THE METABOLIC CONTROL OF NITRITE REDUCTASE IN *NEUROSPORA CRASSA*

AN INVESTIGATION INTO SOME ASPECTS OF THE METABOLIC CONTROL OF NITRITE REDUCTASE IN *NEUROSPORA CRASSA*

By

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SCOPE AND CONTENTS:

Nitrate assimilation is the process by which nitrate is converted into ammonia, and ultimately into organic nitrogenous compounds, which are then made available to organisms which require an exogenous supply of organic nitrogen. Nitrite is an intermediate in this process and the mechanism of its conversion to ammonia, which is catalyzed by the enzyme nitrite reductase, needs clarification.

The purpose of this investigation was to find a suitable assay system for nitrite reductase in N. crassa and to examine some aspects of the metabolic control of the enzyme. A new assay system for nitrite reductase is described and evidence suggesting that the enzyme is derepressible is presented.

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INTRODUCTION

Plants and many microorganisms are capable of utilizing the inorganic salts nitrate, nitrite and ammonia. For most green plants and fungi nitrate is the major source of nitrogen, and the biological process by which it is converted to ammonia is known as nitrate assimilation.

The biological significance of nitrate assimilation is evident from the realization that the ultimate source of nitrogen for all forms of life is inorganic. Many microorganisms and virtually all animals require an exogenous supply of organic nitrogen because they are incapable of assimilating the more oxidized states of inorganic nitrogen. Thus green plants and numerous microoganisms, by virtue of their ability to utilize nitrate, are ultimately responsible for providing nitrogen to many chemo-organotrophic life forms.

Nitrate and nitrite can also be converted to molecular nitrogen, nitrous oxide or nitric oxide by certain microorganisms. This process is known as denitrification or nitrate dissimilation, and is well reviewed by Nason (26). Denitrifying bacteria are facultatively anaerobic organisms, found almost universally in soil and water. They use nitrate and nitrite as electron acceptors for energy yielding oxidative reactions, although the existence of a nicotinamide adenine dinucleotide - flavin

dependent nitrite reductase from Pseudomonas denitrificans has been reported (35), which does not appear to be involved in any energy yielding reactions.

The conversion of ammonia to nitrate (nitrification) is brought about by two specialized groups of aerobic, chemolithotrophic bacteria (26). Ammonia is oxidized to nitrite by members of the Nitrosomonas group, and nitrite is oxidized to nitrate by Nitrobacter. As a result of the combined activities of these bacteria the ammonia liberated during the mineralization of organic matter is rapidly oxidized to nitrate and can thus be assimilated by plants.

The conversion of nitrate to ammonia (or to amine groups) is the result of a series of oxidation - reduction reactions by which nitrate is reduced to ammonia, a reaction involving a net change of 8 electrons. The enzymatic mechanism for the reduction of nitrate to nitrite is well documented (26,27,34,43). This step is catalyzed by a soluble molybdo-flavoprotein, nitrate reductase. However the pathway, intermediates and mechanisms beyond nitrite are still in need of clarification.

If we assume, as has been suggested, that the biological reduction of nitrate proceeds via an inorganic pathway, and that two electron changes are involved for each enzymatic step, then the following sequence of intermediates can be postulated:

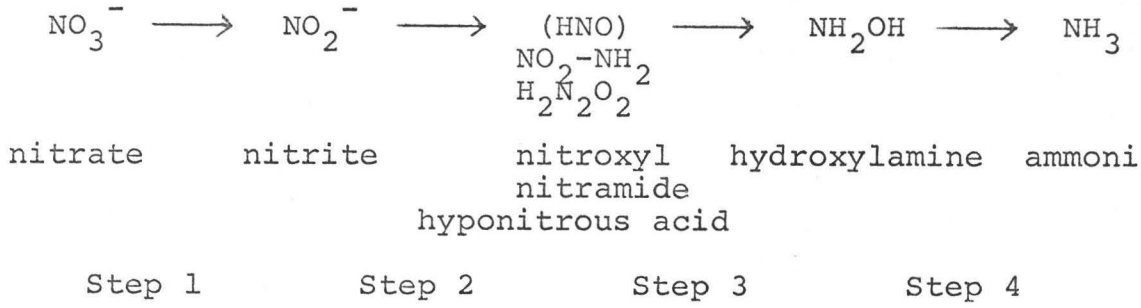

Fig. 1. A possible pathway for the reduction of nitrate (26).

Thus the enzyme concerned with the catalysis of Step 2 would be called nitrite reductase. However evidence, which will be discussed later, concerning the identity of possible intermediates in this sequence is inconclusive, and thus throughout this work the name nitrite reductase refers to the enzyme(s) which catalyse the reduction of nitrite to ammonia.

Yamagata (45) first demonstrated the reduction of nitrite by cell free extracts of Pseudomonas aeruginosa, and subsequently Taniguchi et al. (45) showed the enzymatic reduction of nitrite and hydroxylamine to ammonia by extracts of Bacillus pumilus using reduced methylene blue as an electron donor. More recently, the presence of nitrite reductase in Neurospora crassa (23,28,31), Glycine max. (28,37) and Azotobacter agile (44) has been reported. The observed characteristics of nitrite reductase from these organisms are shown in Table 1. It can be seen from the table that several groups of workers have reported the existence of nitrite reducing enzymes with very similar properties, but before drawing any conclusions it is necessary to examine the ways in which the enzyme was

Table 1. Characteristics of nitrite reductase isolated from different organisms

Organism	Method of measurement of nitrite reductase	Electron Donor	Cofactor	Metal Requirement		Reference
				In vitro[I]	In vivo[II]	
N. crassa	NO_2^- dependent NH_3 production	NADH	Flavin	-	Cu^{++}, Fe^{+++}	Medina & Nicholas (23)
	NO_2^- dependent NH_3 production	NADH or NADPH	FAD	Unidentified	-	Nason, Abraham & Auerbach (28)
	Enzyme dependent NO_2^- loss	NADH	FAD	Fe^{+++}, Cu^{++}	Fe^{+++}, Cu^{++} Zn^{++}, Mg^{++}	Medina & Nicholas (31)
Glycine max	Enzyme dependent NH_3 production	NADH or NADPH	FMN or FAD	Mn^{++}(s)	-	Nason et al. (28)
	Enzyme dependent NO_2^- loss and NH_3 production	NADH or NADPH	Heat stable factor	Unidentified	-	Roussos & Nason (37)
Azotobacter agile	Enzyme dependent NO_2^- loss and NH_3 production	NADPH	FAD	Unidentified	-	Spencer, Takahashi & Nason (44)

Mn^{++}(s) - Observes stimulation of activity when Mn^{++} is added to the assay mixture.

I - Addition of either specific or non-specific metal chelating agents.

II - Reduced specific activity observed in extracts of metal-deficient mycelia.

measured by these workers.

Nason et al. (28) estimated enzyme activity in terms of ammonia production. Their results showed that significant quantities of ammonia were produced even in the absence of added nitrite, although any activity due to endogenous nitrate and nitrite should have been negligible because of the purification procedure used.

A similar non-specific ammonia production was reported by Medina and Nicholas (23) working with N. crassa. In a later paper they measured nitrite loss but the product of the reaction was not identified.

A good stoichiometric relationship between nitrite loss and ammonia production was reported using nitrite reductase from A. agile (44). However insufficient data was given to determine whether any significant ammonia production occurred in the absence of added nitrite.

Finally, Roussos and Nason (37), working with Glycine max. leaf extracts, failed to demonstrate either nitrite loss or ammonia production when using NAD(P)H as an electron donor, thus casting some doubt on the universal application of the reduced nucleotide assay.

It is of interest at this point to examine the work of Silver and McElroy (42) in which enzyme studies on nitrate and nitrite non-utilizing strains of N. crassa were carried out.

Studies with enzyme deficient mutants can provide an indication that an enzyme being measured is the one that is operating in vivo e.g. if a mutant will not use nitrate as its

sole nitrogen source, and if extracts from this same mutant lack nitrate reductase as measured *in vitro*, then this is good evidence that the enzyme being measured is the one responsible for nitrate reduction *in vivo*. Silver and McElroy examined the nitrite (28), nitrate, and m-dinitrobenzene reductase (51) activities in extracts of a number of nitrate and nitrite non-utilizing mutants. All of the nitrate non-utilizing mutants examined lacked nitrate reductase but possessed nitrite reductase as expected. However only one nitrite non-utilizing mutant studied lacked nitrite reductase, and this mutant also lacked nitrate reductase. The possibility that nitrite reduction occurs via a series of organic intermediates has been suggested and thus the ability of mutant extracts to reduce m-dinitrobenzene to nitroaniline was examined. Extracts from nitrite non-utilizing mutants did not reduce dinitrobenzene (DNB) although an active DNB reducing system was present in wild type extracts. However, when grown on ammonium chloride all mutants possessed an active DNB-reductase.

A rather confusing picture emerges from the observations above, and while these observations do not prove that the enzymes being measured by the assays described are not those responsible for *in vivo* nitrite reduction this possibility cannot be ignored.

More recent information on nitrite reductase has come from work with higher plant systems (6,12,16,38). Hageman, Cresswell and Hewitt (12) observed than when reduced benzyl viologen (N-N'-dibenzyl-4,4'dipyridyliumdichloride) was used as an electron donor to nitrate reductase from *Cucurbita pepo* under anaerobic conditions, nitrite sometimes failed to appear.

This suggested to the authors the presence of an active nitrite reductase which could accept electrons from reduced benzyl viologen (BVH). When a system of this type was used to assay nitrite reductase from C. pepo and Zea mays it was found that the degree of dye reduction was critical for the assay. Over-reduced dye caused a non-enzymatic nitrite loss, but 30-65% reduced dye gave an enzyme dependent stiochiometric reduction of nitrite to ammonia. No response of the enzyme to manganese was observed, but it was 80% inhibited by metal chelating agents. NAD(P)H could not serve as an electron donor to this enzyme, either in the presence or absence of added flavins, but when catalytic amounts of benzyl viologen (BV) were added nitrite was lost enzymatically. These findings suggest that NAD(P)H may be the natural electron donor to nitrite reductase in these organisms and that BV is substituting for some natural electron carrier as yet unidentified.

Using the BV assay of Hageman et al. (12), Sanderson and Cocking (38) demonstrated a stoichiometric reduction of nitrite to ammonia with extracts of Lycopersicon aesculentum. They found that the same extracts would not catalyse the reduction of nitrite with NAD(P)H as electron donor, although a number of cofactors and buffer systems were tried. The same authors have shown that when NADH is present in the reaction mixtures used to measure nitrite reductase, it is necessary to make a correction for the ammonia coming from the NAD^+ resulting from NADH oxidation. Ammonia is generally measured by the microdiffusion method of Conway (4).

This consists of treating reaction mixtures in the outer well of a Conway unit with strong alkali. The Conway unit is then sealed. The ammonia is made volatile by this procedure, and is allowed to diffuse into boric acid in the inner well of the Conway unit. The ammonia in the boric acid is then tested colorimetrically. Both NAD^+ and NADH are partially degraded by alkali with the concomitant release of ammonia, NAD^+ being significantly more labile to alkali degradation than NADH. These findings are of relevance to previous work (23,28,31,27,44) in which activity was measured as ammonia production, and where NADH was used as an electron donor. Mainly because of the foregoing considerations, earlier reports of NAD(P)H dependent enzymatic nitrite reduction (23, 28,37) are open to some question (38).

The ammonia production reported by Nason et al. (28) was likely to be due to ammonia released in the degradation of NAD^+ during the microdiffusion procedure. The stimulatory effects of manganese ions and FAD may have been due to their stimulatory effect on NADH oxidase activity, thus increasing the amount of alkali labile NAD^+ present and correspondingly the ammonia released. Similar objections can be raised concerning the results of Medina and Nicholas (23).

The use of reduced dyes in nitrite reductase assay systems was investigated by Joy and Hageman (16), using extracts of Spinacea oleracea and Zea mays. Sodium dithionite ($Na_2S_2O_4$) was found to serve as an electron donor to BV, which subsequently reduced nitrite stoichiometrically to ammonia. The advantage

of this assay system is in its simplicity. The effects of a number of dyes and cofactors on this assay were examined and BVH was the most suitable electron source, because it gave the least amount of non-enzymatic nitrite reduction. Methyl viologen and ferredoxin (Fd) were found to reduce nitrite non-enzymatically to a considerable extent in the above system. Reduced FAD did not stimulate any enzymatic nitrite disappearance.

Illuminated chloroplasts can act as an electron source for nitrite reductase (Cresswell, D. F. and Hewitt, E. J., unpublished work cited by Hagemen et al. (12)). This, and the observation that nitrite reductase is localized in the chloroplasts (36) suggests the presence of an active "photosynthetic nitrite reductase" in higher plant cells. Mortenson, Valentine and Carnahan (25) obtained a water-soluble, non-haem, non-flavin iron-containing protein named ferrodoxin (Fd) from Clostridium pasteurianum. Valentine et al. (46) later showed that Fd and hydrogenase were essential for the reduction by hydrogen of nitrite and hydroxylamine to ammonia with extracts of Clostridium pasteurianum.

A number of workers have reported the reduction of nitrite by illuminated chloroplasts in systems that presumably contained ferrodoxins or to which ferredoxins were added (1,13,16,21,32,38). Because of the foregoing work it was suggested that Fd can serve an as electron carrier in the photosynthetic reduction of nitrite. In all systems the reduction of nitrite was accompanied by oxygen evolution.

