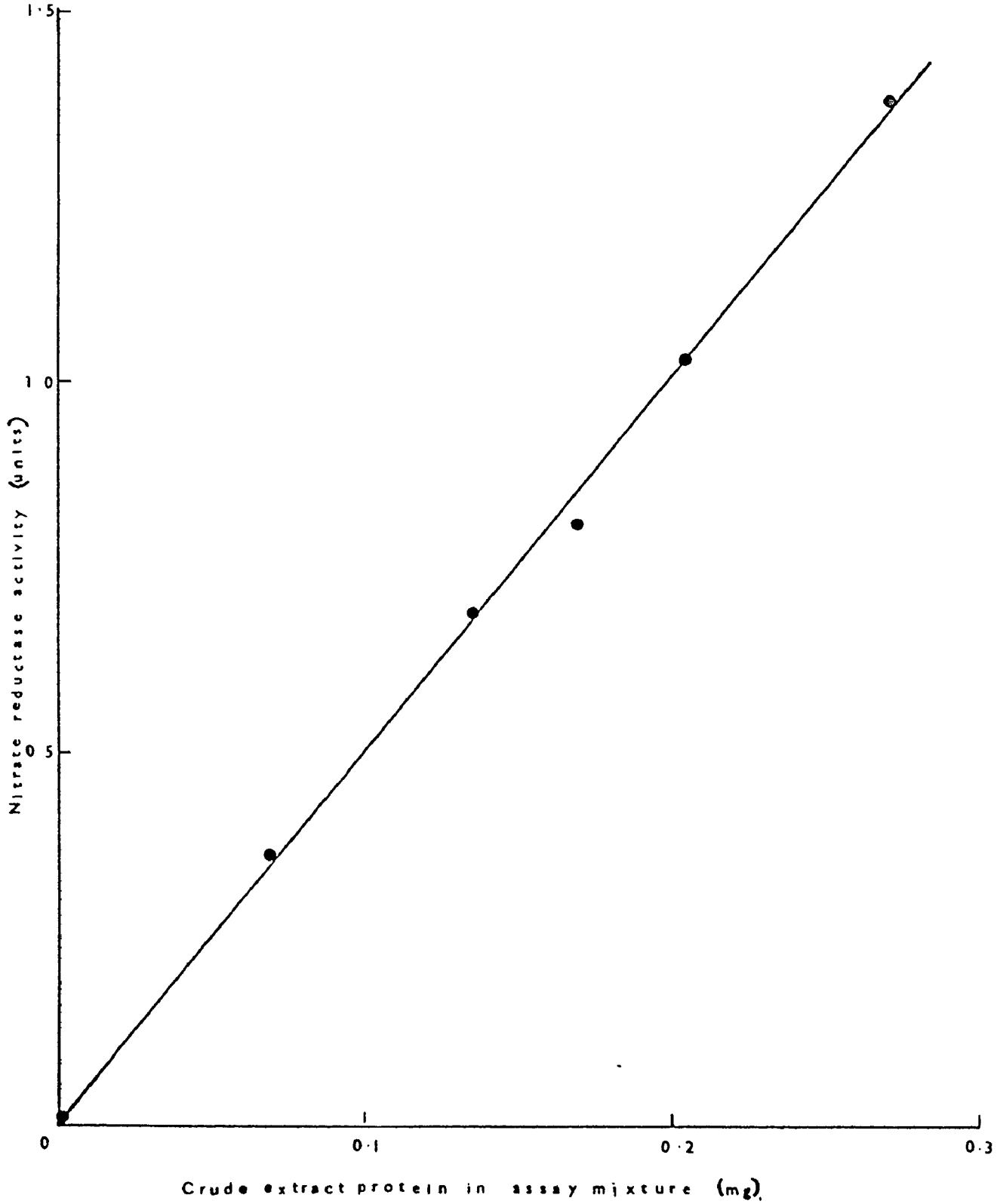
Protease assay

Protease activity was assayed by measuring the trichloroacetic acid (TCA) soluble products of the digestion of casein (Kunitz, 1947; May and Elliott, 1968). The reaction was started by adding 1.0ml of 1% casein (1.0 g casein dissolved in 100ml of 0.1 M KPO_4 buffer, pH 7.6, by heating in boiling water for 15 minutes) to tubes containing extract and KPO_4 buffer (0.1 M, pH 7.6) in a final volume of 2.0ml. (When extracellular protease activity was being assayed, 1.0ml of 1% casein was added to aliquots of culture filtrate and KPO_4 buffer pH 7.6 in a final volume of 2.0ml.) The tubes were incubated for 30 minutes at 37°C and the reaction was stopped with 3.0ml of Hagihara's reagent (.11 M TCA, .22 M sodium acetate, and .33 M acetic acid, May and Elliott, 1968). Blanks were prepared by incubating the extract or the culture filtrate with the KPO_4 buffer alone for 30 minutes at 37°C and then adding Hagihara's reagent, followed by 1.0ml of 1% casein incubated separately. The tubes were refrigerated at 4°C overnight and spun at top speed in a table top centrifuge for 15 minutes. The absorbance of the supernatant was measured at 280nm. One unit of protease activity was defined as the difference in A_{280} / min. x 10^{-3} between the supernatant of the test assay and that of the blank. Specific activity of protease in the extract was expressed as units/mg extract protein; specific

FIGURE 2

Effect of extract concentration on nitrate reductase activity

Mycelial pads, pregrown on $\underline{NH_4^+}$ media for 48 hours, were induced for 4 hours on $\underline{NO_3^-}$ induction media. Cell-free extracts were prepared and subsequently assayed for nitrate reductase activity.


1·5
1·0
0·5
0
0·1
0·2
0·3
Nitrate reductase activity (units)
Crude extract protein in assay mixture (mg)

activity of protease in the culture filtrate was expressed as units/mg dry weight of mycelial pad in the media x 10^{-3}.

The assay was checked for optimum conditions of pH (Figure 3), of salt concentration (Figure 4), of substrate concentration (Figure 5), of length of assay (Figure 6), and of enzyme concentration (Figure 7). It was difficult to test low pH with this assay since casein becomes insoluble in acid. The lack of a discrete optimum pH suggests that there may be two or more proteases present in the extract.

Protein determination

Protein was estimated by the biuret method (Dawson et al., 1959) using bovine serum albumin as a standard.

Materials

Bovine serum albumin, NADPH, and FAD were from Sigma Chemical Company, St. Louis, Mo., U.S.A.; benzyl viologen and phenylmethyl sulfonylfluoride were from Schwarz-Mann Orangeburg, N. Y., U.S.A.; sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were products from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. All other chemicals were reagent grade and were from Fisher Scientific Co., Fairlawn, N.J., U.S.A., or from Baker Chemical Co., Phillipsburg, N.J., U.S.A.

FIGURE 3

Effect of pH of the assay on protease activity

Mycelial pads pregrown on $\underline{NH_4^+}$ media for 48 hours, were induced on $\underline{NO_3^-}$ induction media and then transferred to -N media for 4 hours. The pads were harvested and the extracts were assayed for intracellular protease activity (Δ) and the media assayed for extracellular protease activity (Δ). The pH was adjusted with potassium phosphate buffer; the recorded pH was that of the final assay mixture.

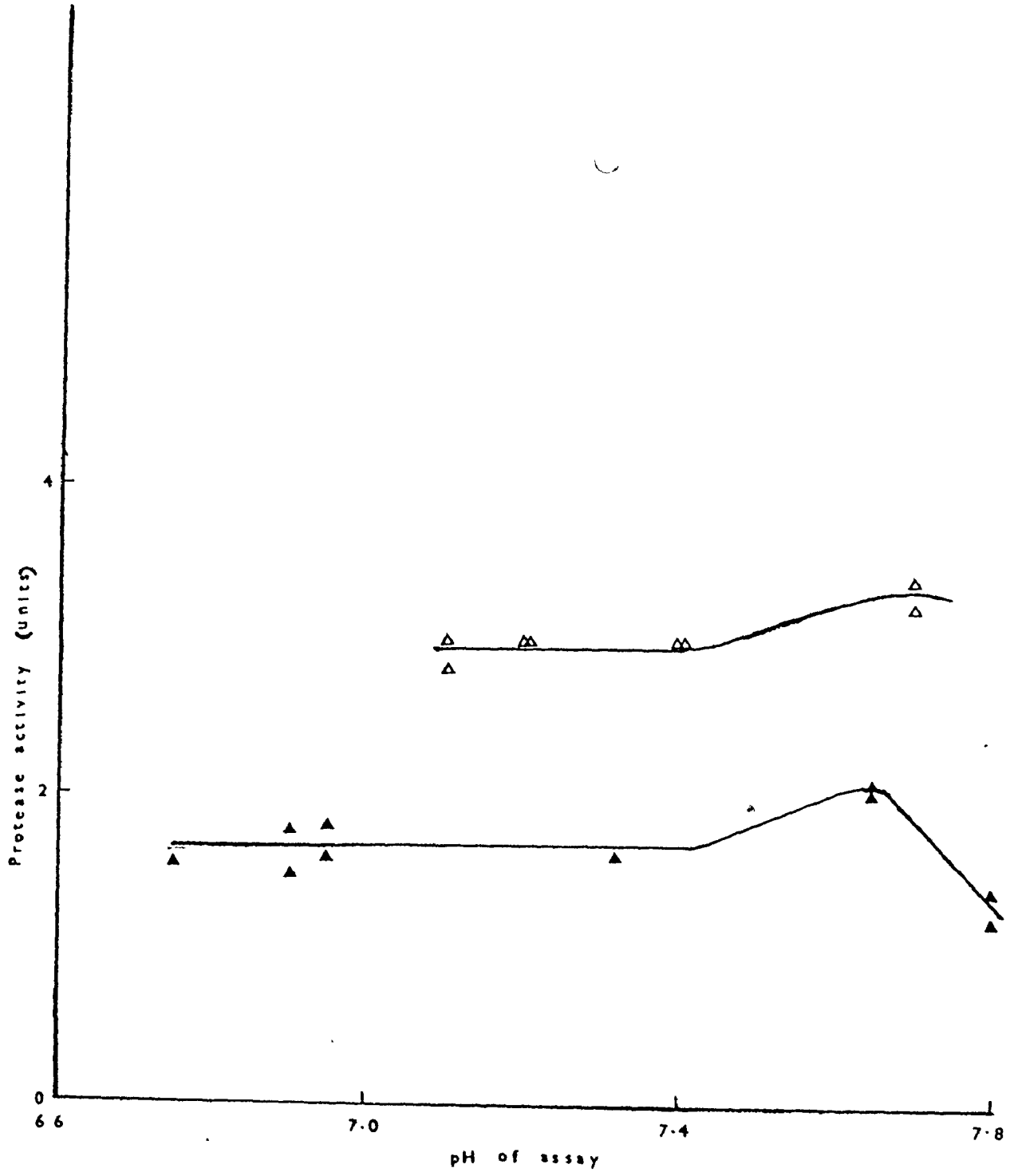

Protease activity (units)
4
2
0
6 6
7·0
7·4
7·8
pH of assay

FIGURE 4

Effect of salt concentration in the assay on extracellular protease activity

Mycelial pads were treated as described in Figure 3. Extracellular protease activity was assayed in the presence of varying concentrations of either NaCl (■) or $CaCl_2$ (□).