In at least one case (32) ammonia formation was accompanied by ATP production, suggesting a type of non cyclic, Fd-dependent photophosphorylation in chloroplasts in which nitrite acts as a terminal electron acceptor.

The data presented on nitrite reduction both in the light and in the dark has been summarized by Joy et al. (16) and Paneque et al. (32) in the following scheme:

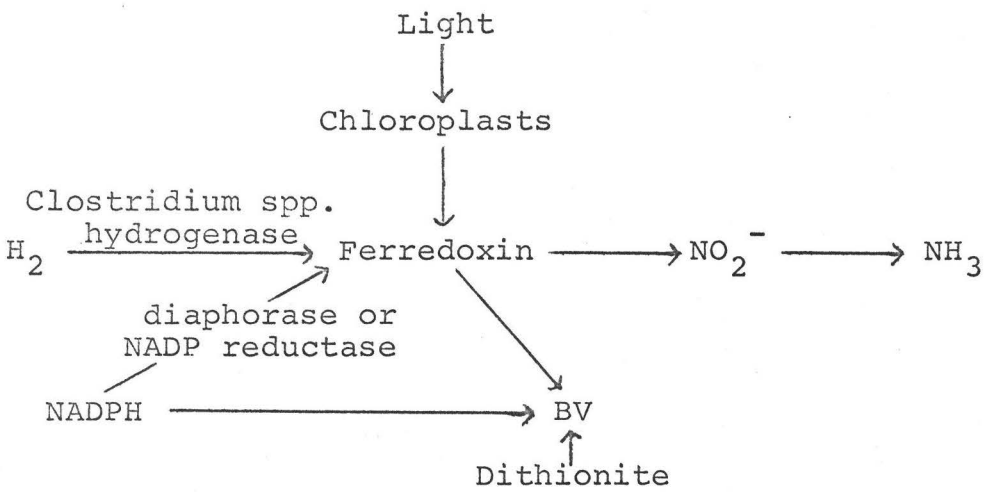

Fig. 2. A general scheme for nitrite reduction.

There is some controversy over the role of hydroxylamine reductase in nitrate assimilation. Cresswell et al. (6) have studied some kinetic aspects of nitrite and hydroxylamine reduction in C. pepo, using BVH as an electron donor to the two enzymes. They have found that in this system nitrite is reduced at a faster rate than is hydroxylamine and they have therefore concluded that free hydroxylamine is not an intermediate in nitrite reduction in plants. These results were later confirmed by Betts and Hewitt (1) who used a photosynthetic

Fd-dependent system to measure nitrite reductase in extracts of Spinacea oleracea.

The possible identity of hydroxylamine reductase with both nitrite and sulphite reductases has been suggested (14,18,20,22,41).

Enzymes capable of catalyzing a hydroxylamine dependent oxidation of NAD(P)H have been reported in extracts of N. crassa (23,28,37,50), higher plants (1,6,12), and bacteria (18,22,40,44,45). The enzyme, designated hydroxylamine reductase, appears to accept electrons from naturally occurring electron donors (NADH, NADPH and reduced Fd) or from reduced dyes (benzyl viologen and methyl viologen). The enzyme has been reported to be flavin-dependent (23,28, 41,44,50) and to be stimulated by manganese ions (23,28,37,44). The apparent stimulation by manganese can perhaps be better explained as a stimulation of NAD(P)H oxidase activity (38).

In Escherichia coli, the reduction of nitrite, hydroxylamine and sulphite is considered to be catalysed by a single enzyme. Mager (22) showed that the hydroxylamine and sulphite reductase activities of E. coli were both repressed by cysteine, an end product of sulphite metabolism (8). Subsequently, it was found that a purified nitrite reductase from E. coli had associated hydroxylamine, sulphite and cytochrome c reductase activities; that sulphite and nitrite reductases showed similar heat inactivation curves; that they were both repressed by cysteine but not by ammonia; and that both were absent from extracts of a sulphite non-utilizing

mutant of E. coli (17,18).

This evidence suggests that nitrite and sulphite reductases, as measured *in vitro*, are identical in *E. coli*, and that the physiological function of the enzyme is concerned with sulphur rather than nitrogen metabolism.

Seigel, Click and Monty (40) and Seigel and Monty (41) concluded from their work on *Salmonella typhimurium* that NADPH specific hydroxylamine reductase is identical with sulphite reductase. They further concluded that both substrates are reduced at a common enzyme site because they competed with each other in the reaction; because different compounds were observed to have similar effects on both enzyme activities; and because both activities were absent from extracts of a sulphite non-utilizing mutant.

There is some evidence that hydroxylamine and nitrite reduction may also be functions of the same enzyme in *C. pepo* and *S. oleracea* (14). Cell-free extracts of *C. pepo* and *Spinacea oleracea* were subjected to gel filtration on G200 Sephadex columns, and each fraction eluted was assayed for the two activities using both reduced BV (6) and reduced Fd (13) as electron donors, and measured as reductant-dependent nitrite loss. Nitrite reductase activity appeared in the same peak irrespective of the assay system used. The pattern of hydroxylamine reductase activity eluted from the column varied with the electron donor used. A number of peaks of enzyme activity were observed, one of which was identical with the nitrite reductase peak.

Zucker and Nason (50) have described a NAD(P)H dependent flavo-protein from N. crassa which catalyzed hydroxylamine-dependent NADH oxidation. The enzyme was reported to be adaptive to nitrate in the growth medium. However, this enzyme activity was also demonstrated in extracts of nitrate and nitrite non-utilizing mutants, a fact leading to some confusion on the role of the enzyme in nitrate assimilation.

The nature of hydroxylamine reductase in N. crassa and its relationship to sulphite reductase was examined recently by Leinweber, Seigel and Monty (20,39), who demonstrated the presence of three different kinds of NAD(P)H dependent hydroxylamine reductase activities. These species were classified on the basis of their sedimentation behaviour in sucrose gradients; their stability to dialysis; their ability to utilize NADH and NADPH as electron donors; their presence or absence in extracts of a sulphite non-utilizing mutant; and their adaptivity to nitrate in the growth medium.

One type of hydroxylamine reductase was stable to dialysis; specific for NADPH as an electron donor; absent from extracts of a sulphite non-utilizing mutant; and not adaptive to nitrate. This was found to have identical sedimentation properties in sucrose gradients as sulphite reductase. The second type of hydroxylamine reductase was stable to dialysis; non-specific for electron donor; present in extracts of a sulphite non-utilizing mutant; and adaptive to nitrate in the medium. It is probably that this second species of hydroxylamine

reductase was the one studied earlier by Zucker and Nason (50). The third type of hydroxylamine reductase studied was non-specific for reduced adenine nucleotide; present in extracts of sulphite non-utilizing mutants; not adaptive to nitrate; and stable to dialysis. This species was considerably less active than the first two discussed and no comment on its significance was made.

Existing evidence from a number of sources (1,6) seems to suggest that hydroxylamine reductase is not involved in nitrate assimilation. However it will be necessary to examine the properties of the different hydroxylamine reductases isolated, their response to different nitrogen sources in the medium, and their presence or absence in extracts of nitrite and hydroxylamine non-utilizing mutants, before its role is fully understood.

From the work on assimilatory nitrite reductase reported above it appears that there are two areas which require further clarification, and the following questions need to be answered:

(1) Is nitrite reductase, as measured using NAD(P)H and reduced dyes as electron donors, the enzyme which operates in vivo?

(2) Is hydroxylamine reductase an enzyme operative in nitrite assimilation, and if so is it different from nitrite reductase?

It is desirable to demonstrate that an assay system is measuring the enzyme which operates in vivo, preferably

by showing the absence of activity in an appropriate mutant, and by considering the mechanism of enzymic control by molecules involved in the pathway in which the enzyme is believed to be operative.

The genetic and metabolic control of nitrate reductase has been studied with this aim in mind (5,34,43,52,53) and similar studies have been made on nitrite reductase (15,34) but these are not extensive.

Pateman, Rever and Cove (34) have demonstrated the absence of nitrite reductase in two nitrite non-utilizing mutants of Aspergillus nidulans, one of which also lacks nitrate reductase. Nitrite reductase was reported by these investigators as being induced by both nitrate and nitrite, repressed by ammonia, and constitutive in a number of nitrate reductase-lacking mutants. This led them to suggest that nitrate reductase itself may be a component of the regulatory system of the nitrate assimilation pathway. They have also suggested that nitrate assimilation may only involve two enzymes, nitrate and nitrite reductase, because they found only two structural genes which appeared to be concerned with enzymes involved in nitrate assimilation. They used an assay system in which NADPH was used as an electron donor and FAD as a cofactor, a system about which there is some doubt in other organisms (12,38). Furthermore, the assay was indirect in that nitrite-dependent NADPH oxidation was measured.

Ingle, Joy and Hageman (15), working with excised

radish cotyledons, demonstrated that nitrite reductase was induced by both nitrate and nitrite, although kinetic studies suggested that nitrate probably induced merely by virtue of its subsequent conversion to nitrite, which then functioned as a true inducer. Only low specific activities for the enzyme were reported when cells were grown on ammonium chloride and nitrogen deficient media. However cells were in nitrogen deficient media for periods of up to 5 days before extracts were made. It is questionable whether sufficient useable nitrogen reserves to allow synthesis of new enzymes were present in the cells at this time. In this work no inhibition by postulated end products of nitrite assimilation was observed. The absence of enzyme activity in extracts of cells which had been exposed to inhibitors of protein and RNA synthesis suggested that de novo protein synthesis was occurring. No mutants were used in these studies.

In the present investigation the role of nitrite reductase in N. crassa was examined, the purpose of the investigation being:

(1) To develop a suitable assay system for the measurement of nitrite reductase, and,

(2) To study the physiological role of the enzyme by consideration of its metabolic control and by the use of mutants.

METHODS AND MATERIALS

Fungal Strains

Neurospora crassa strains 74A and 3.1a were used as the wild type strains. Mutant O, which cannot utilize nitrite, was isolated in this laboratory by Miss C. Dyer from a pantothenic acid requiring strain B36/pan2A. Strain nit-1-8A is a nitrate non-utilizing mutant in 74A background and was from Dr. G. J. Sorger's stock collection.

Media

The basic medium used was that described by Sorger and Giles (53). The nitrogen sources used were added to this basic medium in the following amounts (g/l): NH_4Cl, 3.9; casamino acids (Difco), 5.0; KNO_3, 5.0 and $NaNO_2$, 0.1.

Where glycerol or lactose were required as carbon sources these were used to replace sucrose at a concentration of 20 g/l. For solid media 1 1/2% agar (Difco) was added. Sterilization was with steam at 15 psi and 121 to 132°C for 15 minutes unless otherwise stated.

All media will be subsequently referred to by the name of the nitrogen source which they contain.

Culture conditions

Liquid cultures, in Erlenmeyer flasks filled to two-fifths capacity, were incubated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) at

27 ± 2°C with constant moderate agitation. Cultures on solid media were incubated in a standing incubator (Model 805, Precision Scientific Co., Chicago, Illinois) at 27 ± 2°C.

"Induction"[1]

N. crassa was grown from a conidial inoculum, into stationary phase in a liquid casamino acids medium. This was known as the "pre-incubation" stage.

Mycelial pads were then harvested by filtration, washed with distilled water, and cut into sectors. Weighed sectors were then placed in liquid media containing an appropriate nitrogen source, or in media completely lacking a nitrogen source, and incubated for 15-18 hours. This was known as the "induction" stage, and mycelia so treated are designated as being "induced".

Extraction

"Induced" mycelial pads which had been harvested by filtration were washed in distilled water and blotted between paper towels until no more moisture could be squeezed out by application of manual pressure. The blotted pads of mycelium were immersed in liquid nitrogen for 5-10 minutes

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[1]Mycelia subjected to the treatment described in this section are subsequently referred to as "induced". However as nitrite reductase appears to be a derepressive, and not an inducible enzyme, the word induction is written in inverted commas when applied to nitrite reductase.

and subsequently ground in an ice cold mortar with 1 volume of silica per volume of mycelial pad as an abrasive. The resulting paste was then suspended in three volumes of cold potassium phosphate buffer (0.1M, pH 7.0) and the slurry centrifuged at 3020g for 20 mins. in a refrigerated centrifuge (Sorvall, Model RC2B, Norwalk, Conn.). The resulting supernatant was kept on ice and used within 3 hours as the crude extract.

Dialysis of 1.0 ml of crude extract, when necessary, was against three changes of 2.0 l of potassium phosphate buffer (0.1M, pH 7.0) for a total time of 3 hours.

Enzyme assays

(1) Nitrite reductase

The assay mixture contained in a volume of 3.0 ml (µmoles): potassium phosphate buffer pH 7.5, 120; benzyl viologen, 0.5; $NaNO_2$, 3.3. Between 0.2 and 0.4 ml. of crude extract was then added, and the reaction initiated by adding, with minimum turbulence, 7.5 µmoles of sodium dithionite in 0.6 ml of water. The assay mixtures were then briefly shaken and incubated in a water bath at 30°C for 10 mins. in open test tubes. At this time the reaction was terminated by vigorously shaking the tubes on a mechanical mixer (Scientific Products, Evanston, Illinois) for at least 5 seconds after the blue colour of the reduced benzyl viologen had disappeared. Samples (0.1 ml) were then pipetted into 7.9 ml of double distilled water and 1.0 ml each of sulphanilamide and N-1-napthylethylenediamine

dihydrochloride reagents were added. After 20 mins. the colour was estimated using a Klett-Summerson colourimeter (Klett Manufacturing Co., Inc., New York) with a number 54 filter. Reagent blank and zero time controls were included. One unit of activity of nitrite reductase is defined as the reduction of 1 mμmole of nitrite per minute at 30°C. The specific activity of crude extracts of fully "induced" cells (units/mg. protein) is constant between 0 to 1.3 mg of crude extract protein in the assay mixture. All readings were made in this linear range. The results are expressed as units of activity per ml of undiluted extract.