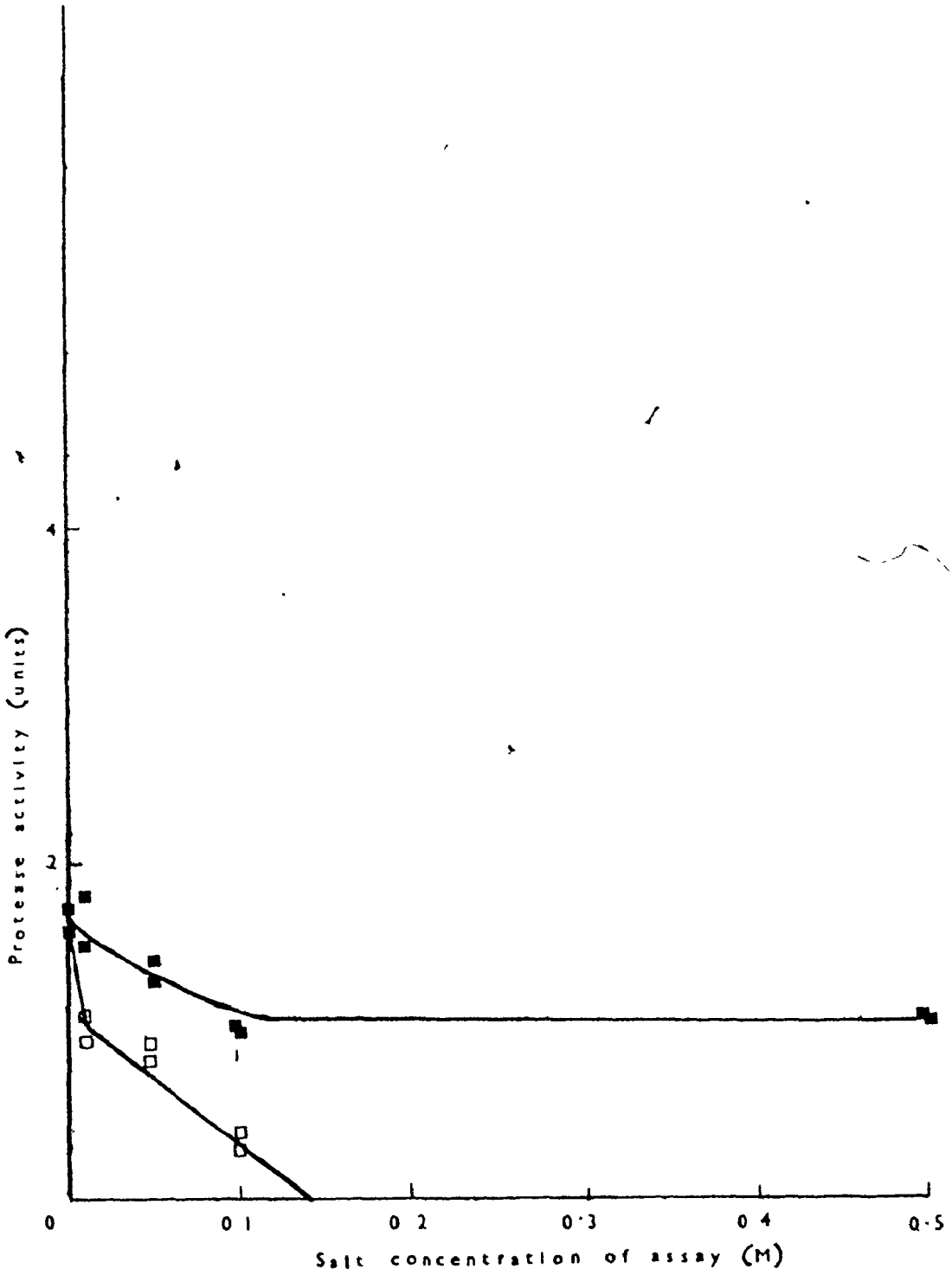

Protease activity (units)
4
2
0
0·1
0·2
0·3
0·4
0·5
Salt concentration of assay (M)

FIGURE 5

Effect of substrate concentration on protease activity

Mycelial pads were treated as described in Figure 3. Intracellular (▲) and extracellular (△) protease activities were assayed with varying amounts of casein.

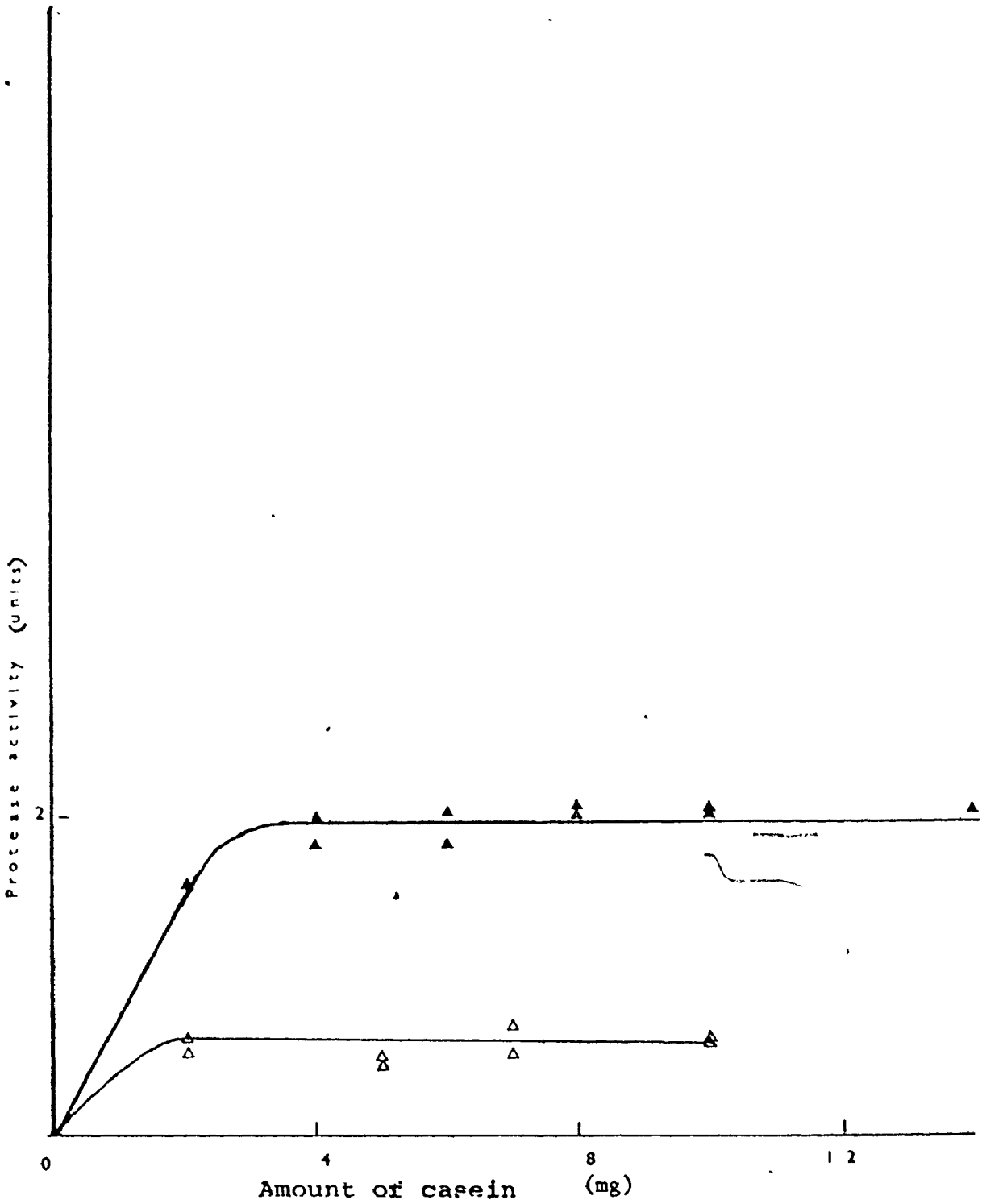

Protease activity (units)
2
0
4
8
12
Amount of casein (mg)

FIGURE 6

Effect of length of assay on protease activity

Mycelial pads were treated as described in Figure 3. Intracellular (▲) and extracellular (△) protease activities were assayed for the times shown.

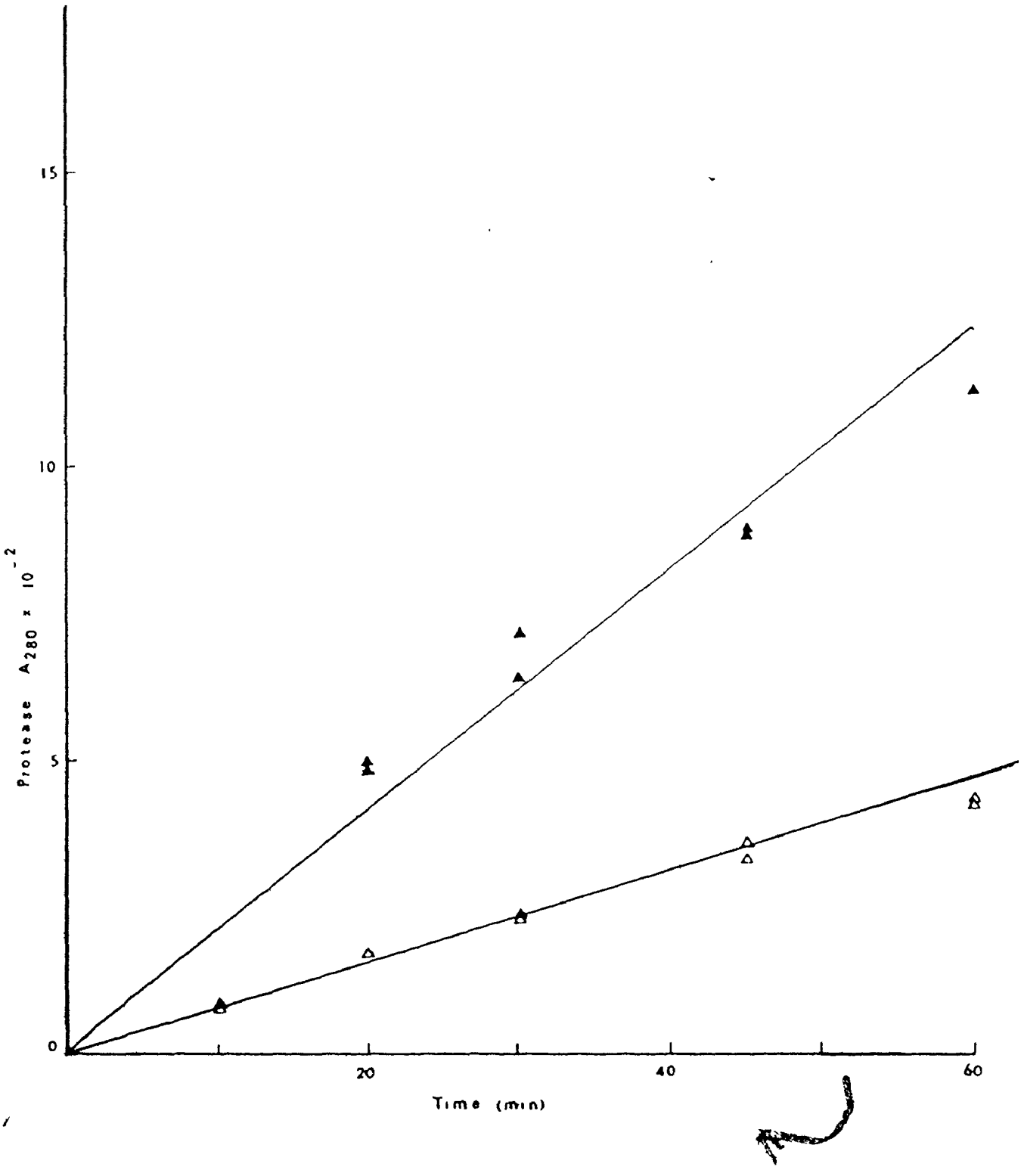
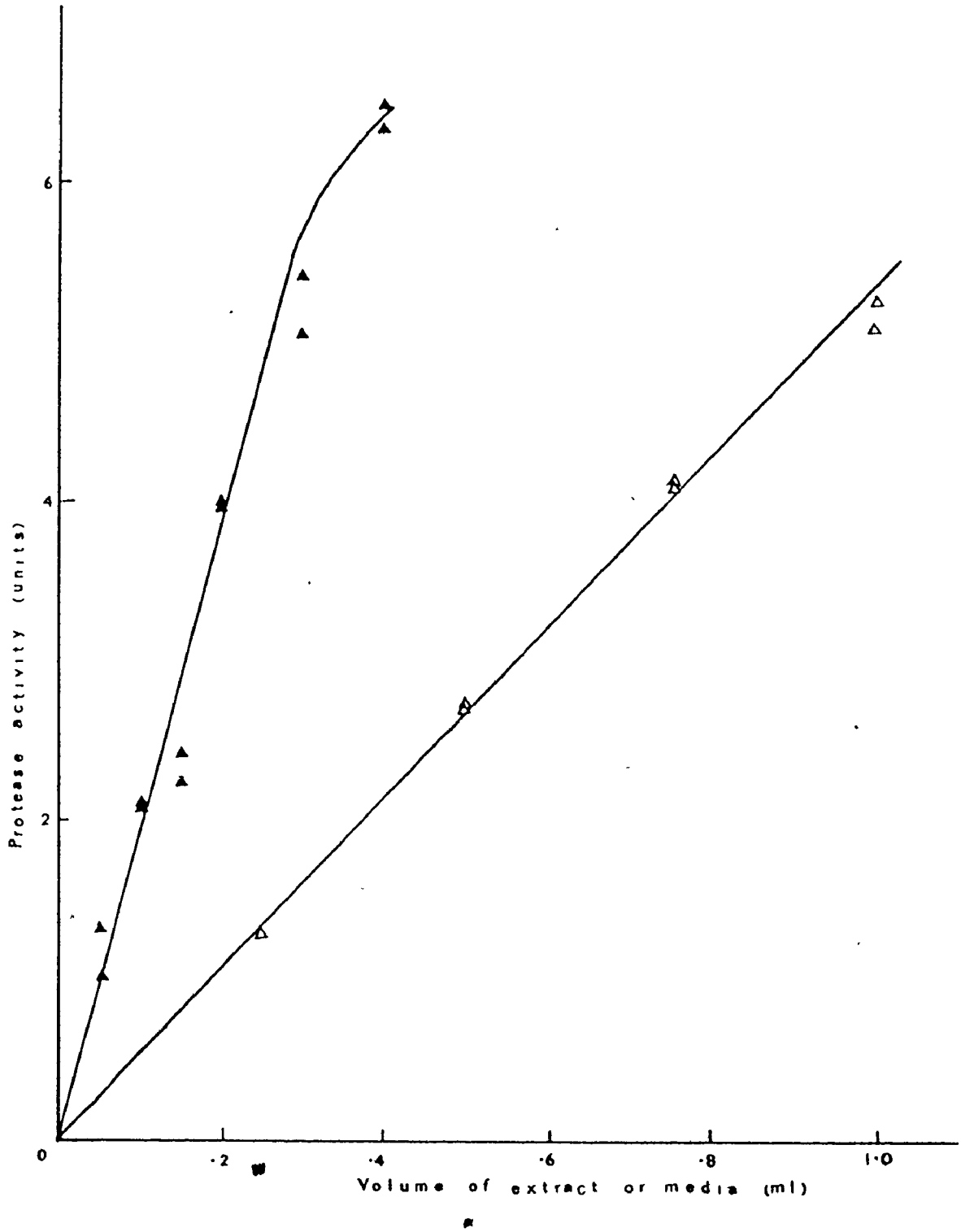

15
10
5
0
Protease $A_{280} \times 10^{-2}$
20
40
60
Time (min)

FIGURE 7

Effect of extract or culture filtrate concentration in the assay on protease activity

Mycelial pads were treated as described in Figure 3. Intracellular (▲) and extracellular (∧) protease activities were assayed with varying amounts of extract and media respectively. The extract contained 8.0mg protein/ml, and the media was obtained from a flask containing a mycelial pad, dry weight 58mg.


Protease activity (units)
0
2
4
6
·2
·4
·6
·8
1·0
Volume of extract or media (ml)

RESULTS

Stability in vitro

Studies in several different organisms show that nitrate reductase is an enzyme that turns over in vivo (Ferguson, 1969; Lewis and Fincham, 1970; Zielke and Filner, 1971; Syrett and Hipkin, 1973; Orebamjo and Stewart, 1974; Sorger et al., 1974). If a protease is involved in this turnover, then evidence for it may be found in cell free extracts. The instability of tryptophan synthase activity in extracts of Neurospora (Yu et al., 1973) and nitrate reductase activity in cell free extracts of corn (Wallace, 1973, 1974) has been shown to be correlated with a specific protease activity in the respective extracts.

The results in Table I show that the specific activity of nitrate reductase in cell free extracts of log phase mycelia induced for 4 hours on NO_3^- media is higher than that in comparable preparations of older mycelia induced in the same way. Contrary to the situation in corn (Oaks et al., 1972; Wallace, 1973, 1974), the enzyme from younger mycelia is less stable in cell free extracts at 30°C than the enzyme from older mycelia under comparable conditions (Table I). The addition of the substrate $NaNO_3$ and of 2% bovine serum albumin (BSA) to cell free preparations appears to partially protect nitrate reductase from inactivation in vitro (Table I, Table II). Schrader et al. (1974) also found that casein and BSA stabilized nitrate reductase activity in cell free extracts of corn.

In early experiments the cell free nitrate reductase appeared to be more unstable than in later ones (Table I). The reason for this cannot be explained. Nitrate reductase in extracts of 28 hour old mycelia

TABLE I

Stability of nitrate reductase activity in cell free preparations of mycelia of different ages

Age of mycelial pad	Specific activity of nitrate reductase	Rate of decay of nitrate reductase (Δ log of specific activity / h)		
		Expt 1 +0.4ml $NaNO_3$	Expt 2 +0.4ml $NaNO_3$	Expt 2 +0ml $NaNO_3$
28 hours	16.0	-1.68	0	-9.92
48 hours	5.4	-0.50	0	-2.41
72 hours	1.7	-0.31	0	-1.56

Mycelial pads, pregrown on $\underline{NH_4^+}$ media for 28 hours, 48 hours or 72 hours, were induced on $\underline{NO_3^-}$ induction media for 4 hours prior to being harvested. The extracts were assayed for nitrate reductase as described in the Methods. The stability of nitrate reductase activity was measured by incubating aliquots of the extract and buffer at 30°C for intervals of up to 30 minutes in the presence and absence of 0.4ml substrate (see Methods). At the end of the appropriate interval, substrate was added if necessary and the assay was begun with NADPH. The rate of decay of nitrate reductase activity was determined using linear regression analysis. Experiment 1 is representative of several experiments carried out in the initial stages of the enquiry, experiment 2 is representative of those carried out several months later.

TABLE II

Effect of BSA and nitrate ions on the stability of nitrate reductase activity in cell free preparations

Age of mycelial pad		Rate of decay of nitrate reductase (Δ log of specific activity / h)	
		+ 2% BSA	- BSA
28 hour	$+NO_3^-$	0	0
	$-NO_3^-$	-0.27	-9.92
48 hour	$+NO_3^-$	0	0
	$-NO_3^-$	-1.34	-2.41
72 hour	$+NO_3^-$	0	0
	$-NO_3^-$	-1.34	-1.56

Mycelial pads were treated as described in Table I. One half of each pad was extracted with buffer containing 2% BSA and the other half of the pad was extracted with buffer without this addition. The stability of nitrate reductase activity in the presence and absence of BSA and NO_3^- was measured as described in Table I.

was unstable in the early experiments, even in the presence of NO_3^-, thus it was necessary to add 2% BSA to the extraction buffer. Comparisons of the level of enzyme activity in extracts of mycelia of different ages are thus most accurate in Table I.

Decay of nitrate reductase in vivo

Previous work in Chlorella (Morris and Syrett, 1965), in duckweed (Ferguson, 1969), in Ustilago (Lewis and Fincham, 1970), in cultured tobacco cells (Heimer and Filner, 1971) and in Neurospora (Subramanian and Sorger, 1972a; Sorger et al., 1974) indicates that the rate of decay of nitrate reductase in vivo varies with the composition of the culture medium. In Neurospora, the rate of decay of nitrate reductase in vivo is relatively fast in young fully induced mycelia transferred to -N medium, is intermediate in comparable mycelia transferred to NH_4^+ medium and relatively slow in similarly pre-treated mycelia transferred to NO_3^- medium (Sorger et al., 1974). The results in Figure 8a confirm this previous work. In older mycelia the stability of nitrate reductase in mycelia transferred from full induction conditions to NH_4^+ medium remains constant, whereas it increases with increasing age of mycelia similarly transferred to -N and NO_3^- media (Figure 8b, 8c, Table III).

Addition of tungstate ($WO_4^=$) to NO_3^- medium prevents the formation of active nitrate reductase; hence, it has been used to measure the rate of decay of nitrate reductase formed prior to $WO_4^=$ addition in mycelia incubated with NO_3^-(Sorger et al., 1974). The addition of $WO_4^=$ to -N or NH_4^+ medium has no effect on the rate of decay of nitrate reductase activity in vivo in 28 hour old mycelia (Table IV). In 48 and 72 hour old mycelia however, the rate of decay of nitrate reductase is greater in the presence of $WO_4^=$ in all conditions.

FIGURE 8

Decay of nitrate reductase activity *in vivo*

a) 28 hour old mycelia

b) 48 hour old mycelia

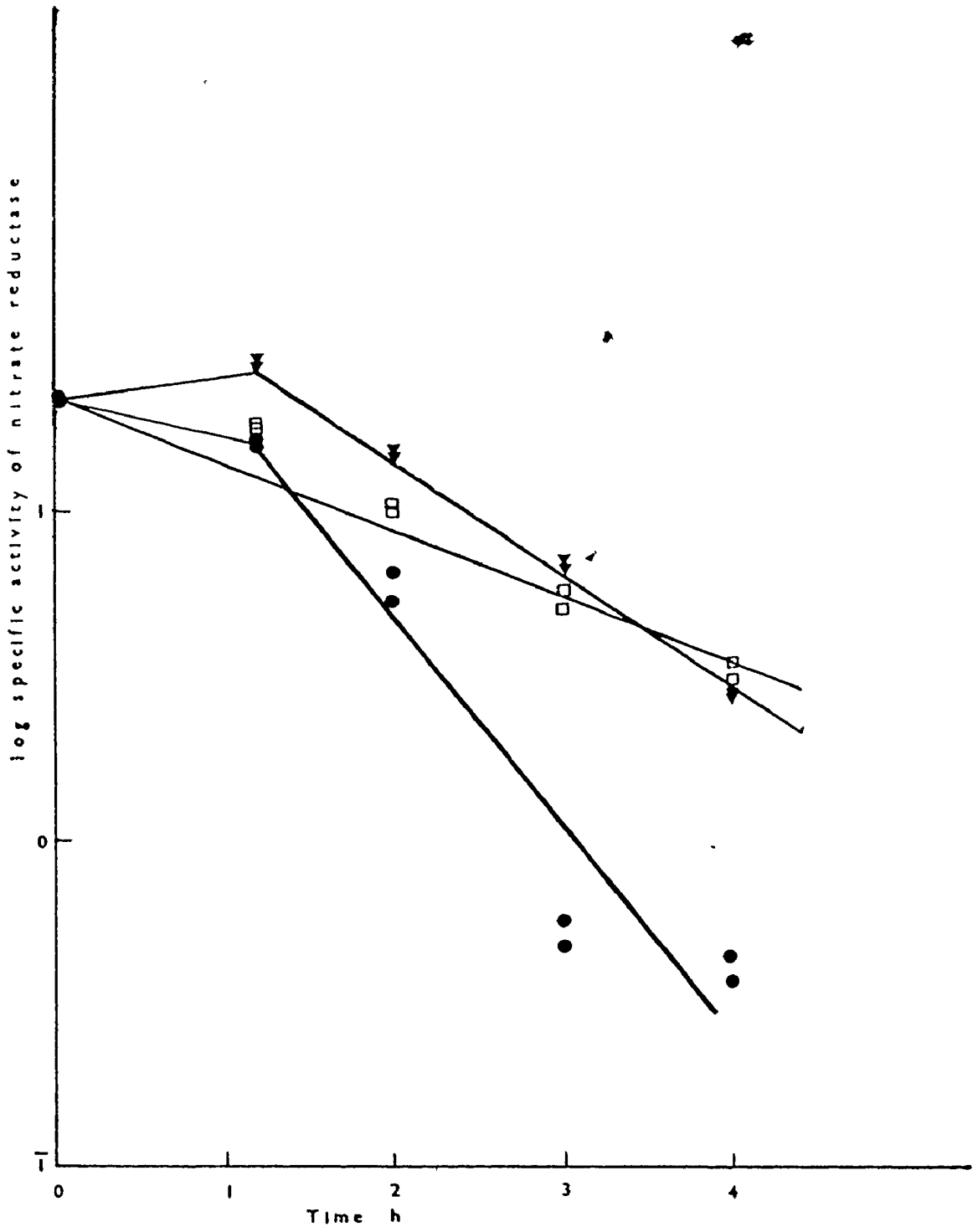
c) 72 hour old mycelia

Mycelial pads pregrown on NH_4^+ media for 28 hours (a), 48 hours (b) or 72 hours (c), were induced for 4 hours on NO_3^- induction media and then transferred at 0 hours to -N media (●), to NO_3^- media (□), or to NH_4^+ media (▼). These three media all contained 6mM Na_2WO_4. The pads were harvested at the indicated times, cell free extracts prepared and assayed for nitrate reductase activity. The extraction buffer for the 28 hour old mycelia contained 2% BSA. The mycelial contents of protein in mg/pad are given below:

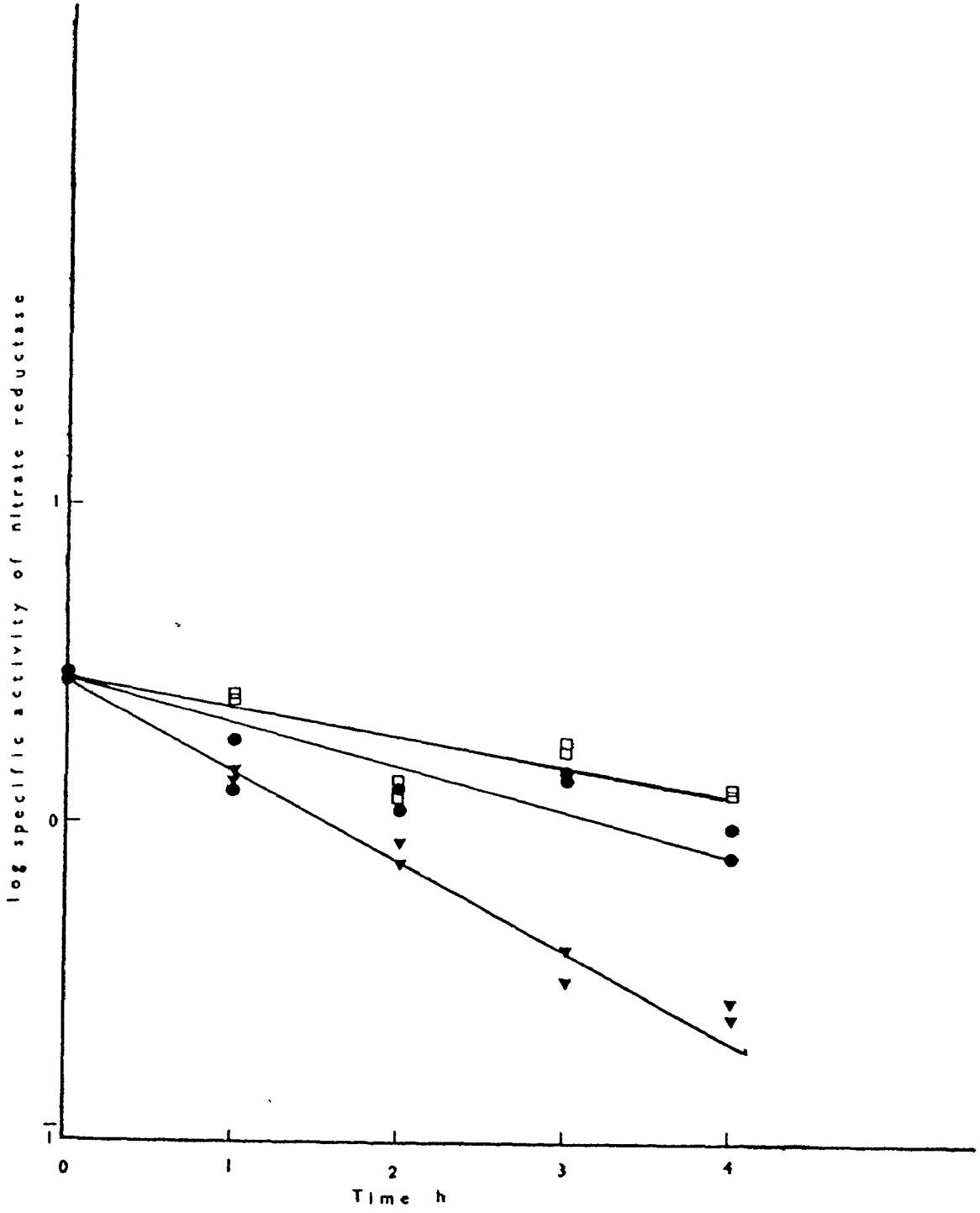
(Figure 8, cont.)

Hours exposure to decay conditions	mg protein/pad				
	0 hours	1 hour	2 hours	3 hours	4 hours
28 hour old mycelia					
-N medium	4.7	4.0	3.0	4.3	3.3
NO_3^- medium		3.7	3.7	4.4	5.8
NH_4^+ medium		2.7	3.3	4.6	5.8
48 hour old mycelia					
-N medium	13.2	13.8	16.4	13.4	13.6
NO_3^- medium		17.0	14.8	16.0	15.4
NH_4^+ medium		16.6	15.6	19.4	16.0
72 hour old mycelia					
-N medium	13.6	17.8	19.2	13.6	18.6
NO_3^- medium		20.0	18.6	17.6	19.4
NH_4^+ medium		15.4	18.6	19.6	20.2

a) 28 hour old mycelia



b) 48 hour old mycelia



(c) 72 hour old mycelia

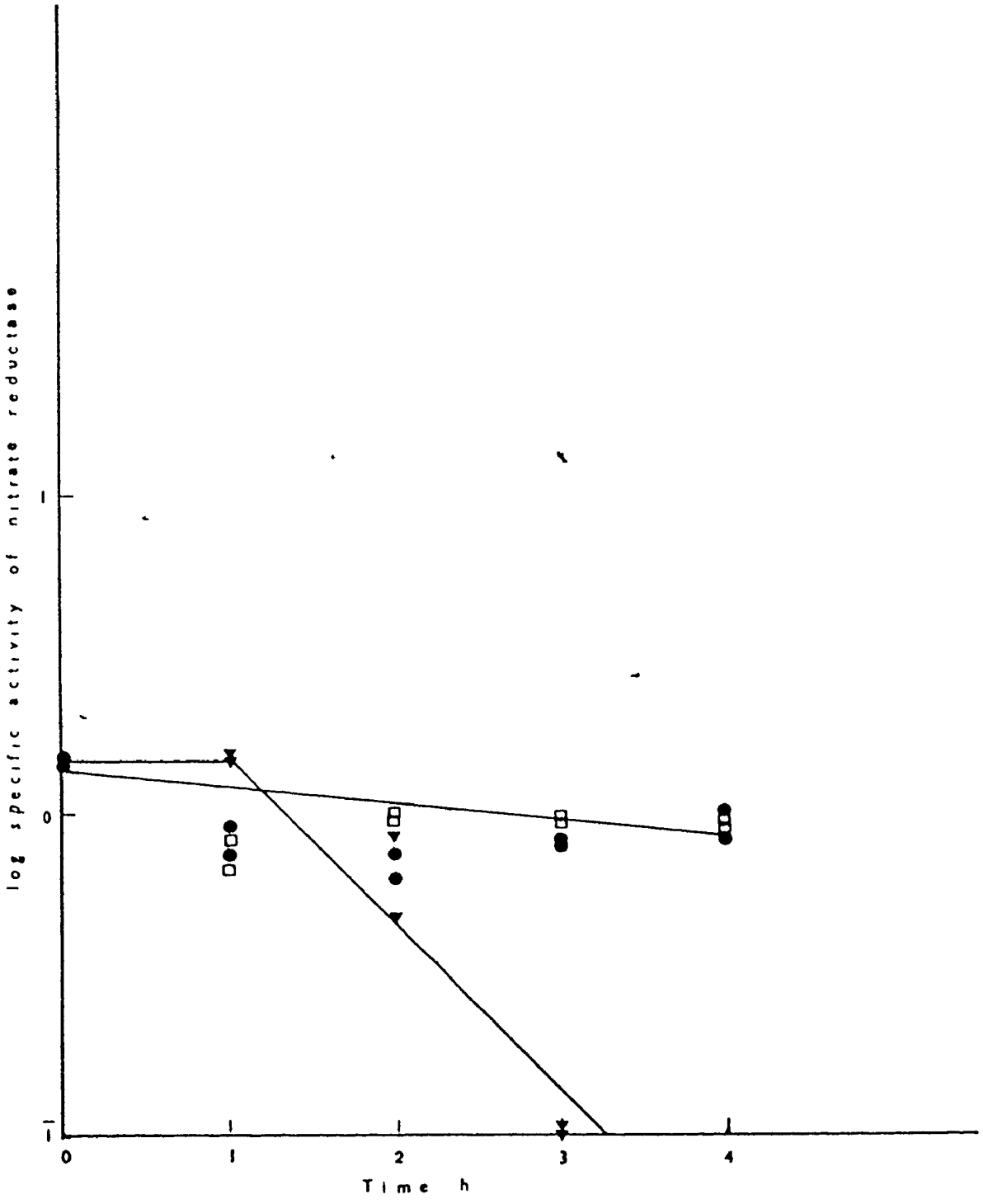


TABLE III

Decay of nitrate reductase activity *in vivo*

Age of mycelial pad	Rate of decay of nitrate reductase (Δ log of specific activity / h)		
	-N media	NO_3^- media	NH_4^+ media
28 hour	-0.59	-0.25	-0.33
48 hour	-0.10	-0.09	-0.27
72 hour	-0.04	-0.04	-0.33

Mycelial pads, pregrown on NH_4^+ media for 28 hours, 48 hours, or 72 hours, were induced on NO_3^- induction media for 4 hours, and then transferred to -N media, to NO_3^- media, or to NH_4^+ media. These three media all contained 6mM Na_2WO_4. Mycelial pads were harvested at hourly intervals for four hours and the extracts were assayed for nitrate reductase activity. The extraction buffer for the 28 hour old mycelia contained 2% BSA. The rate of decay of nitrate reductase activity was determined using linear regression analysis. The initial reading at 0 hours was omitted from the linear regression analysis when it was not in alignment.

TABLE IV

Effect of tungstate on the rate of decay of nitrate reductase *in vivo*

Age of mycelial pad	Decay medium	Rate of decay of nitrate reductase (Δ log of specific activity / h)	
		$+WO_4^=$	$-WO_4^=$
28 hour	- N	-0.59	-0.71
	NH_4^+	-0.33	-0.30
48 hour	- N	-0.10	+0.06/-0.51 * (3 hrs / 1 hr)
	NH_4^+	-0.27	-0.14
72 hour	- N	-0.04	+0.20/0 * (2 hr / 2 hr)
	NH_4^+	-0.33	-0.04

Mycelial pads, pregrown on NH_4^+ media for 28 hours, 48 hours or 72 hours, were induced on NO_3^- induction media for 4 hours and then transferred to -N media or to NH_4^+ media in the presence and absence of 6mM Na_2WO_4. Mycelial pads were harvested at hourly intervals for four hours and the extracts assayed for nitrate reductase activity. The extraction buffer for the 28 hour old mycelia contained 2% BSA. The rate of decay of nitrate reductase activity was determined using linear regression analysis. The initial reading at 0 hours was omitted from the linear regression analysis when it was not in alignment.

* These readings represent a biphasic rate of decay.

Nitrate reductase in 48 hour and 72 hour old mycelia transferred from NO_3^- medium to -N or to NO_3^- medium has a slower rate of decay in vivo than enzyme in younger mycelia treated in the same way (Figure 8b, 8c, Table III). Nitrate reductase in mycelia similarly transferred to NH_4^+ medium has the same rate of decay irrespective of the age of the mycelium (Figure 8, Table III). The pattern of stability of nitrate reductase in cell free preparations of mycelia of different ages parallels that of the enzyme in vivo (Table II, Table III).

Nitrate reductase in fully induced mycelia transferred to NH_4^+ medium at 27°C is more stable in vivo than in comparable mycelia transferred to NH_4^+ medium at 37°C (Figure 9, Table III). When fully induced mycelia are transferred to -N or NO_3^- medium the rate of decay of nitrate reductase in vivo is the same at 37°C and 27°C though the lag period before decay begins is shorter at the higher temperature (Figure 9, Table V).

The rate of decay of nitrate reductase in vivo is influenced by the source of nitrogen in the culture medium, by the age of the mycelium and by temperature.