(2) Glucose-6-phosphate dehydrogenase.

This enzyme was assayed by the procedure of Glock and McLean (11), with Tris-chloride instead of glycyl-glycine buffer. Activity was measured as an enzyme and substrate-dependent increase in optical density at 340 mμ in a 1 cm light path in a silica cuvette, and recorded by a Beckman DBG recording spectrophotometer. A unit of activity is defined as the reduction of 1 μmole of nicotinamide adenine dinucleotide phsophate per minute. Measurements of activity were made in a range of protein concentration where the specific activity (units/mg. of protein) was constant.

(3) β-galactosidase

The assay used was basically that described by Hestrin, Feingold and Schram (3) using β-o-nitrophenyl-galactoside as substrate. The reaction was followed by observing the increase in optical density at 420 mμ in a

Beckman DBG recording spectrophotometer in a one cm light path at room temperature. This increase was due to the production of o-nitrophenol from β-o-nitrophenylgalactoside. One unit of activity of β-galactosidase is defined as the production of 1 mμ mole of o-nitrophenol per min. A reference curve for o-nitrophenol is given in Fig. 3. There is a linear relationship between optical density at 420 mμ and o-nitrophenol up to 250 mμ moles of o-nitrophenol. The specific activity (units/mg of protein) of β-galactosidase in the reaction mixture was constant between 0 and 2.0 mg. of protein from extracts of fully induced cells (Fig. 4).

Nitrite estimation

This was by a modification of the method described by Sanderson and Cocking (38). The method was as follows: 1.0 ml of 1% (w/v) sulphanilamide in 1 N HCl and 1.0 ml of 0.01% (w/v) of N-1-napthylethylenediamine dihydrochloride were added to a test solution of nitrite in 8.0 ml of double distilled water. The resulting magenta colour was then estimated after 20 mins. using a Klett-Summerson colourimeter with a number 54 filter, and related to a standard curve. A linear relationship exists between Klett units and nitrite concentration up to 300 mμ moles of nitrite in the test mixture (Fig. 5). In the linear range indicated one Klett unit is equivalent to 0.4 mμ moles of nitrite. Samples to be estimated for nitrite content were diluted, where necessary, in order to contain less than 300 mμ moles of nitrite.

Fig. 3. Reference curve for o-nitrophenol. A number of different concentrations of o-nitrophenol were prepared in a final volume of 2.5 ml. The pH of these solutions was 7.0. The optical density of the samples was then determined at 420 mμ, in a 1 cm. light path in a Beckman DBG spectrophotometer, and related to o-nitrophenol concentration as shown.

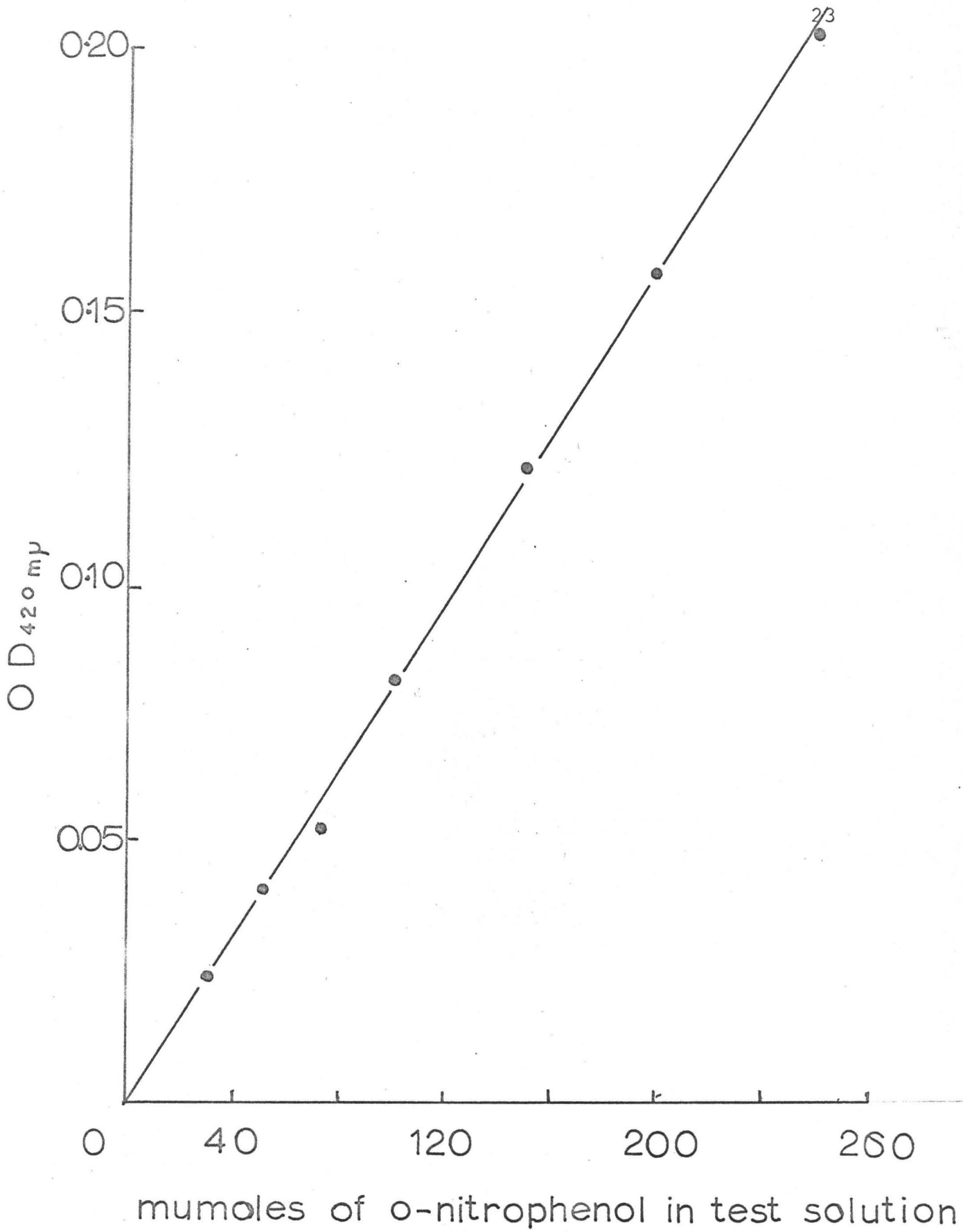
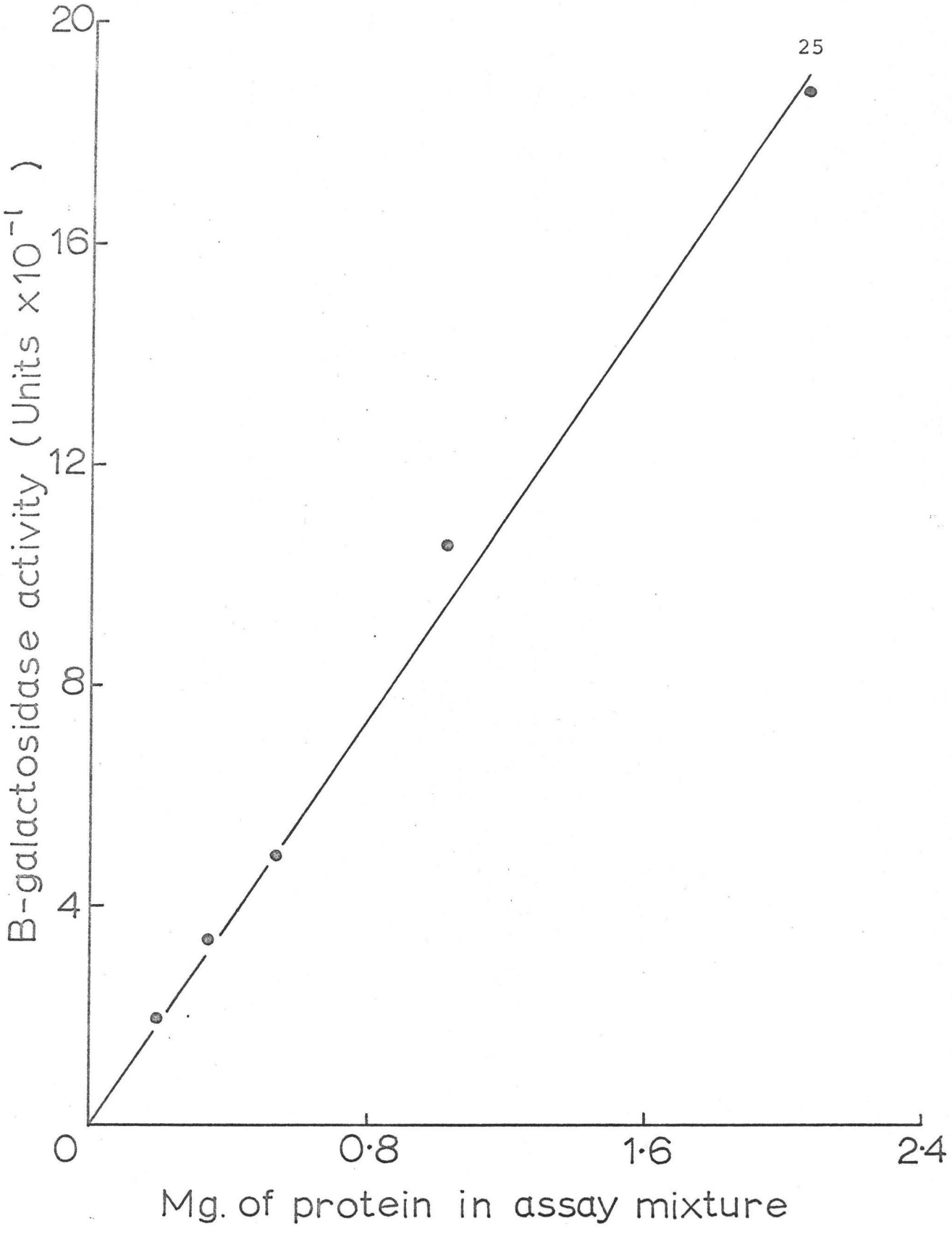

2/3
0·20
0·15
0·10
0·05
O D 420 mμ
0
40
120
200
260
mumoles of o-nitrophenol in test solution

Fig. 4. Effect of extract concentration on β-galactosidase activity. Cells were grown for 72 hrs. on basic medium containing KNO_3 and glycerol (see METHODS). The resulting mycelial pad was washed, filtered and cut into sectors which were subsequently induced for 24 hrs. on nitrate medium containing glycerol and lactose (10 g/l each) as carbon sources. At this time pads were harvested and the cell free extracts assayed for β-galactosidase activity.


20
16
12
8
4
0
25
B-galactosidase activity (Units x10⁻¹)
0·8
1·6
2·4
Mg. of protein in assay mixture

Ammonia estimation

The microdiffusion method of Conway was used (4). The test solution (3.0 ml) was placed in the outer well of a plastic Conway dish. The inner well contained 1.0 ml of 2% boric acid. Saturated K_2CO_3 (1.5 ml) was added to the solution in the outer well and the dishes were sealed. Volatile ammonia, released from the test solution by addition of K_2CO_3, is allowed to diffuse into the boric acid. After 3-6 hours the boric acid (1.0 ml) is reacted with 2.0 ml of commercially prepared Nessler's reagent (Paragon Co., Bronx, New York) and the optical density at 440 mμ (in a Beckman DBG recording spectrophotometer in a 1 cm. light path) compared to a standard. A reference curve for ammonia is shown in Fig. 6. There is a linear relationship between optical density at 440 mμ and NH_4Cl concentration when between 0 and 1 μ mole of NH_4Cl is estimated by this method. All subsequent measurements were made within this linear range.

Protein estimation

This was by the Biuret test as described by Dawson et al. (49) using bovine serum albumin as a standard.

Materials

Sources were as follows: benzyl viologen was from Mann Research Labs., New York, New York; FMN, FAD, NAD^+ and pyridoxal phosphate were from Nutritional Biochemicals Co., Cleveland, Ohio; glucose-6-phosphate and NADPH were from Sigma Chemical Co., St. Louis, Missouri, β-o-nitrophenyl-

Fig. 5. Reference curve for nitrite. Samples (3.0 ml) of $NaNO_2$ at different concentrations were prepared. Aliquots of 0.1 ml were then removed from these samples and estimated for nitrite as described in METHODS.