Relationship between levels of nitrate reductase and protease activity

It has been shown above that the rate of decay of nitrate reductase in cell free extracts varies in parallel fashion with the age of the mycelium from which the extract was prepared, and that the decay of the enzyme in vivo varies in parallel fashion with the age of the mycelium, with the nitrogen source of the medium, and with the temperature. If the decay of nitrate reductase is due to destruction of the enzyme by protease(s), then the level of protease(s) may also vary with these different conditions of decay.

FIGURE 9

Decay of nitrate reductase activity *in vivo*

at 27°C and 37°C

Mycelial pads pregrown on NH_4^+ media for 28 hours, were induced for 4 hours on NO_3^- induction media and then transferred at 0 hours to -N media (● , ○), to NO_3^- media (■ , □), or to NH_4^+ media (▼ , ▽) and incubated at either 27°C (● , ■ , ▼) or 37°C (○ , □ , ▽). The pads were harvested at the indicated times and nitrate reductase activity assayed. The extraction buffer contained 2% BSA. The mycelial contents of protein in mg/pad are given below:

(Figure 9, cont.)

Hours exposure to decay conditions	mg protein/pad				
	0 hours	1 hour	2 hours	3 hours	4 hours
27°C					
-N media	4.7	4.0	3.0	4.3	3.3
NO_3^- media		3.7	3.7	4.4	5.8
NH_4^+ media		2.7	3.3	4.6	5.8
37°C					
-N media	4.7	5.7	4.4	6.3	6.3
NO_3^- media		4.7	3.6	3.8	6.3
NH_4^+ media		5.3	4.5	7.6	5.6

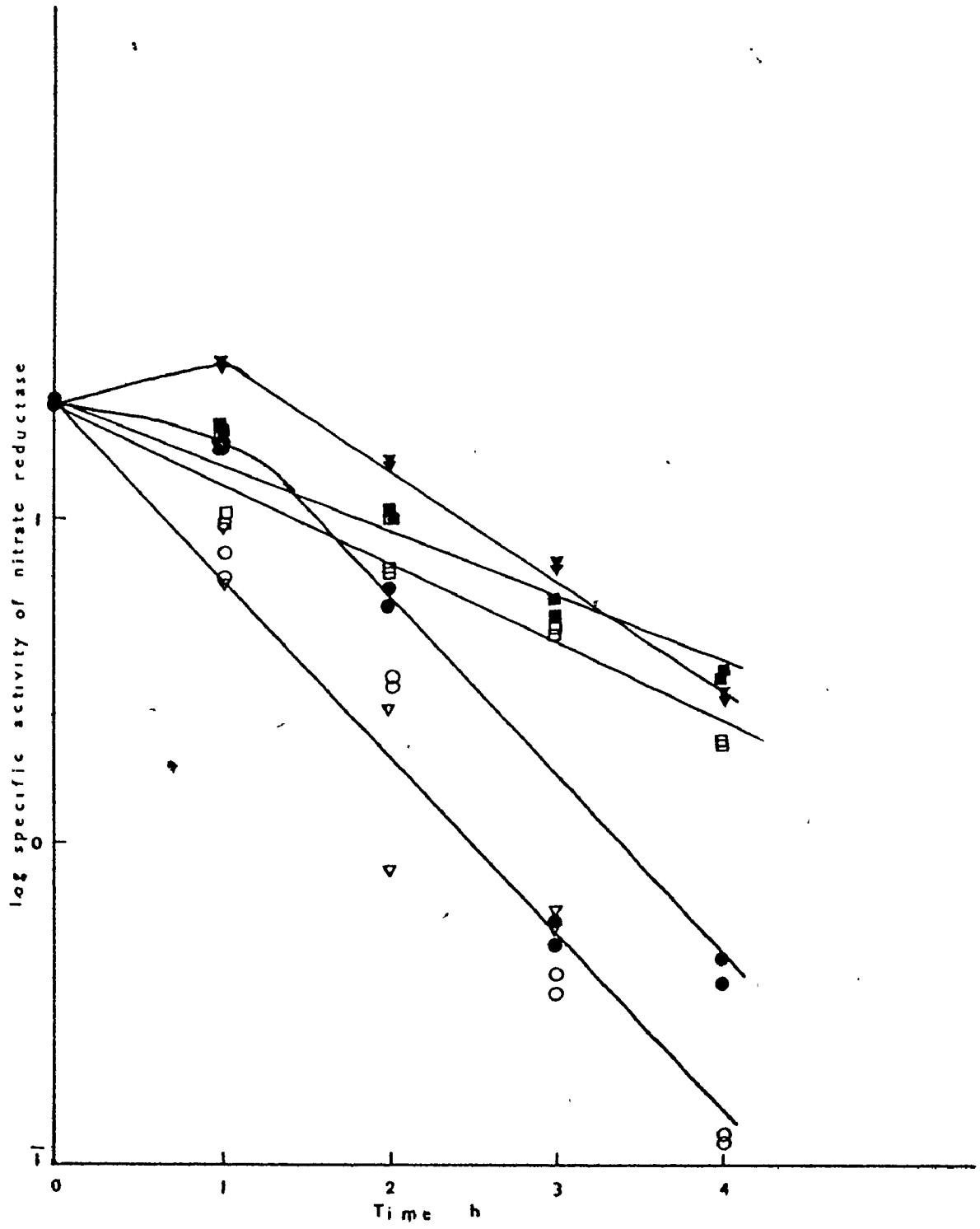

log specific activity of nitrate reductase
1
0
-1
0
1
2
3
4
Time h

TABLE V

Effect of temperature on the rate of decay of nitrate reductase activity *in vivo*

Decay media	Rate of decay of nitrate reductase (Δ log of specific activity / h)	
	27°C	37°C
-N media	-0.59	-0.57
NO_3^- media	-0.25	-0.24
NH_4^+ media	-0.33	-0.62

Mycelial pads, pregrown on *NH_4^+ media* for 28 hours, were induced on *NO_3^- induction media* for 4 hours and then transferred to *-N media*, to *NO_3^- media*, or to *NH_4^+ media* at 27°C or 37°C. These three media all contained 6mM Na_2WO_4. Mycelial pads were harvested at hourly intervals and the extracts assayed for nitrate reductase activity. The extraction buffer for all of the pads contained 2% BSA. The rate of decay of nitrate reductase activity was determined using linear regression analysis.

The results in Table VI show that the intracellular level of protease does not vary significantly in mycelia of different ages induced with NO_3^-. The results of Table VII indicate that there is no obvious correlation between the effects of the culture conditions on the rate of decay of nitrate reductase *in vivo*, and on the level of intracellular protease activity of the mycelium.

Cohen (1973) has shown that there is an NH_4^+ repressible extracellular protease in the fungus *Aspergillus niger*. The rate of decay of nitrate reductase *in vivo* was slower on NH_4^+ medium than on -N medium (Figure 8). Was this perhaps due to repression of a protease that can be measured as an extracellular protease?

The level of extracellular protease activity in inducing NO_3^- medium is highest when the medium is inoculated with 28 hour old mycelia, and becomes progressively lower in medium inoculated with mycelia of increasing age (Table VI). There is a relatively low level of extracellular protease activity in NH_4^+ medium at 27°C or 37°C inoculated with mycelia of different ages (Table VIII). As the age of mycelium transferred to -N or to NO_3^- medium increases, the level of extracellular protease activity decreases. The level of extracellular protease activity is higher in NO_3^- medium containing 28 hour old mycelia inoculated at 37°C than in similar medium inoculated at 27°C. The above effects do not appear to be due to the direct action of NH_4^+ or of NO_3^- on protease activity (Figure 10).

Twenty-eight hour old mycelia induced on NO_3^- medium, contain a less stable nitrate reductase and display a higher level of extracellular protease activity in the medium than do 72 hour old mycelia induced in the same fashion. This might appear to indicate a possible correlation between the stability of nitrate reductase and the level of extracellular protease activity; however, when the rate of decay of nitrate reductase

TABLE VI

Levels of intracellular and extracellular protease activity in nitrate-induced mycelia of different ages

Age of mycelial pad	Specific activity of intracellular protease (A_{280}/min/mg protein x 10^{-3})	Specific activity of extracellular protease (A_{280}/min/mg dry weight of mycelium x 10^{-6})
28 hour	3.4	51
	3.5	51
48 hour	3.1	24
	3.2	23
72 hour	2.8	17
	3.3	18

Mycelial pads, pregrown on $\underline{NH_4^+}$ media for 28 hours, 48 hours or 72 hours, were induced on $\underline{NO_3^-}$ induction media for 4 hours. The pads were harvested and the extracts were assayed for intracellular protease activity and the culture filtrate assayed for extracellular protease activity.

Table VII

Mycelial pads, pregrown on NH_4^+ media for 28 hours, 48 hours, or 72 hours, were induced on NO_3^- induction media and then transferred to -N media, to NO_3^- media, or to NH_4^+ media and incubated for 4 hours at 27°C or 37°C. The pads were harvested and the extracts were assayed for intracellular protease activity.

TABLE VII

Levels of intracellular protease activity in extracts of mycelia of different ages exposed to media containing different nitrogen sources and incubated at different temperatures

Age of mycelial pad	Specific activity of intracellular protease (A_{280}/min/mg protein x 10^{-3})		
	-N media	NO_3^- media	NH_4^+ media
28 hour	5.9	3.8	4.9
	5.4	3.8	5.3
48 hour	2.7	-	2.6
	3.0	-	2.6
72 hour	2.2	1.8	1.8
	2.0	2.3	1.7

b)	NH_4^+ media 27°C	NH_4^+ media 37°C
28 hour	2.4	2.4
	2.5	2.0
48 hour	3.9	3.8
	3.9	3.8
72 hour	2.5	2.6
	2.3	

FIGURE 10

Effect of nitrate and ammonium ions on extracellular protease activity

Mycelial pads were treated as described in Figure 3. Extracellular protease activity was assayed in the presence of varying concentrations of either $NaNO_3$ (■) or of ammonium tartrate (▽).

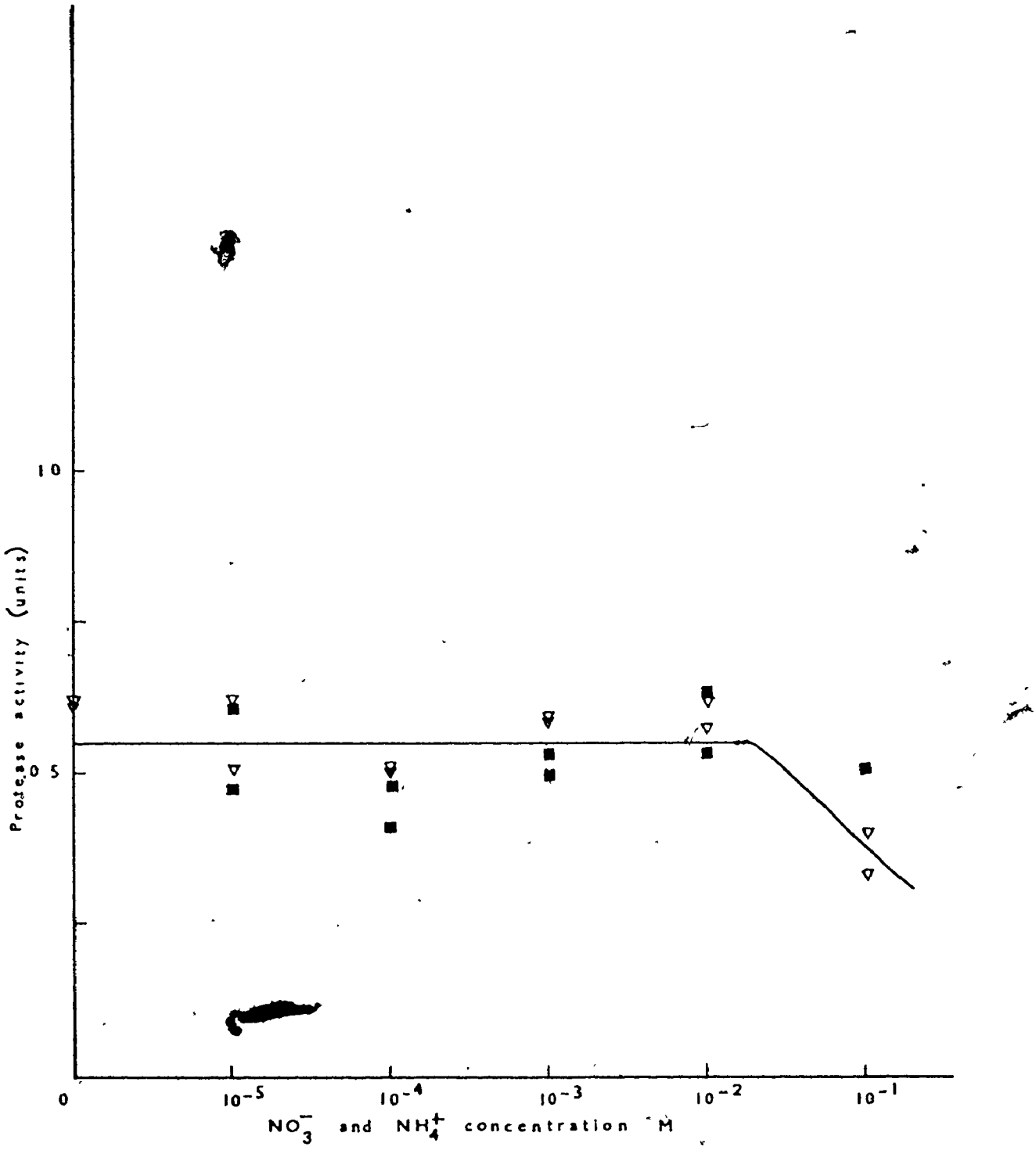

Protease activity (units)
10
5
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
NO_3^- and NH_4^+ concentration M

Table VIII

Mycelial pads, pregrown on NH_4^+ media for 28 hours, 48 hours or 72 hours, were induced on NO_3^- induction media for 4 hours and then transferred to -N media, to NO_3^- media or to NH_4^+ media at 27°C or 37°C. These three media all contained 6mM Na_2WO_4. The pads were harvested at hourly intervals for four hours and the extracts were assayed for nitrate reductase activity. The extraction buffer for the 28 hour old mycelia contained 2% BSA. The rate of decay of nitrate reductase activity was determined using linear regression analysis. The culture filtrate was assayed for extracellular protease activity.

TABLE VIII

Extracellular protease activity and rate of decay of nitrate reductase activity *in vivo* under different conditions

-N media	Age of mycelial pad	Specific activity of extracellular protease (A_{280}/min/mg dry weight of mycelium x 10^{-6})	Rate of decay of nitrate reductase (Δ log specific activity/h)
	28 hours	193	-0.59
		193	
	48 hours	88	-0.10
		84	
	72 hours	78	-0.04
		78	
	27°C 28 hours	193	-0.59
		193	
	37°C 28 hours	170	-0.57
		180	

TABLE VIII cont.

NO_3^- media	Age of mycelial pad	Specific activity of extracellular protease (A_{280}/min/mg dry weight of mycelium x 10^{-6})	Rate of decay of nitrate reductase (Δ log specific activity/h)
	28 hours	103	-0.25
		141	
	48 hours	85	-0.09
		81	
	72 hours	116	-0.04
		113	
	27°C 28 hours	103	-0.25
		141	
	37°C 28 hours	200	-0.24
		214	

TABLE VIII cont.

NH_4^+ media	Age of mycelial pad	Specific activity of extracellular protease (A_{280}/min/mg dry weight of mycelium x 10^{-6})	Rate of decay of nitrate reductase (Δ log specific activity/h)
	28 hours	5	-0.33
		5	
	48 hours	0	-0 27
		0	
	72 hours	0	-0.33
		0	
	27°C 28 hours	5	-0.33
		5	
	37°C 28 hours	20	-0.62
		33	

in vivo was compared with the level of extracellular protease activity in the medium under different conditions, the correlation was weak (Table VIII). The rate of decay of nitrate reductase in vivo, and the level of extracellular protease activity in the medium both decrease with increasing age of mycelia incubated in -N and NO_3^- media at 27°C (Table VIII). The rate of decay of nitrate reductase in vivo is about the same in mycelia of different ages incubated in NH_4^+ medium, but the extracellular protease activity is repressed to a very low level (Table VIII). When the temperature is raised to 37°C, the rate of decay of nitrate reductase in vivo is relatively fast only in mycelia incubated in NH_4^+ medium, whereas the level of extracellular protease activity is relatively high mainly in NO_3^- medium (Table VIII). These observations would suggest that the rate of decay of nitrate reductase in vivo might be influenced in parallel fashion with extracellular protease activity, but the correlation is not a simple one.

Induction of nitrate reductase

The accumulation of nitrate reductase during induction is a function both of the rate of synthesis and the rate of degradation of the enzyme. The rate of decay of nitrate reductase in vivo varied with the nitrogen source of the medium, the temperature and the age of the mycelium. Consequently it was of interest to see how these three variables affected the rate of induction of nitrate reductase. The 28 hour old mycelia contained the highest level of nitrate reductase (Table I) and displayed the fastest rate of induction of the enzyme (Figure 11). As the mycelia increased in age, the rate of induction of nitrate reductase became progressively less (Figure 11). Nitrate reductase was synthesized more rapidly initially at 37°C than it was at 27°C. The rapid induction at 37°C was followed by a decrease in the level of nitrate

FIGURE 11

Induction of nitrate reductase activity on $\underline{NO_3^-}$ induction media

a) 28 hour old mycelia

b) 48 hour old mycelia

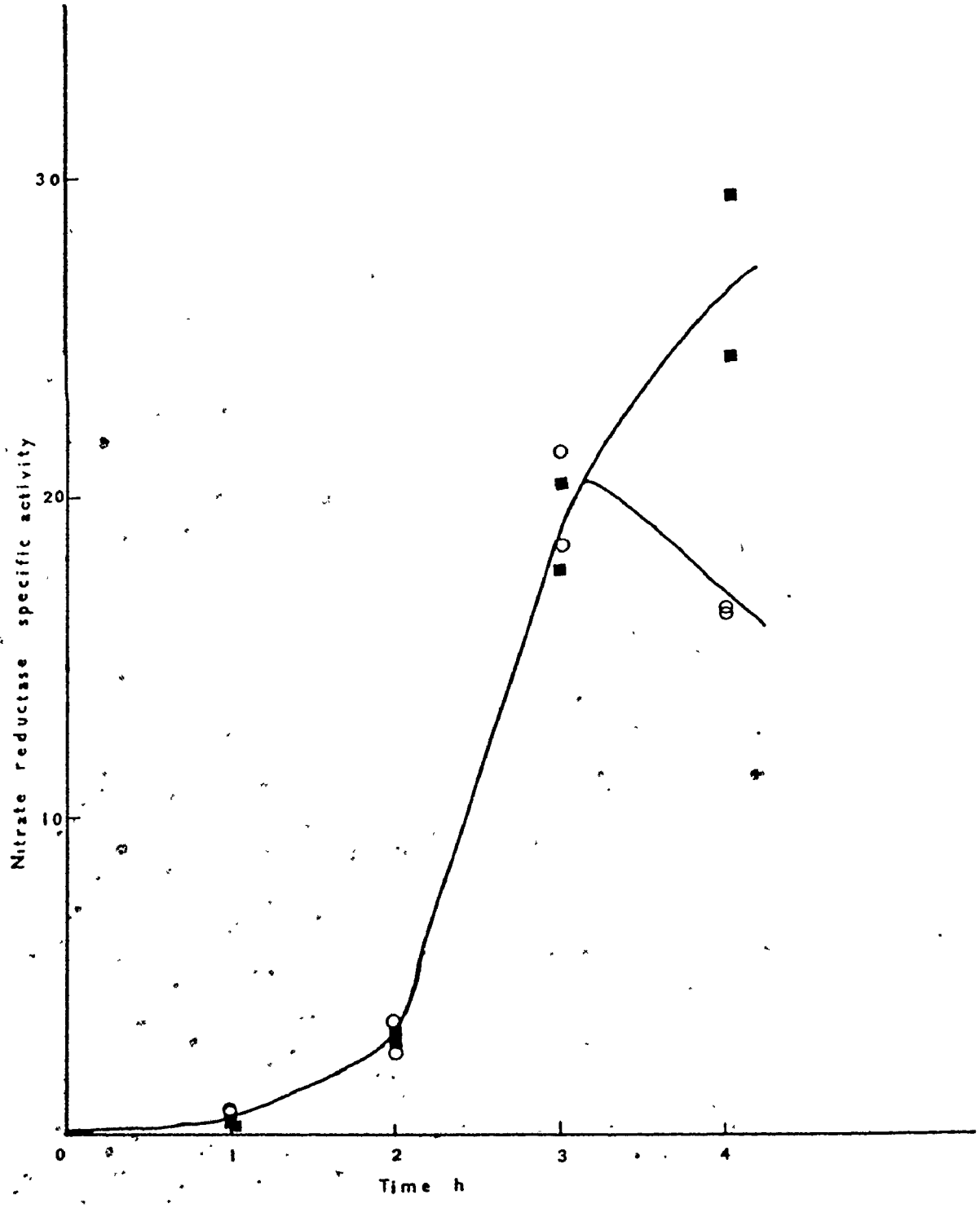
c) 72 hour old mycelia

Mycelial pads pregrown on $\underline{NH_4^+}$ media for 28 hours (a), 48 hours (b), or 72 hours (c) were transferred to $\underline{NO_3^-}$ induction media at 0 hours and incubated at either 27°C (■) or at 37°C (o). The pads were harvested at the indicated times and assayed for nitrate reductase activity. The extraction buffer for the 28 hour old mycelia contained 2% BSA. The mycelial contents of protein in mg/pad are given below:

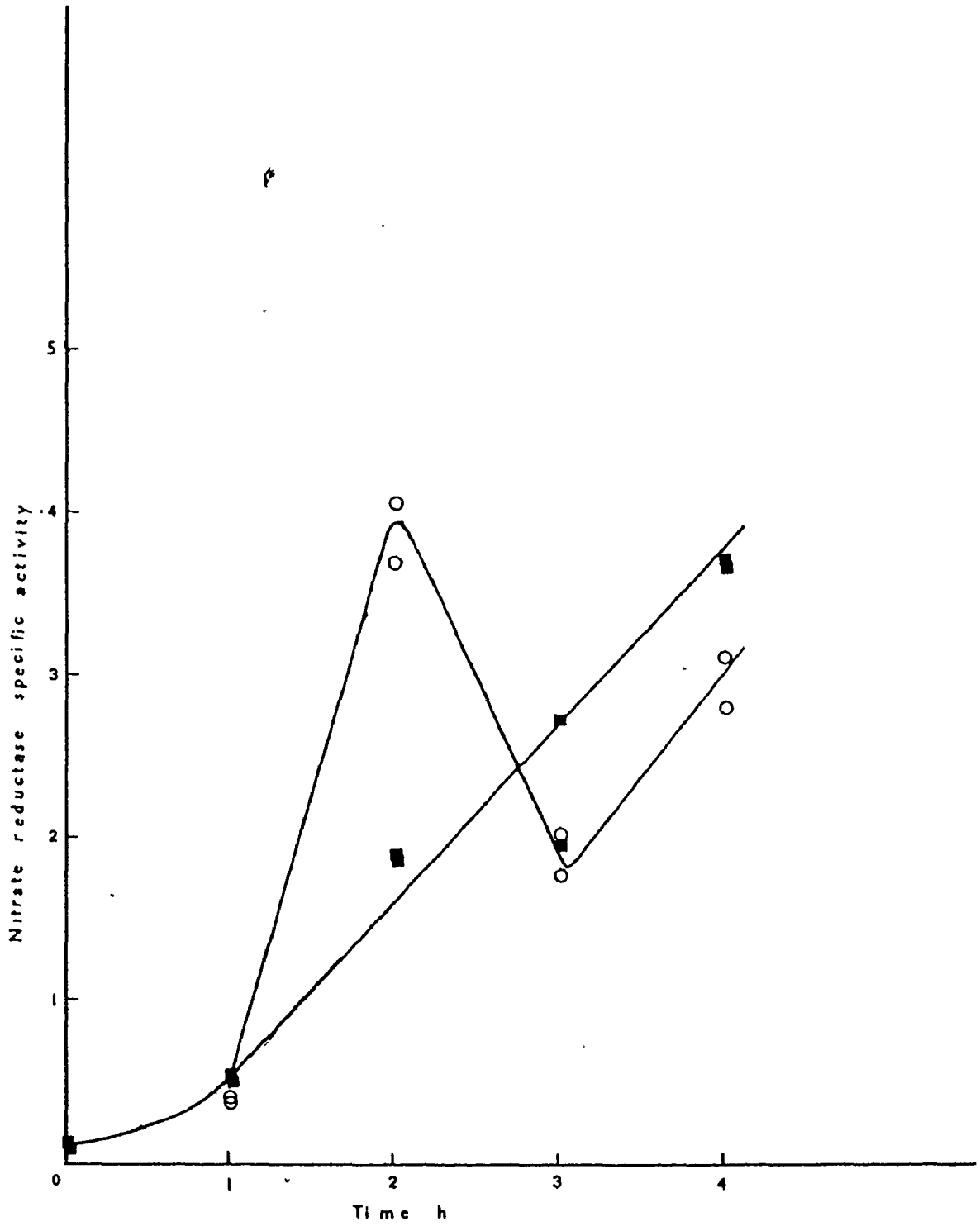
(Figure 11, cont.)