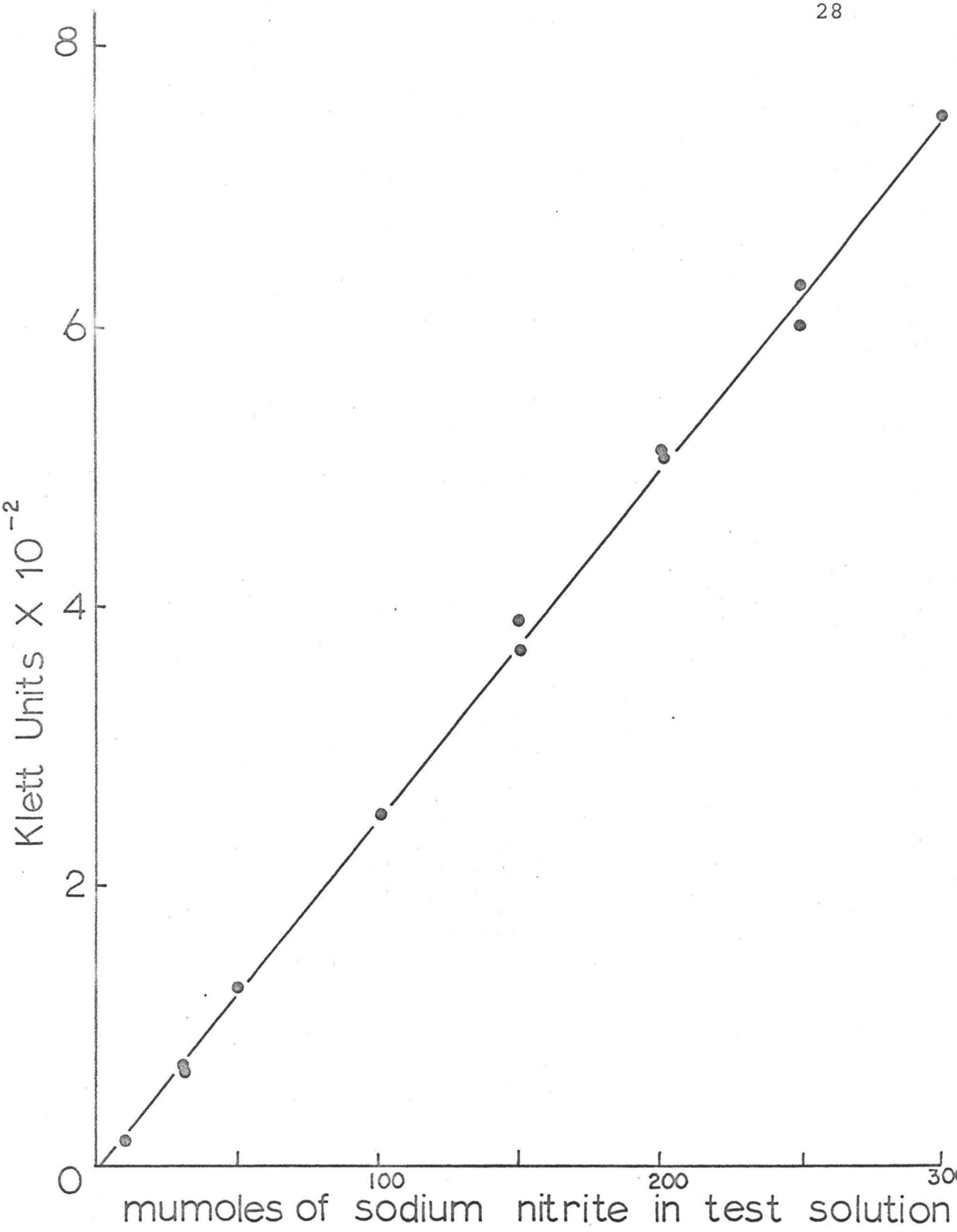
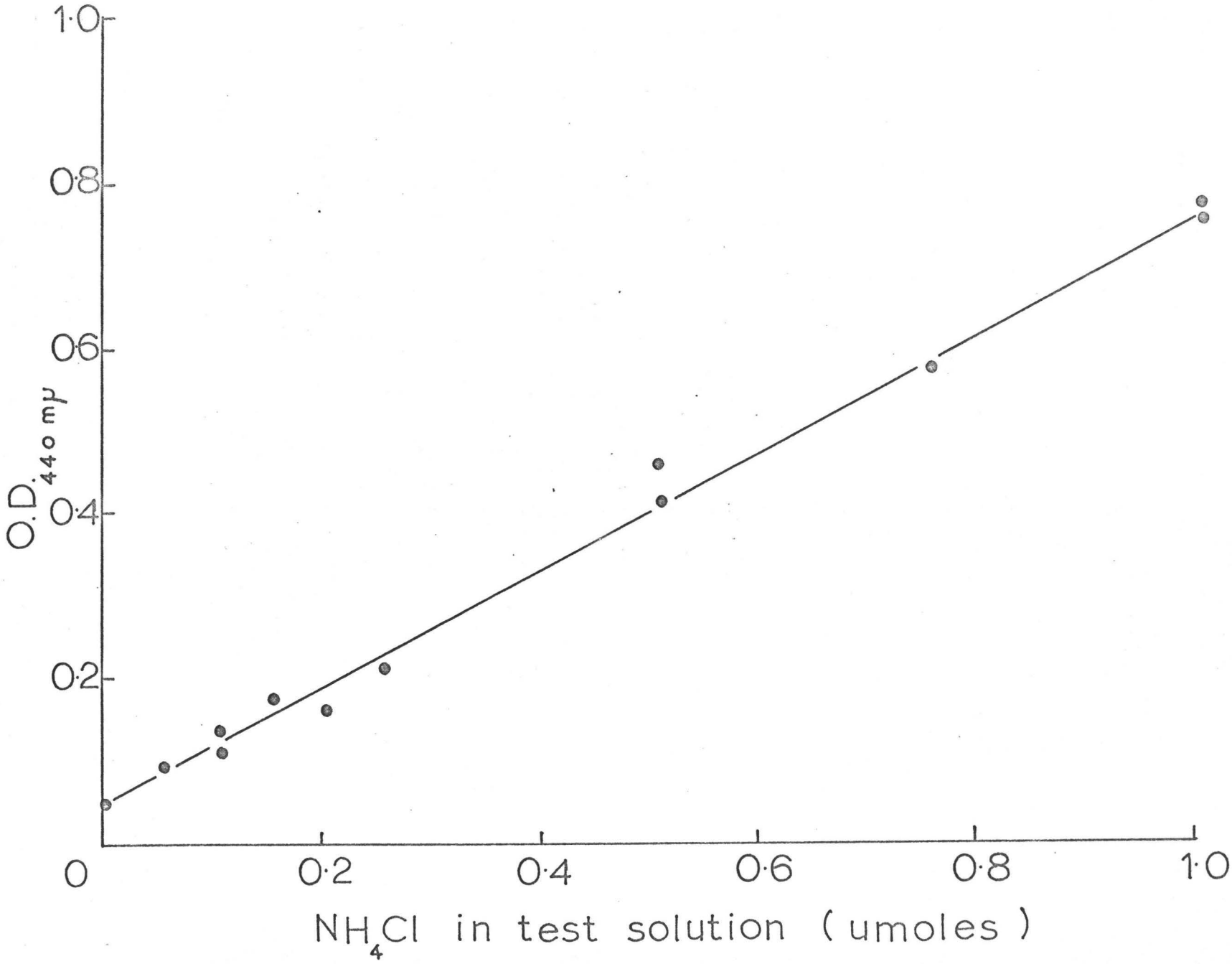

8
6
4
2
0
Klett Units X 10^{-2}
100
200
300
mumoles of sodium nitrite in test solution

Fig. 6. Reference curve for ammonia as estimated by the microdiffusion method. Three ml. aliquots of ammonium chloride solutions of known concentration were introduced into the outer well of a plastic Conway unit. The inner well contained 1.0 ml of 2% boric acid. The reaction was initiated by adding 1.5 ml of saturated K_2CO_3 to the outer well and closing the lid of the unit. After 3-8 hrs. the boric acid had absorbed the volatile ammonia released from the test solutions. The acid solution was then reacted with Nessler's reagent (see METHODS) and the $OD_{440\ m\mu}$ estimated.


1·0
0·8
0·6
0·4
0·2
O.D.440 mμ
0
0·2
0·4
0·6
0·8
1·0
NH4Cl in test solution (umoles)

galactoside was from Calbiochem, Los Angeles; Nessler's reagent was from Paragon Co., Bronx, New York.

All inorganic reagents were of analytical grade and were obtained from Fisher Scientific Co., Fairlawn, New Jersey and Baker Chemical Co., Phillipsburg, New Jersey.

RESULTS

Section 1. Assay of Nitrite Reductase

The purpose of the first part of this investigation was to find an assay system for nitrite reductase (E.C.1.6.6.4.) in N. crassa. It was thought that the assay described by Joy et al. (16) depending on the use of sodium dithionite as an electron donor, might be suitable. When Joy's assay was used to measure nitrite reductase in N. crassa it was found that the enzyme was not saturated with respect to nitrite and consequently the conditions of the assay were investigated. In preliminary experiments the concentrations of $NaNO_2$, benzyl viologen and dithionite were varied until maximum enzyme activity was obtained. Each assay parameter was then varied separately, keeping all other conditions constant, until the combination of conditions giving maximum enzyme activity was arrived at. A crude extract from N. crassa was used in all of these experiments.

Enzyme activity was measured in terms of reductant and enzyme dependent nitrite disappearance. The assay procedure and the colourimetric test used for nitrite estimation are described in METHODS.

Development of the assay system

The apparent K_m of nitrite reductase is 0.3 mM for nitrite (Fig. 7) and V_{max} is reached at a concentration of

1 mM nitrite. The final concentration of nitrite in the assay is 1.1 mM. The results shown in Fig. 8 show that nitrite reductase activity is at a maximum when 0.5 µmoles of BV are present in the assay mixture. As the amount of BV present is increased, a decrease in enzyme activity is observed, possibly because the BV is no longer sufficiently reduced to act as an efficient electron donor. Dithionite appears to be capable of donating electrons directly to nitrite in this assay mixture, without the intervention of BV, although BV increased the activity by about 50%.

A significant non-enzymatic reduction of nitrite occurs when more than 8 µmoles of sodium dithionite are present in the reaction mixture (Fig. 9). There is also some chemical nitrite reduction when less that 8 µmoles of dithionite are present but the extent of this reduction is usually negligible. The non-enzymatic nitrite reduction is compensated for by using a control assay system to which no extract is added.

The results shown in Fig. 10 demonstrate that chemical reduction of nitrite can occur at low pH values. The extent of this chemical reduction is negligible between pH 6.0 to 8.0. While there appears to be no sharp pH optimum for the enzyme maximum activity is observed at about pH 7.5. The concentration of buffer in the assay giving maximum activity is 0.04M (Fig. 11).

The results in Fig. 12 indicate that the rate of nitrite disappearance is constant for 10 mins at the enzyme concentrations shown, when the conditions described above are satisfied. The specific activity of crude extracts of fully

"induced" cells is constant when 0 to 1.3 mg of extract protein are present in the assay mixture (Fig. 13). In subsequent experiments all measurements were made within this linear range. Extracts containing more than 1.3 mg of protein were appropriately diluted before their activity was measured. The observed activity was then multiplied by the appropriate dilution factor to give the total activity in x mls. of the original extract.

The final assay mixture is shown in Table 2, and contains saturating amounts of nitrite, dithionite and benzyl viologen, and is at optimum pH and strength of buffer. This assay differs from that of Joy in the concentrations of nitrite and benzyl viologen and in the time of the assay. The disappearance of nitrite measured by this assay is also higher than in most previous systems (see DISCUSSION).

Stoichiometry and dialysis of the extract

The natural product of assimilatory nitrite reduction is thought to be ammonia (6,12,16,38). An attempt was made to determine whether nitrite was reduced to ammonia in this system.

The relationship between nitrite loss and ammonia production is shown in Table 3. In this experiment, BV was omitted from the reaction mixtures as there is some indication that it may be degraded, with the release of ammonia, under the conditions of microdiffusion. The results show that no significant ammonia production from nitrite can be detected under the conditions of the assay. The possibility that

ammonia produced in the assay mixture was being removed was investigated (Table 4). In all cases studied no ammonia was lost under the conditions described. It should be mentioned that in all these experiments the background level of ammonia was high, due to its presence in the extracts used, and this tended to obscure the results obtained.

In view of this problem the extract was subjected to dialysis (see METHODS) in an attempt to remove endogenous ammonia. The results in Table 5 show that extract treated in this way lost virtually all of its activity, and none of the additions made to the reaction mixture would restore this activity. The inactivation of the enzyme was not due to storage at a low temperature, as crude extract which had been stored under the same conditions did not loose activity. Thus the problems associated with the measurement of ammonia were not resolved by these experiments. The inability to demonstrate ammonia production from nitrite in this system is considered in the DISCUSSION.

Assay of nitrite reductase using NADPH as an electron donor

In view of earlier reports of nitrite reduction using NADPH as an electron donor (23,28,37) the use of an assay system utilizing NADPH was examined (Table 6). No nitrite loss was observed in any of the cases studied. In this experiment it can be seen that in some cases nitrite accumulated in the assay mixtures. The significance and compensation for this accumulation are considered below.

Accumulation of nitrite in reaction mixtures

Under appropriate conditions (i.e. the presence of NADPH and FAD in the reaction mixture) nitrate reductase will reduce endogenous nitrate present in cell extracts to nitrite. This confuses the measurement of nitrite reductase activity as some of the nitrite that disappears is replaced by nitrite produced from nitrate. In cases where this problem was likely to be encountered the nitrite accumulation was accounted for by using a control reaction mixture to which no nitrite was added. Any nitrite produced from endogenous nitrate can thus be detected. Endogenous nitrite in cell extracts can also be accounted for, although this is usually found to be negligible. With the information obtained using these control systems the true nitrite disappearance due to nitrite reductase can be calculated from the formula:

$$\text{Nitrite reductase activity} = \text{Nitrite lost from complete assay mixture} + \text{nitrite accumulated in assay mixtures minus nitrite}$$

e.g. if 40 mμ moles of nitrite were lost from the complete assay mixture, and if 10 mμ moles accumulated in the assay mixture minus nitrite, then from the formula, the disappearance of nitrite due to nitrite reductase is 40 + 10 = 50 mμ moles.

Fig. 7. Effect of nitrite on nitrite reductase activity. Mycelial were pregrown, harvested and cut into sectors which were subsequently "induced" for 18 hrs. on nitrate medium. Crude extracts were then assayed for nitrite reductase as described in METHODS. The sodium nitrite was varied as indicated. The reaction mixtures contained 1.75 mg of extract protein.

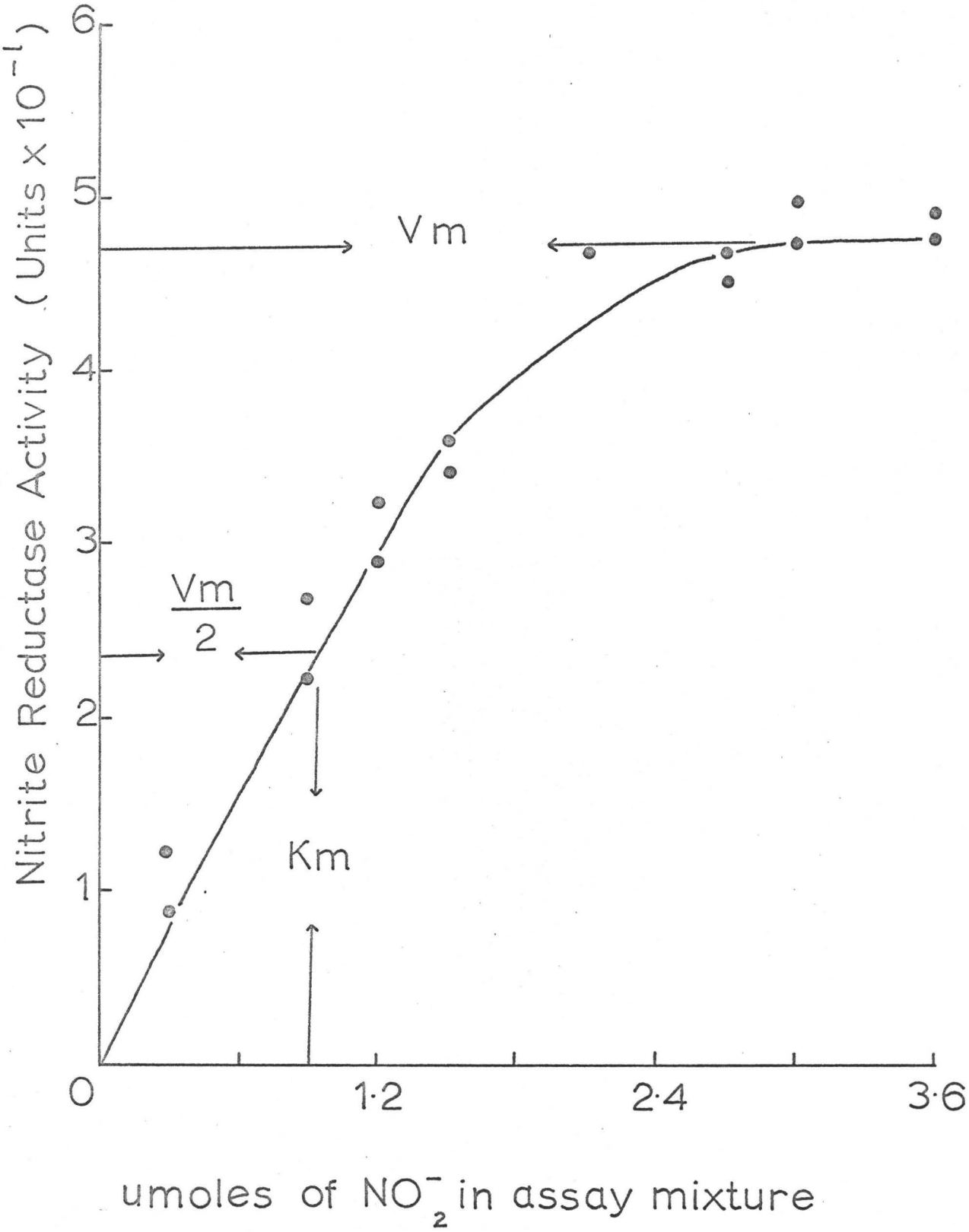

Nitrite Reductase Activity (Units x 10^{-1})
6
5
4
3
2
1
0
Vm
Vm/2
Km
1·2
2·4
3·6
umoles of NO_2^- in assay mixture

Fig. 8. Effect of benzyl viologen on nitrite reductase activity. The growth and "induction" conditions were described in Fig. 7. The benzyl viologen concentration was varied as shown. The assay mixture contained 1.31 mg of extract protein.

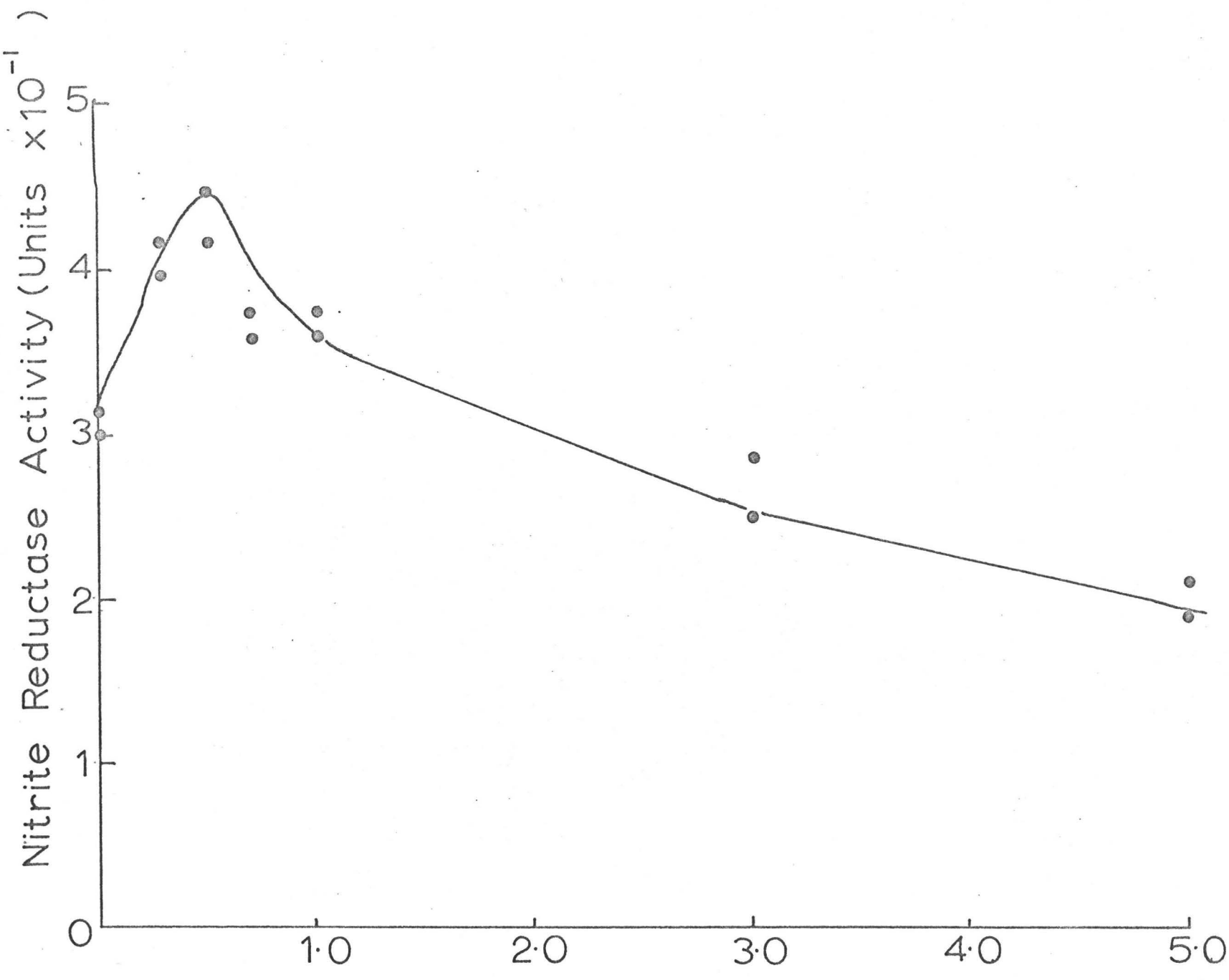

Nitrite Reductase Activity (Units x10⁻¹)
0
1
2
3
4
5
1·0
2·0
3·0
4·0
5·0
umoles of benzyl viologen in assay mixture

Fig. 9. Effect of sodium dithionite on nitrite disappearance with and without extract. The growth and "induction" conditions were described in Fig. 7. The assay mixture contained 1.31 mg. of extract protein where indicated. ●–● , total reduction of nitrite; ○–○ , reduction of nitrite with no extract in the assay; ■–■ , extract-dependent nitrite reduction (the difference between ●–● and ○–○).

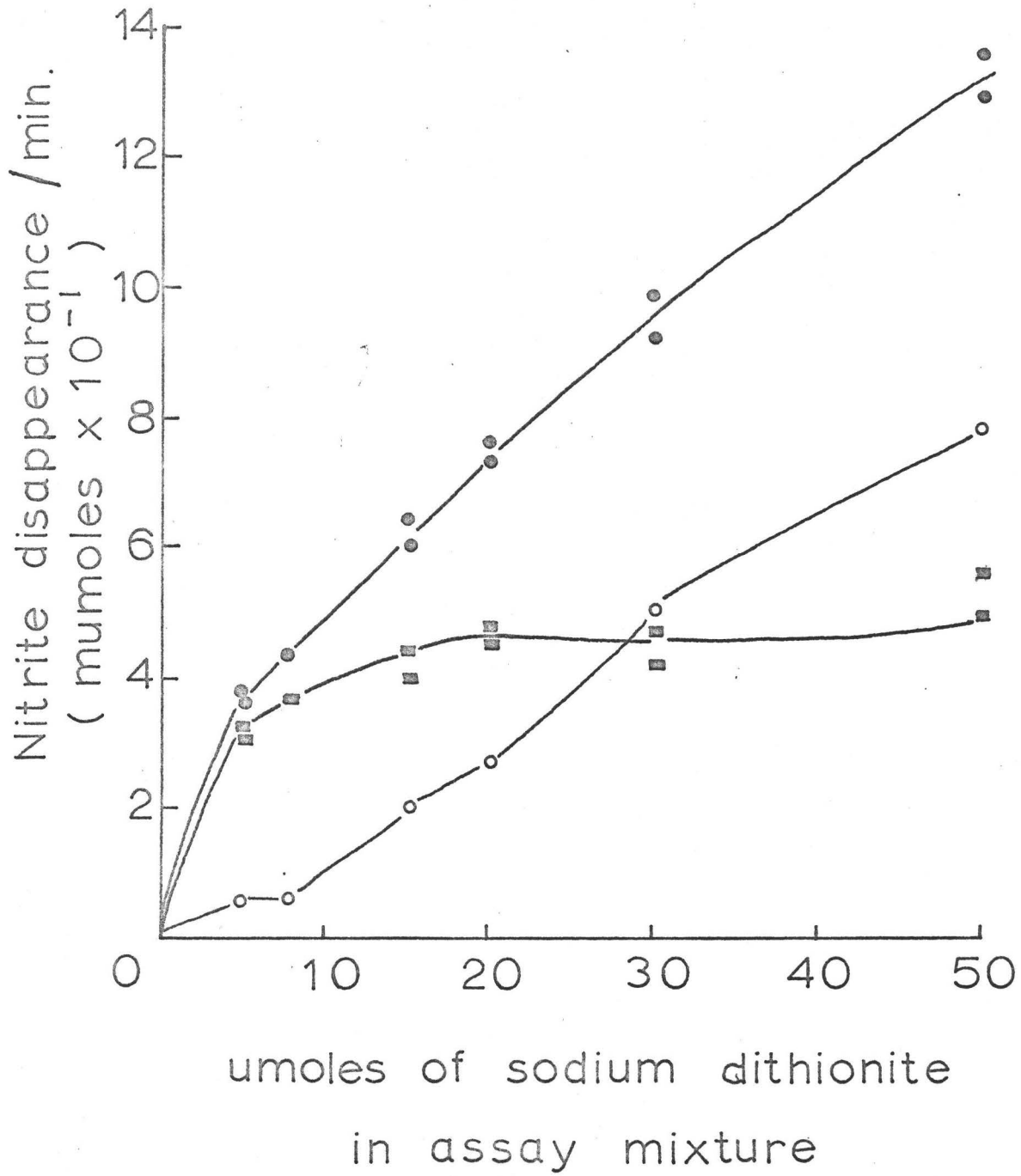

Nitrite disappearance / min.
(mumoles x 10^{-1})
14
12
10
8
6
4
2
0
10
20
30
40
50
umoles of sodium dithionite
in assay mixture

Fig. 10. Effect of pH on nitrite disappearance with and without extract. Mycelia were grown, "induced" and extracted as described in Fig. 7. The pH of the assay mixture was varied by using 1 M potassium phosphate buffer of different pH. The assay mixture contained 0.75 mg. of extract protein where indicated ●–● , total reduction of nitrite; ○–○ , reduction of nitrite with no extract in the assay mixture; ■–■ , enzyme dependent nitrite reduction (the difference between ●–● and ○–○).