Hours exposure to induction media	mg protein/pad				
	0 hours	1 hour	2 hours	3 hours	4 hours
28 hour old mycelia					
27°C		3.3	3.7	2.9	3.4
37°C		3.3	3.7	3.7	2.8
48 hour old mycelia					
27°C	11.4	11.4	12.0	12.4	12.2
37°C		12.0	11.8	14.6	13.2
72 hour old mycelia					
27°C	15.8	17.4	16.6	15.2	15.4
37°C		16.6	15.8	16.6	15.8

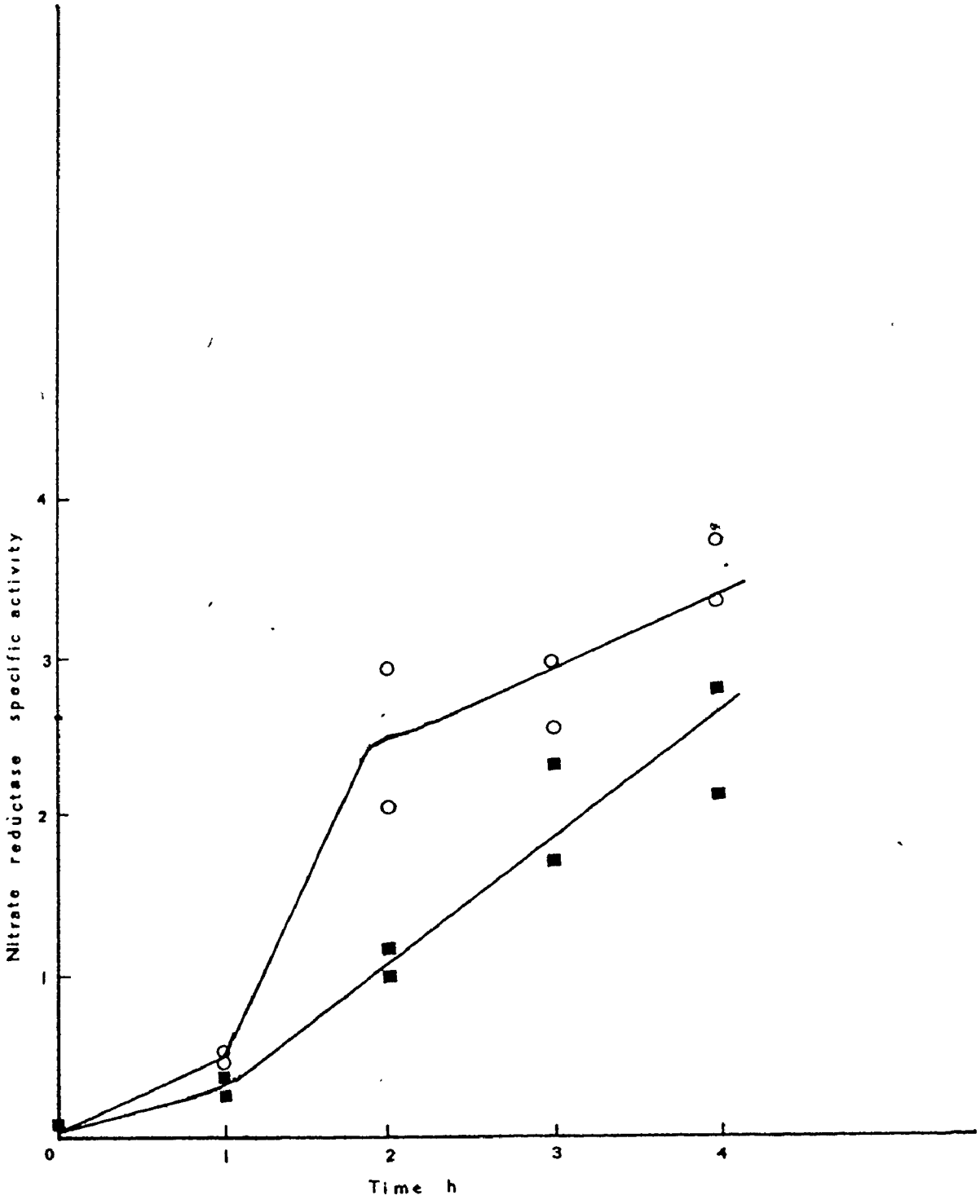
a) 28 hour old mycelia



b) 48 hour old mycelia



c) 72 hour old mycelia



reductase in the third or fourth hour of induction, while at 27°C the level of nitrate reductase continued to increase during the entire period examined (Figure 11).

The general pattern of derepression of nitrate reductase on -N medium is similar to that of the induction of the enzyme on NO_3^- medium. The most rapid derepression of the enzyme occurred in 28 hour old mycelia, while older mycelia had progressively slower rates of derepression (Figure 12). In all cases the level of nitrate reductase fell after the fourth hour of derepression. The lack of derepression when cycloheximide was present in the culture media suggests that de novo synthesis of nitrate reductase was involved (Figure 12).

FIGURE 12

Induction of nitrate reductase activity on -N media

a) 28 hour old mycelia

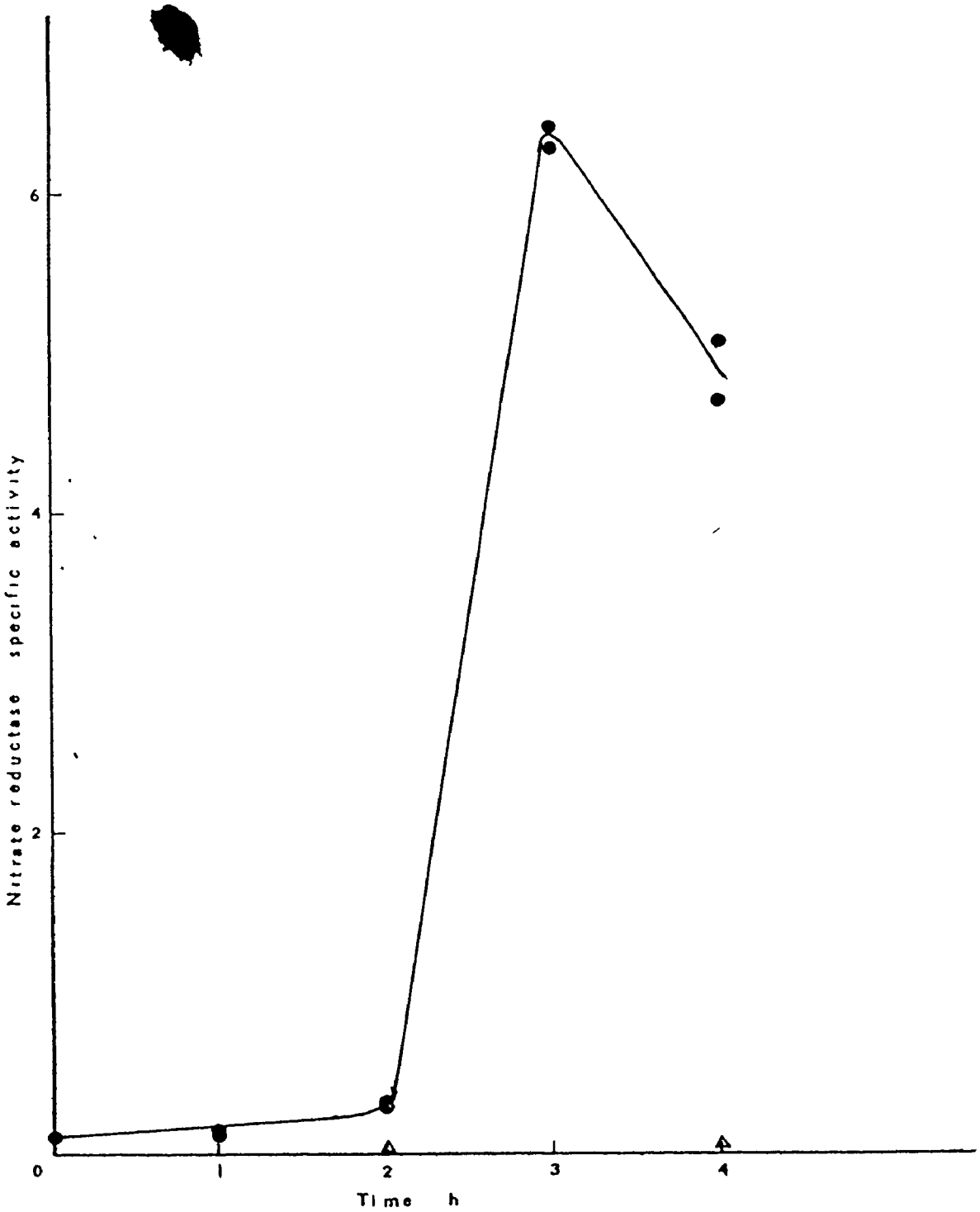
b) 48 hour old mycelia

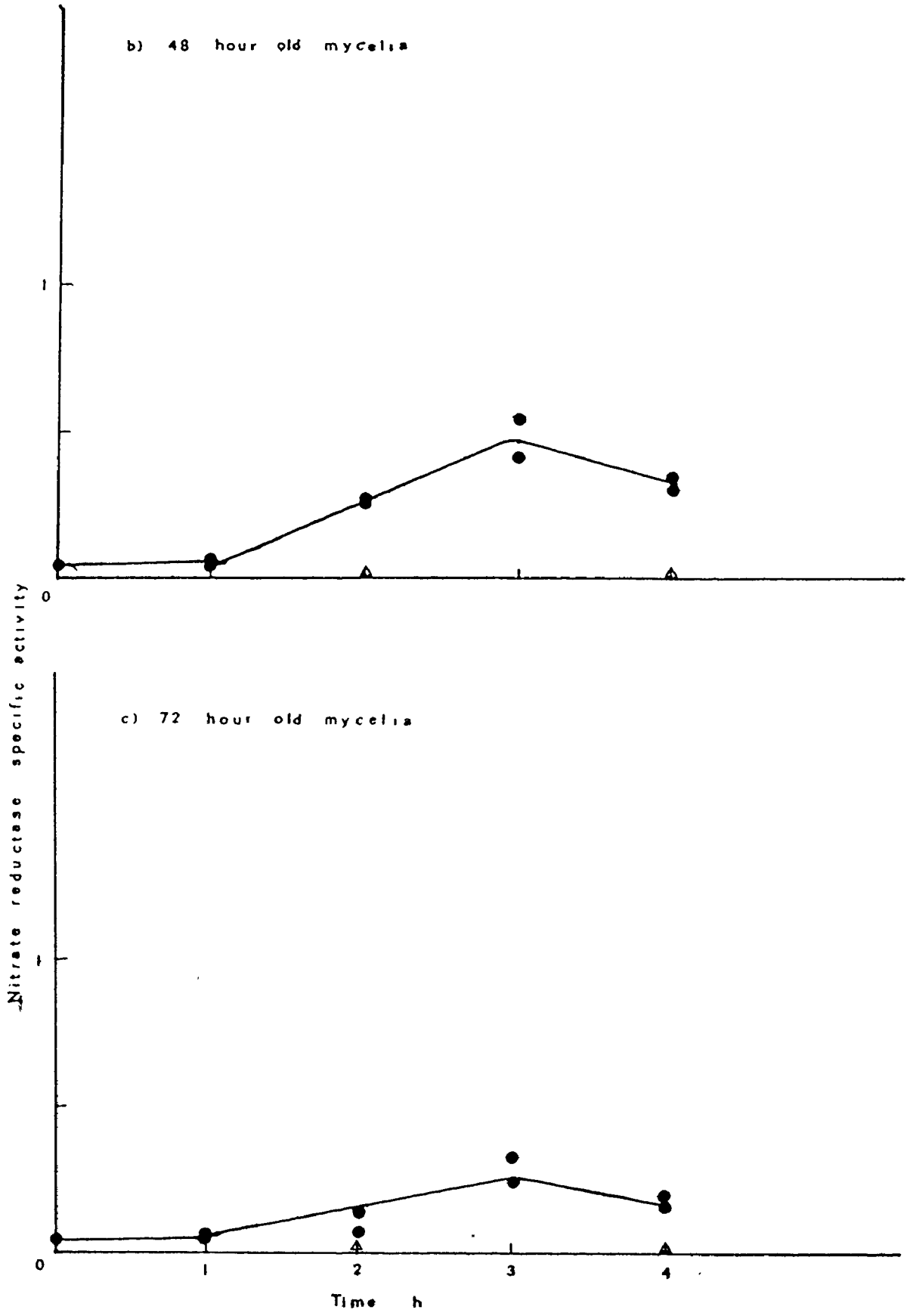
c) 72 hour old mycelia

Mycelial pads, pregrown on NH_4^+ media for 28 hours (a), 48 hours (b) or 72 hours (c), were transferred at 0 hours to -N media (●) or to -N media plus cycloheximide (1 µg/ml) (△). The pads were harvested at the indicated times and assayed for nitrate reductase activity. The extraction buffer for all of the pads contained 2% BSA. The mycelial content of protein in mg/pad is given below:

Hours exposure to -N media	mg protein/pad			
	1 hour	2 hours	3 hours	4 hours
28 hours	3.7	4.3	2.8	1.7
48 hours	12.6	14.6	17.4	11.4
72 hours	14.6	16.8	16.4	19.6

a) 28 hour old mycelia




b) 48 hour old mycelia
1
0
c) 72 hour old mycelia
1
0
1
2
3
4
Time h
Nitrate reductase specific activity

DISCUSSION

In this study there is some evidence for the involvement of a protease(s) in the disappearance of nitrate reductase in Neurospora crassa. While intracellular levels of protease activity do not seem to be correlated in any simple way with the rate of decay of nitrate reductase activity in vivo, the levels of extracellular protease activity seem to show some correlation with the rapidity of decay of nitrate reductase activity in vivo and in vitro.

Sorger et al. (1974) showed that nitrate reductase protein in Neurospora crassa, when measured by immunological techniques, disappears in parallel with nitrate reductase activity during non-inducing conditions. This evidence is consistent with the hypothesis that a protease(s) degrades the nitrate reductase in vivo. An inactivating enzyme that appears to be specific for nitrate reductase has been isolated from mature roots of corn (Wallace, 1973, 1974). This inactivating enzyme digests casein and is inhibited by phenylmethylsulfonyl fluoride (PMSF), suggesting that it is a serine protease. Cohen (1973) found that three neutral or alkaline proteases were secreted into the media by the fungus Aspergillus niger. All three of these enzymes digested casein when incubated at 45°C, at pH 8-9, and all were repressed by 50mM NH_4^+. If similar proteases are present in Neurospora, the assay used in this study would measure a mixture of the three.

The levels of extracellular protease from 28 hour old mycelia incubated on -N media are higher than those from similar mycelia incubated on NO_3^- media. This is perhaps similar to the higher level of protease activity in bacteria incubated on media deficient in carbon or

nitrogen than in bacteria on growth media (Mandelstam, 1958, Goldberg, 1971; Nath and Koch, 1971).

The instability of nitrate reductase in cell free extracts and the rate of decay of nitrate reductase activity *in vivo* when mycelia are transferred to either -N or NO_3^- media, decrease with increasing age of the mycelia. The level of extracellular protease activity also decreases with increasing age of mycelia incubated under these conditions. In apparent contrast to these observations, it is usual for bacteria in log phase of growth to have less protease activity than bacteria in stationary phase (Mandelstam, 1958; Goldberg, 1971; Nath and Koch, 1971). The specific activity of most enzymes is highest in extracts of cells in their exponential stage of growth, hence it is uncertain whether the correlation of extracellular protease activity with the age of the mycelia and hence with the rate of decay of nitrate reductase activity is significant. If a protease is involved in the decay of nitrate reductase activity, the correlation might have been obscured in this study because the assay for protease may have measured the activity of more than one protease.

The protective effect of BSA on nitrate reductase in cell free extracts at 30°C, is consistent with the hypothesis that nitrate reductase is destroyed by protease(s). BSA has been shown to decrease the instability of nitrate reductase activity during purification (Leclair and Grant, 1972, Oaks, personal communication) and during extraction of the enzyme (Schrader *et al.*, 1974) from mature roots of corn. Schrader *et al.* (1974) suggested that the BSA protected the activity either by preventing the dissociation of nitrate reductase, by preventing the action of an inhibitor on nitrate reductase or by preventing the binding of nitrate reductase to broken plastids or organelles. While this last alternative could explain how BSA might increase levels of activity in the extract, it does not explain how BSA would make nitrate reductase activity more

stable when incubated at 30°C.

The presence of the substrate NO_3^- also increased the stability of nitrate reductase activity in cell free extracts. This observation is in contrast to reports by Wallace (1973) that 30mM NO_3^- did not protect nitrate reductase from inactivation. In this study NO_3^- seems to partially protect nitrate reductase activity from decay *in vivo*, since the rate of decay is less on NO_3^- media than on -N media. The protective effect of NO_3^- is in agreement with the observations of Sorger *et al* (1974). These investigators found that the presence of WO_4^- in the culture medium did not affect the rate of decay of nitrate reductase on -N or NH_4^+ media. In this study, addition of WO_4^- had no effect on the rate of decay of nitrate reductase in 28 hour old mycelia incubated on -N and NH_4^+ media, but when WO_4^- was added to 48 hour or to 72 hour old mycelia on these media, the rate of decay of nitrate reductase was increased by its presence. Since 48 and 72 hour old mycelia are in the stationary phase of growth, the rate of metabolism in these mycelia is slower than in 28 hour old mycelia and this may mean that pools of NO_3^- might disappear more slowly than in the younger mycelia. Thus, induction might be occurring after transfer from NO_3^- media to -N or to NH_4^+ media. When WO_4^- is added to the media an inactive enzyme is synthesized, and continuing induction would result in no active nitrate reductase. This may explain the apparent effect of WO_4^- on the rate of decay of nitrate reductase in 48 and 72 hour old mycelia.

The rate of decay of nitrate reductase in mycelia transferred to NH_4^+ media was uniformly high in 28 hour, 48 hour and 72 hour old mycelia; in mycelia transferred to -N or NO_3^- media the rate of decay of the enzyme decreased with increasing age of the mycelium. With a rise in incubation temperature from 27°C to 37°C the rate of decay of nitrate reductase activity increased in mycelia on NH_4^+ media; in mycelia transferred to -N or NO_3^- media there was no increase in the rate

of decay when the temperature was raised.

It seems likely that in mycelia incubated on NH_4^+ media the mechanism of decay of nitrate reductase is different from that in mycelia incubated on -N or NO_3^- media. Several workers have suggested that NH_4^+ may help to regulate nitrate reductase activity by its effect on the redox state of the fungus, or by its effect on the level of pyridoxamine and pyridoxal phosphate (Davies, 1972, Herrera et al., 1972, Losada et al., 1973, Solomonson, 1974). These changes could alter the susceptibility of nitrate reductase to inactivation or to degradation by a protease. Pan and Marsh (1972) found evidence for two inhibitors of nitrate reductase activity in corn, one that acts slowly and is temperature dependent and another that acts more rapidly. It is possible that these inhibitors may correspond with two mechanisms of decay of nitrate reductase in Neurospora crassa.

Since the level of nitrate reductase activity is a function of the rate of degradation and the rate of synthesis of the enzyme, it was of interest to examine the rate of induction in conditions similar to those in which decay was measured. There is some evidence in the literature for a higher level of nitrate reductase activity in younger tissue of cotton, cauliflower and Chlamydomonas than in older tissue (Hewitt and Afridi, 1959, Herrera et al., 1972; Bilal and Rains, 1973). This could be a result of greater induction or a slower decay of the enzyme in young tissue compared with older tissue. Oaks et al. (1972) showed that the initial rate of induction of nitrate reductase activity in young root tips of corn was slightly faster at 29 units/hour than in the mature root at 16 units/hour. In this study the rate of induction in 28 hour old mycelia was approximately twenty times greater than that in 48 and 72 hour old mycelia on both NO_3^- and -N media. Oaks et al. (1972) showed that the rate of decay of nitrate reductase in young root tips was slower than that in the mature root. Thus the higher level of enzyme activity

in the root tip of corn was due to both a faster induction and slower decay of the activity than in the mature root. In Neurospora crassa however, the nitrate reductase in 28 hour old mycelia has the fastest rate of decay as well as the most rapid rate of induction of activity. The enzyme in 28 hour old mycelia is therefore subject to very rapid rates of turnover.

The derepression of nitrate reductase activity measured after transfer from ammonia growth media to -N media was much faster in 28 hour old mycelia than in 48 hour or in 72 hour old mycelia. The absence of derepressed activity in the presence of cycloheximide suggests that nitrate reductase was made de novo. This synthesis of nitrate reductase in 28 hour old mycelia on -N media is slow compared with the rate of decay of the enzyme. When $WO_4^=$ was added to -N media the rate of decay of nitrate reductase did not increase as one would expect if derepressed synthesis was contributing significantly to the level of the enzyme. The synthesis of nitrate reductase was very limited in 48 hour and 72 hour old mycelia transferred from the growth media to -N media. However, when $WO_4^=$ was added to -N media containing these older mycelia, the rate of decay of nitrate reductase increased, indicating that some synthesis of the enzyme was occurring when $WO_4^=$ was absent. Since this synthesis of nitrate reductase in 48 hour and 72 hour old mycelia occurred on NH_4^+ media as well as -N media, it is probably more likely that it is a result of induced synthesis from slowly disappearing pools of NO_3^- rather than from derepressed synthesis.

Zielke and Filner (1971) also found evidence for the synthesis of nitrate reductase during decay conditions in -N media. In order to measure the actual rate of synthesis and degradation of nitrate reductase, they used a technique dependent on density and radioactive labelling. They were unable to calculate the actual rates of turnover because general protein turnover was high enough to supply ^{15}N-amino

acids to the pool after the cells had been transferred to a ^{14}N-amino acid medium. The data was sufficient however, to show that both synthesis and degradation occurred in both exponential and stationary phase cells growing on nitrate media and also in exponential phase cells transferred from nitrate to no nitrate medium.

Sorger et al. (1974) looked at the effect of NO_3^- on the turnover of nitrate reductase activity in Neurospora crassa. They found that NO_3^- had a greater effect on the rate of induction than on the rate of decay of the enzyme activity. This present work shows that the exact age of the mycelia is an important consideration when measuring rates of turnover. In 28 hour old mycelia, the rate of induction of nitrate reductase activity on NO_3^- induction media is about three times greater than that in mycelia on -N media. When 28 hour old induced mycelia is transferred to NO_3^- media (containing $WO_4^=$), the rate of decay of nitrate reductase activity is about one third that of induced mycelia transferred to -N media. In 48 hour and 72 hour old mycelia, there is no correlation of the effect of NO_3^- on rates of induction and decay of the enzyme. In these older mycelia the rate of decay of nitrate reductase is very slow and the resulting measurements are too inaccurate for a valid comparison between -N and NO_3^- media. In the exponentially growing mycelia, it would appear that the effect of NO_3^- on the rate of induction of nitrate reductase might be able to be explained in terms of stabilizing the enzyme. Further work would be necessary to validate this suggestion.

In this study then, there seems to be some evidence for two mechanisms of decay of nitrate reductase activity, with a possibility, but little evidence, for a protease involvement with one of these. It has been shown that the rate of induction and decay of the enzyme are very dependent on the age of the mycelia, with the rates of both being highest

in exponentially growing mycelia. Synthesis of nitrate reductase occurs in non-inducing conditions and the higher levels of activity found in $\underline{NO_3^-}$ $\underline{media}$ can perhaps be explained by the protective effect of NO_3^- on the stability of the enzyme.

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