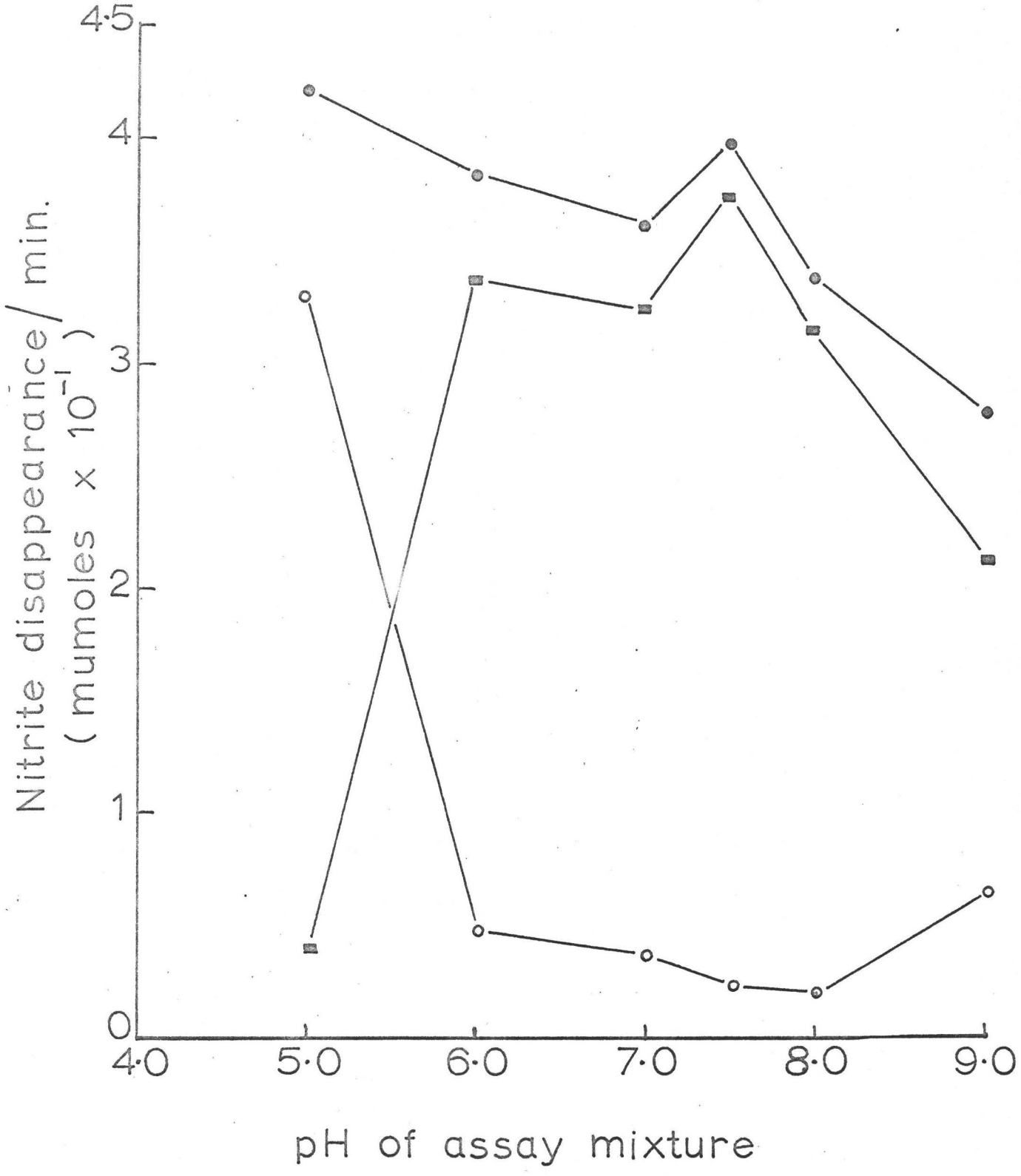

4·5
4
3
2
1
0
Nitrite disappearance/ min.
(mumoles x 10^{-1})
4·0
5·0
6·0
7·0
8·0
9·0
pH of assay mixture

Fig. 11. Effect of strength of buffer on nitrite reductase activity. Mycelia were "induced" and extracted as described previously (Fig. 7). The strength of the assay mixture was changed by varying the concentration of potassium phosphate buffer (pH 7.5). The assay mixture contained 0.9 mg. of extract protein.

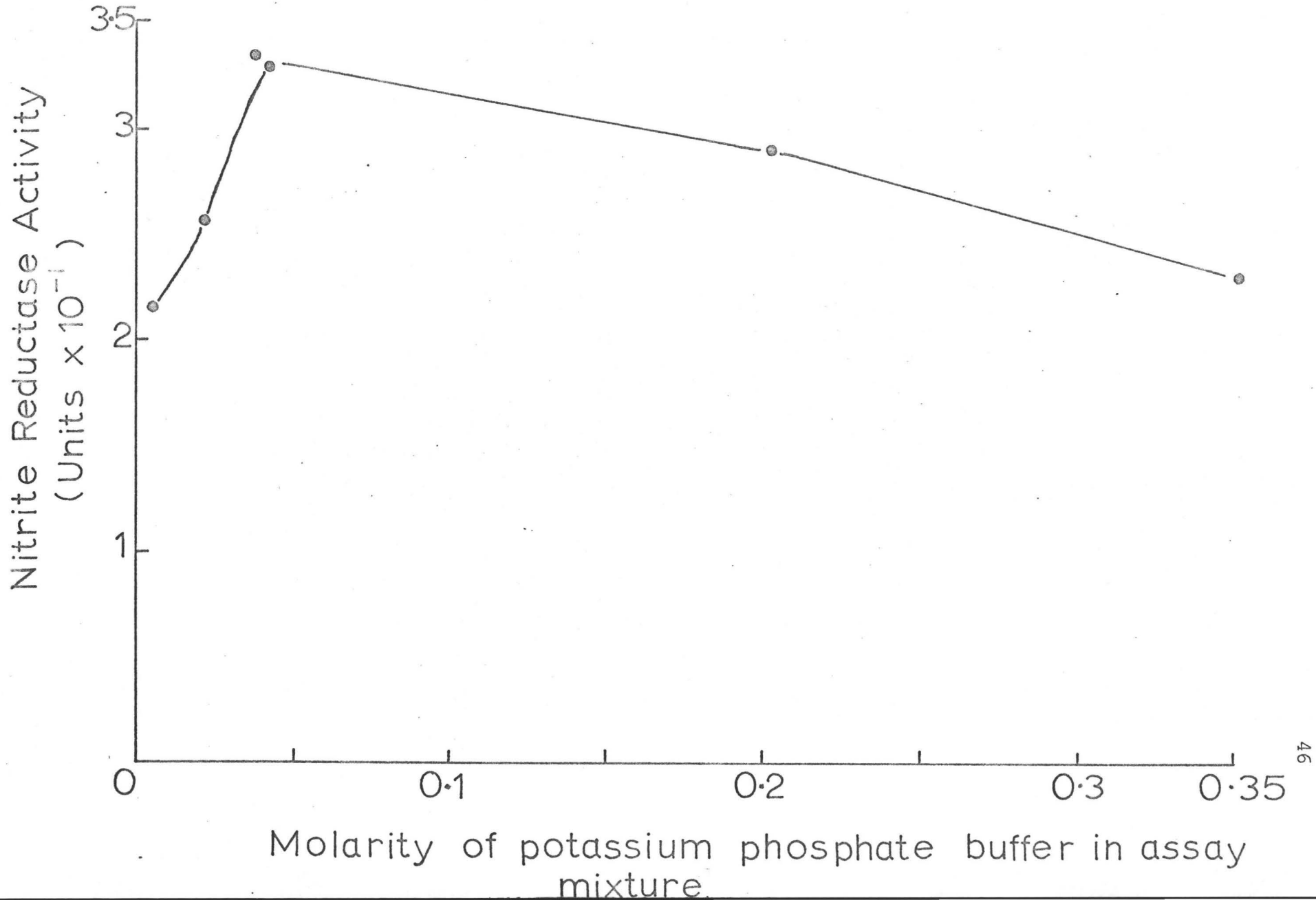

Nitrite Reductase Activity
(Units x 10⁻¹)
3·5
3
2
1
0
0·1
0·2
0·3
0·35
Molarity of potassium phosphate buffer in assay mixture.

Fig. 12. Effect of time on the extract-dependent loss of nitrite at different protein concentrations. Mycelia were grown and "induced" as described in Fig. 7. The amount of extract protein (mg.) in the assay mixture was varied and nitrite loss was estimated at the time intervals indicated. ●—● , ○—○ , ■—■ , □—□ represent assay mixtures containing 1.42, 1.07, 0.71 and 0.36 mg. of extract protein, respectively.

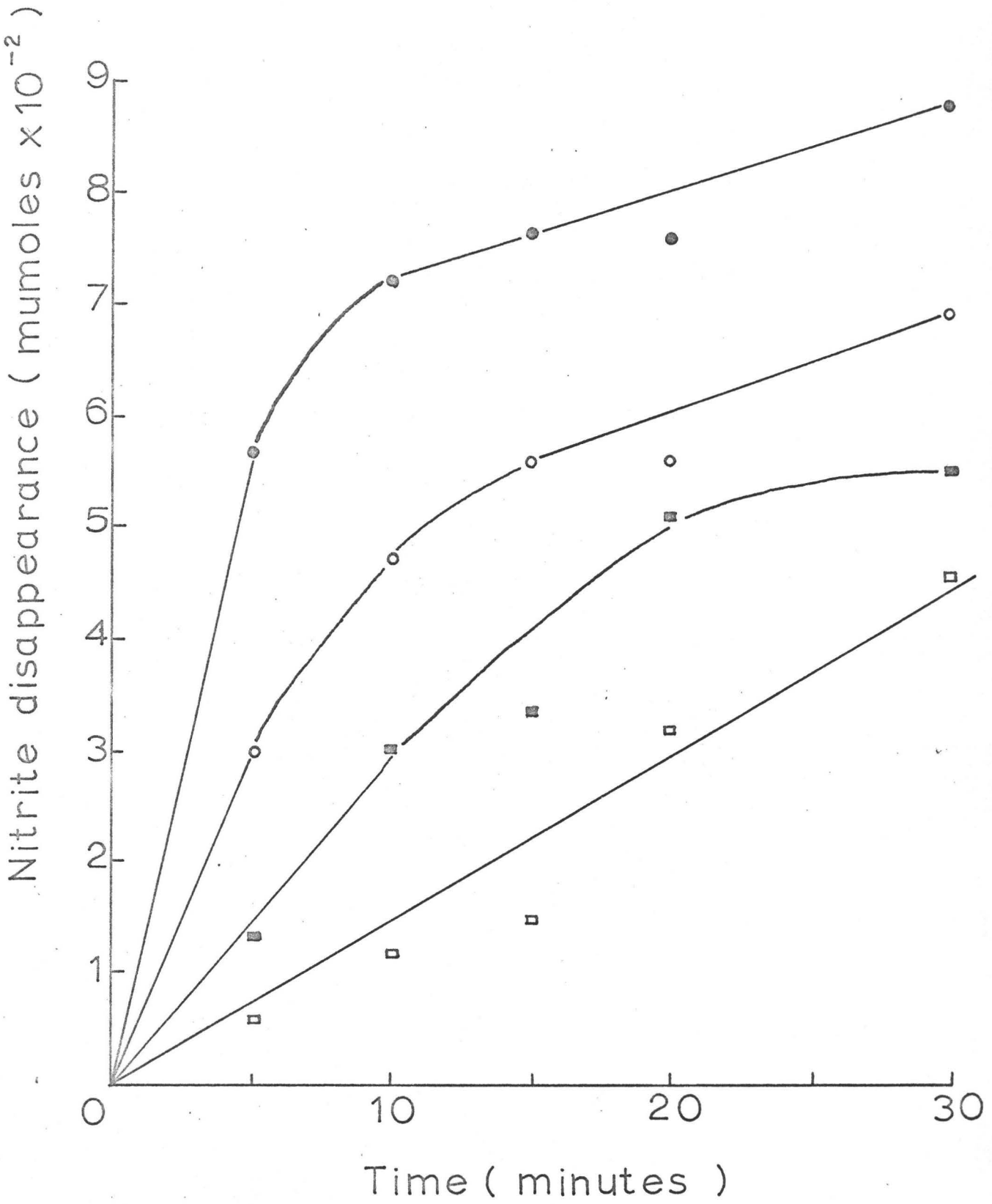

Nitrite disappearance (mumoles x10^{-2})
0
1
2
3
4
5
6
7
8
9
0
10
20
30
Time (minutes)

Fig. 13. Effect of extract concentration on nitrite reductase activity. Mycelia were grown and "induced" as described in Fig. 7. ●—● , ▲—▲ and □—□ represent results obtained from independent experiments.

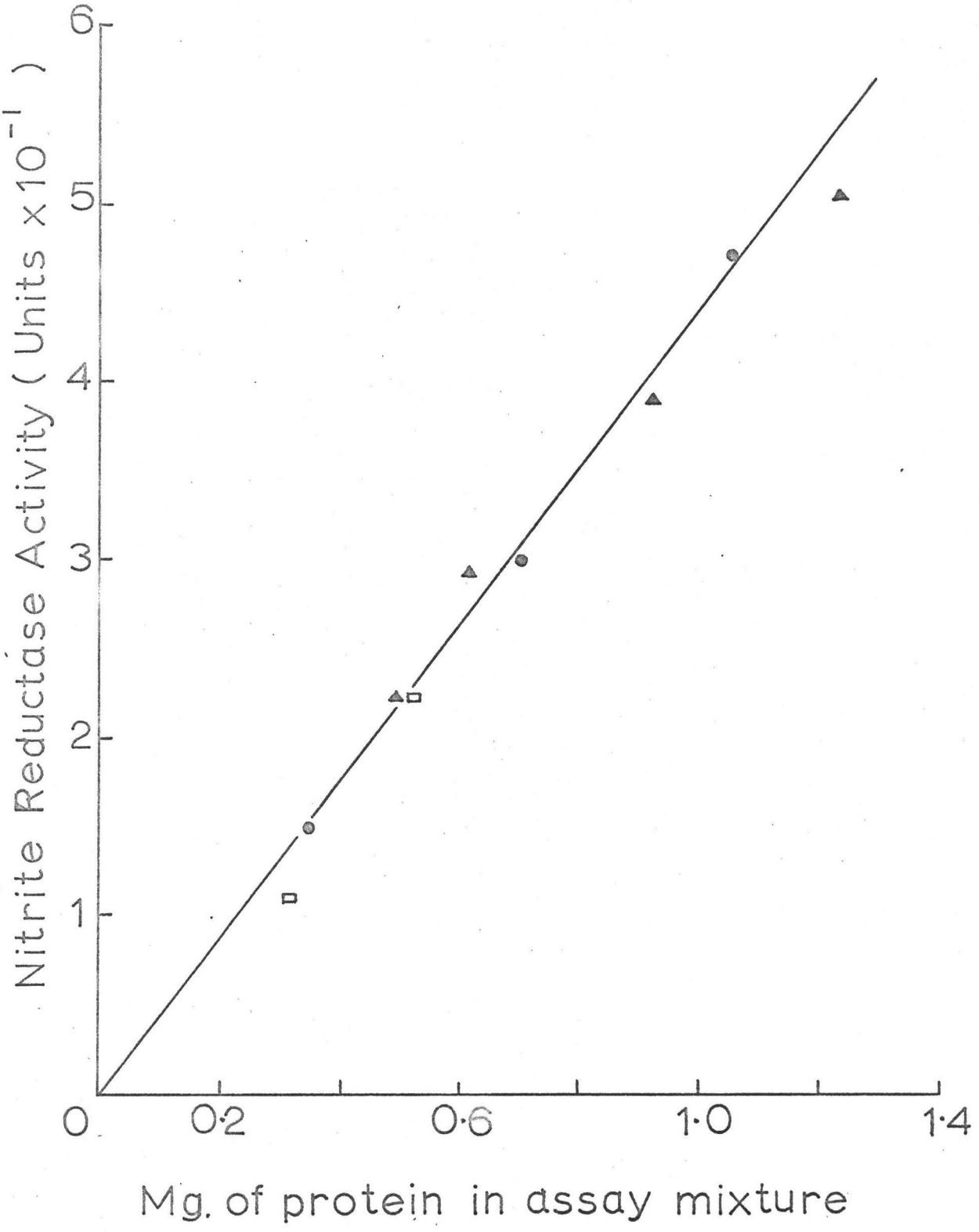


Table 2. Nitrite Reductase Assay Mixture

The final volume was adjusted to 3.0 ml. by the addition of double distilled water.

Potassium phosphate buffer (pH 7.5)	120 µmoles
Benzyl viologen	0.5 µmoles
Sodium dithionite	7.5 µmoles
Sodium nitrite	3.3 µmoles
Neurospora extract	0.2-0.4 ml.
Final Volume	3.0 ml.

Table 3. Relationship Between Nitrite Reduction and Ammonia Production in the Nitrite Reductase Assay

Mycelia were pre-grown, "induced" for 18 hrs. on nitrate, extracted, and assayed for nitrite reductase as described in METHODS. Benzyl viologen was omitted from these reaction mixtures. The assay mixtures used in Expt. 1 to 6 contained 1.67, 3.3, 1.1, 1.6, 2.2 and 3.3 mg protein, respectively. As appreciable amounts of ammonia were present in the extracts, ammonia production is defined as the difference between the total amount of ammonia in the assay mixture at the end of the reaction, and the ammonia produced from a control assay mixture to which no sodium dithionite was added.

Expt. No.	Nitrite lost from reaction mixture (mμmoles)	Ammonia produced during the enzymatic reduction of nitrite (mμmoles)
1	324	29.0
2	684	125.0
3	396	0
4	600	0
5	288	0
6	396	0

Table 4. Recovery of Added Ammonia from the Nitrite Reductase Assay Mixture

Growth, "induction" and ammonia estimation were as described in Table 3. The indicated quantities of ammonium chloride were added to assay mixtures to which no sodium nitrite had been added. Benzyl viologen was omitted from these reaction mixtures. All final assay volumes were adjusted to 3.0 ml. by the addition of double distilled water. After termination of the assay the entire 3.0 ml. reaction mixture was analyzed for ammonia. The percentage of ammonia recovered was calculated from the formula:

$$\frac{\text{mμ moles of ammonia present at end of assay}}{\text{mμ moles of ammonia present at start of assay}} \times 100$$

The reaction mixtures used in Expt. 1 and 2 contained 0.55 and 2.23 mg. protein respectively.

Expt. No.	NH_4Cl added to assay mixture (mμ moles)	% Ammonia recovered
1	500	99.0
2	750	100.0
	1000	96.0

Table 5. Effects of Various Additions on the Nitrite Reductase Activity of Dialysed Extracts of *N. crassa*

The mycelium was "induced" on nitrate medium for 18 hrs. and extracts from this dialysed as described in METHODS. The reaction mixtures used contained 1.4 mg. of protein.

Extract	Addition[2]	Amount of addition in reaction mixture (µmoles)	Nitrite reductase activity (Units)
Undialysed[1]	-	-	45.6
Dialysed	-	-	6.0
Dialysed	FAD	0.1	4.8
Dialysed	FMN	0.1	6.0
Dialysed	Pyridoxal phosphate	0.1	3.6
Dialysed	NAD^+	0.1	3.6
Dialysed	$MgCl_2$	0.015	3.6
Dialysed	AA	0.1	1.2

[1] Extract was subjected to all conditions of the experiment other than dialysis, i.e., storage at 5°C for 6 hrs.

[2] When additions were made the volume of water in the assay mixture was reduced to compensate for the addition.

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NAD, nicotinamide adenine dinucleotide; AA, casamino-acids digest.

Table 6. Enzymatic Reduction of Nitrite with Sodium Dithionite or Reduced Nicotinamide Adenine Dinucleotide Phosphate as Electron Donors

Mycelia were grown, "induced" for 18 hrs. on nitrate medium, extracted and assayed using sodium dithionite as an electron donor (see METHODS). When NADPH was used as an electron donor the reaction mixture contained (μ moles): NADPH, 0.36; FAD, 0.22; $NaNO_2$, 3.3; potassium phosphate buffer (1.0 M, pH 7.5), 120; and 0.4 ml of extract containing 0.75 mg. of protein.

Assay mixture	Nitrite reductase activity (Units)	
Electron donor	Reduced BV	NADPH
Complete	57.6	4.8+
-extract	0	0
-$NaNO_2$	0	4.8+
-FAD	-	0

+ Nitrite accumulation above level initially added. The true activity is obtained using the formula below:
Activity = Disappearance of nitrite observed in complete assay mixture + nitrite accumulation observed in control with no $NaNO_2$ added.

Section 2. Metabolic Control of Nitrite Reductase. How is nitrite reductase activity affected by different nitrogen sources in the "induction" medium?

The purpose of this section was to attempt to answer two questions:

(1) Which compounds thought to be involved in the pathway of nitrate assimilation will induce nitrite reductase?

(2) Which postulated products of nitrate assimilation will repress nitrite reductase?

Nitrite is presumably the natural substrate of nitrite reductase and it is of interest to know whether nitrite alone will induce this enzyme, or if it can also be induced by nitrate. In order to study this problem, it was necessary to "induce" mycelia on media containing these different nitrogen sources and to examine the activity of nitrite reductase in mycelial extracts obtained from these cultures.

N. crassa does not grow well with nitrite as sole nitrogen source (Fig. 14). The yield of mycelium and the rate of growth are low when compared to growth on other nitrogen sources, e.g. nitrate and casamino-acids. The results given in Fig. 15 show that there is a marked inhibition of the growth of N. crassa on casamino-acids by nitrite, suggesting some toxic effect.

If nitrite is toxic by virtue of its effects on protein synthesis then it should inhibit the synthesis of an inducible enzyme. The results in Table 7 show that the presence of nitrite in a nitrate induction medium does not

significantly affect the activities of either nitrite reductase or glucose-6-phosphate dehydrogenase. The latter enzyme is constitutive in N. crassa and thus its specific activity in mycelial extracts would not necessarily be affected to any great extent by an inhibitor of protein synthesis. It was possible that in the case of nitrite reductase, the nitrate present in the medium was overcoming the effects of nitrite to some degree in this experiment. The effects of nitrite on the activity of an inducible enzyme not involved in nitrogen metabolism was therefore examined. The results described in Table 8 show that the presence of nitrite in the induction medium for β-galactosidase drastically reduces the level of this enzyme in mycelial extracts. Obviously nitrite cannot be used in "induction" experiments if this effect cannot be overcome.

The results in Fig. 16 show that filter sterilized nitrite gives a greater inhibition of growth of N. crassa on casamino-acids medium than does nitrite which has been sterilized by steam. This suggests that nitrite is degraded to some extent under the conditions of steam sterilization.

When filter sterilized nitrite is added to neutralized media containing casamino-acids, little inhibition of growth is observed (Fig. 16). The specific activity of β-galactosidase in extracts of mycelia induced in neutralized induction media is the same whether nitrite is present or not (Table 9). Thus neutralization of the induction medium appears to relieve the

toxic effects of nitrite on growth of N. crassa, and on the synthesis of an inducible enzyme. In all subsequent experiments where nitrite was used, it was filter sterilized and added to neutralized medium.

The results described in Table 10 show the effects of different nitrogen sources in the "induction" medium on the activity of nitrite reductase in mycelial extracts. Nitrate and nitrite were examined as possible inducers of the enzyme, and casamino-acids as possible repressors. As a control, mycelia were also "induced" in media containing no nitrogen source. In a true induction process the presence of an inducing molecule(s) is required to promote enzyme synthesis. Thus if nitrate and nitrite are true inducers in this system no nitrite reductase synthesis should be observed when mycelia are "induced" in a medium with no nitrogen source.

The specific activity of glucose-6-phosphate dehydrogenase is found to be fairly constant irrespective of the nitrogen source in the medium (Table 10). Thus the final results in these experiments are presented as a ratio of the activity of nitrite reductase to glucose-6-phosphate dehydrogenase per ml. of mycelial extract. In this way nitrite reductase activity is expressed relative to active protein in the extract rather than to total protein as measured by the Biuret test, which does not distinguish between active and inactive protein. The ratio of nitrite reductase to glucose-6-phosphate dehydrogenase is subsequently referred to simply as the N/G activity ratio.

Examination of the N/G activity ratios obtained in this experiment shows that the lowest N/G activity ratio is observed in extracts of mycelia isolated from casamino-acids medium. An increase in this ratio is observed in extracts of mycelia "induced" on nitrate, nitrite, and no nitrogen source, respectively. From these results a number of points are immediately apparent:

(1) Nitrite reductase is not completely repressed by a mixture of casamino-acids.

(2) Both nitrate and nitrite in the "induction" medium stimulate the formation of nitrite reductase, although to different degrees.

(3) The absence of a nitrogen source in the "induction" medium appears to stimulate enzyme formation to an even greater extent than did the presence of nitrate and nitrite, making it unlikely that nitrite reductase is truly induced.

It was necessary at this point to examine the N/G activity ratios observed in extracts prepared from mycelia which had been "induced" for different periods of time. In this way it should be possible to determine whether the differences between the N/G ratios on different media were due to the effects on the time necessary to achieve maximum "induction". When nitrate, nitrite, and no nitrogen source were present in the "induction" medium the N/G activity ratio increased for a period of 7 to 10 hrs. and then remained fairly constant, before finally decreasing after about 24 hrs. (Fig. 17). When casamino-acids were used as a nitrogen source no appreciable

increase in enzyme activity was observed throughout the course of the experiment (Fig. 17).

From the results described in the two previous experiments it would seem that the N/G activity ratio in extracts of mycelia "induced" in casamino-acids medium is significantly lower than the N/G ratio in extracts of mycelia "induced" in the other media used. This N/G ratio is fairly constant. It appears therefore that nitrite reductase is only partially repressed by casamino-acids. The effects of a number of possible repressors on the enzyme were thus examined. Methylalanine is an analogue of alanine, and hence an analogue of a possible end product of nitrite assimilation. It is a known repressor of nitrate reductase (52), and may thus be expected to repress nitrite reductase. Similarly in view of reports that sulphite and nitrite reductase activities are sometime associated (17,18,22) it is possible that the activity being measured in extracts of mycelia "induced" on casamino-acids media could be due to sulphite reductase activity. Therefore the effects of a level of cysteine known to repress sulphite reductase (8) were also examined. The results shown in Table 11 indicate that with all the possible repressors studied the N/G activity ratio is similar to that found in extracts of mycelia "induced" on casamino-acids medium. The significance of these results will be considered in the DISCUSSION.

The high N/G activity ratio observed in extracts of mycelia "induced" without a nitrogen source suggests that a

derepression may be occurring i.e., that de novo synthesis of the enzyme may require the absence of repressing molecules rather than the presence of inducing molecules. If nitrite reductase is induced by nitrate then nitrate should relieve the repression caused by amino-acids to some extent. However the results given in Table 12 show that no such effect is observed, making it improbable that nitrate is an inducer of nitrite reductase. If however, nitrite reductase is a derepressible enzyme then it should be synthesized in the presence of any compound which does not actively repress the enzyme, or in the absence of any nitrogen source, providing that the metabolic reserve of nitrogenous compounds is of sufficient size to support enzyme synthesis. This was in fact shown to be the case in previous experiments where nitrite reductase is synthesized by mycelia in media containing nitrate, nitrite, or no nitrogen source. The magnitude of the N/G activity ratios found in extracts of mycelia "induced" in these different media was found to differ, and this needs to be explained in the light of the derepression hypothesis.

The discrepancy in N/G activity ratios could be due to the accumulation of nitrite in extracts of mycelia "induced" on nitrate or nitrite, which would tend to obscure measurements of nitrite reductase activity. However there is not enough accumulation of nitrite in wild type mycelia grown on these media to interfere with the measurement of nitrite reductase activity. A more likely explanation for the differences in these N/G activity ratios is as follows.

Where no N source was used in the "induction" medium no end products of nitrate assimilation would build up, and thus no potential repressors would accumulate. Nitrate and nitrite are both assimilated and the end products of this assimilation could repress nitrite reductase, once they had been produced in sufficient quantities. As nitrate was present at a higher concentration in the "induction" medium than was nitrite, repression by the products of nitrate assimilation in these experiments would be greater. This hypothesis was tested using a nitrate non-utilizing mutant. In this mutant nitrate cannot be metabolized, and thus no end products could be formed, and consequently no repression would be expected. The results given in Table 13 show that when mutant nit-1-8A was "induced" on media containing a number of different N sources the resulting N/G activity ratios in cell free extracts did not differ to any great extent. These results support the hypothesis that the different N/G activity ratios encountered in wild type extracts of mycelia "induced" on different N sources are due to repression of nitrite reductase by metabolic end products.

In conclusion it appears that nitrite reductase is a derepressible, rather than an inducible enzyme.

Ammonium ions are known to have an effect on a number of physiological processes in N. crassa. The effects of "preinducing" mycelia on basic medium containing ammonium chloride was thus examined (Table 14). The N/G activity ratios observed were similar to those found when mycelia were

"preinduced" on media containing casamino-acids.

It was necessary to examine the effects of nitrate, ammonium chloride and casamino-acids on the in vitro assay of nitrite reductase to determine whether these compounds could inhibit the enzyme activity. The results summarized in Table 15 show that NH_4^+ has little effect on nitrite reductase activity, and that casamino-acids appear to give a slight stimulation when added in substrate amounts. However this stimulation was not observed when smaller amounts of casamino-acids were added to a dialysed extract (Table 5). The apparent low specific activity of the enzyme when nitrate was added to the assay mixture was due to the activity of nitrate reductase in the extract used. This activity can be compensated for as described in Section 1.

Section 3. Nitrite Reductase in a Nitrite Non-Utilizing Strain of *N. crassa*

A nitrate and nitrite non-utilizing strain of *N. crassa*, designated O, selected on the basis of its inability to grow on nitrate or nitrite and its ability to accumulate nitrite when grown on nitrate, was tested for nitrite reductase activity (Table 16). The mutant was shown to have an active nitrite reductase and to respond to the presence of nitrate and casamino-acids in the "induction" medium in a manner similar to the wild type strain.

Preliminary experiments have also indicated that certain nitrate non-utilizing mutants will grow quite well on nitrate after pre-induction on a casamino acids medium. The significance of the work on these mutants will be considered in the DISCUSSION.

Fig. 14. Growth of N. crassa on nitrite medium. Aliquots (0.5 ml.) of conidial suspension of N. crassa were inoculated into flasks, each containing 50 ml. aliquots of unneutralized basic medium containing varying amounts of autoclaved sodium nitrite. The cultures were then incubated on a rotary shaker (see METHODS) for 48 hrs. At this time mycelia were filtered free of the media on a Buchner funnel, dried in an oven at 60°C for 12 hrs., and weighed.

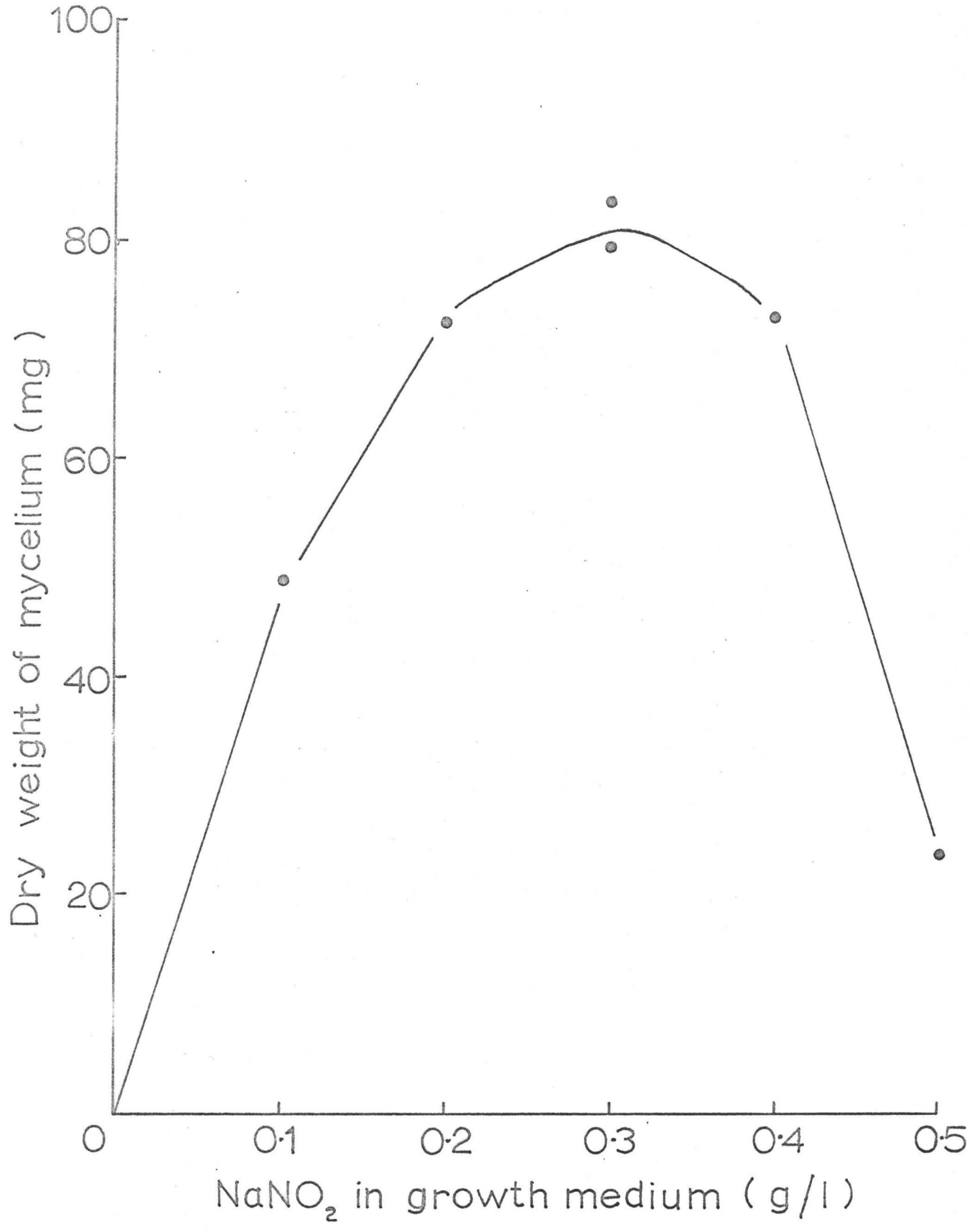

100
80
60
40
20
0
Dry weight of mycelium (mg)
0·1
0·2
0·3
0·4
0·5
NaNO2 in growth medium (g/l)

Fig. 15. Effect of autoclaved sodium nitrite on the growth of N. crassa in unneutralized casamino-acids medium. The growth procedure, isolation and drying of mycelial pads was as described in Fig. 14. Nitrite was added to basic medium containing 5 g/l casamino-acids as indicated, and cultures were incubated for 24 hrs.

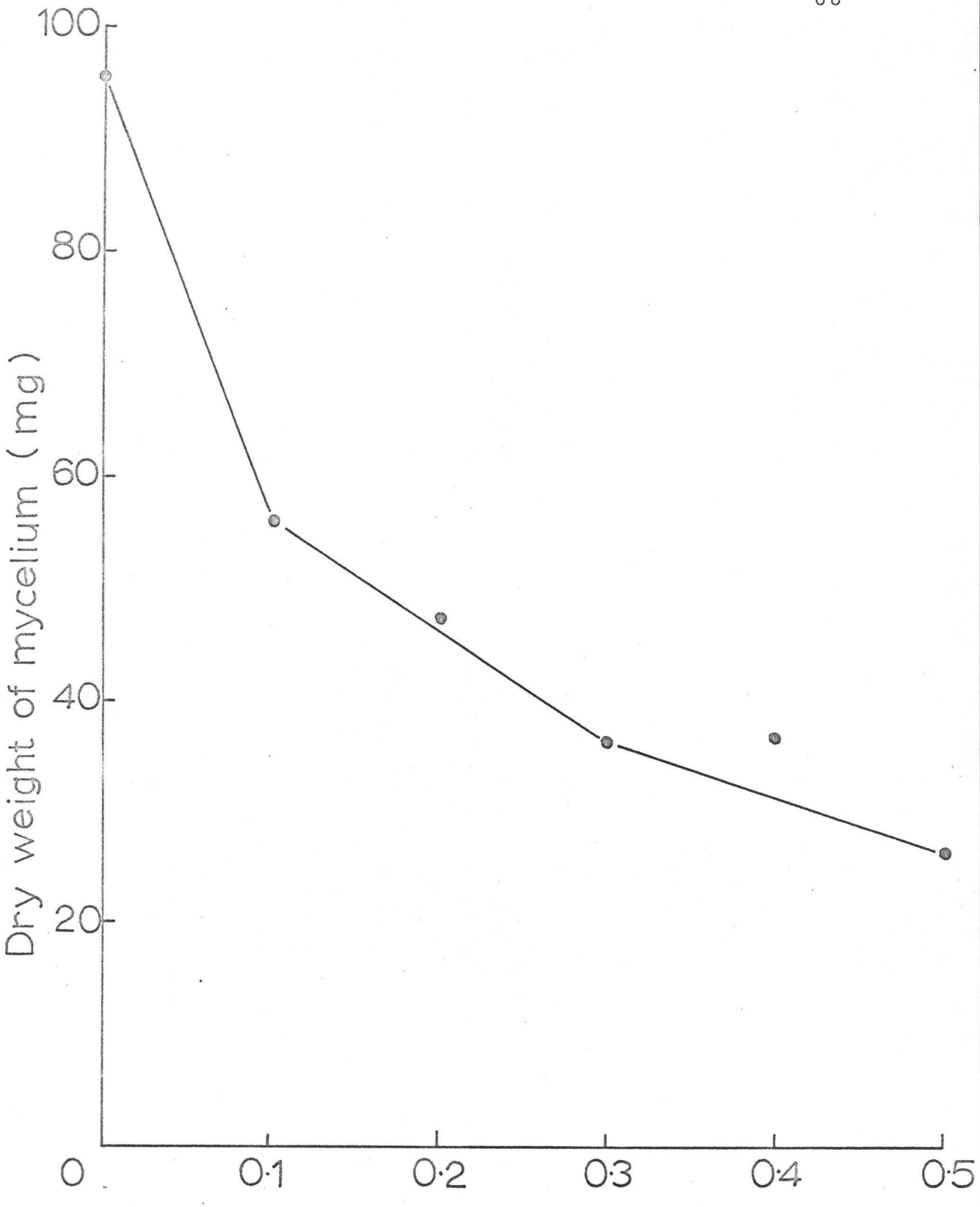

100
80
60
40
20
0
0·1
0·2
0·3
0·4
0·5
Dry weight of mycelium (mg)
NaNO2 added to casamino acids growth medium (g/l)

Table 7. Relative Effects of Autoclaved Sodium Nitrite in Unneutralized "Induction" Medium on the Specific Activity of Nitrite Reductase and Glucose-6-Phosphate Dehydrogenase

The growth conditions used are described in METHODS. Sectors of mycelial pads were "induced" for 17 hrs. on unneutralized media containing the nitrogen sources indicated. Extracts were subsequently prepared and assayed for nitrite reductase and glucose-6-phosphate dehydrogenase activities.

N Source in "Induction" Medium	Specific activity (Units/mg. protein)	
	Nitrite Reductase	Glucose-6-phosphate dehydrogenase
Casamino-acids	11.77	0.335
Nitrate	30.1	0.302
Nitrate + 0.1 g/l nitrite*	34.3	0.342

* Sterilized by autoclaving.

Table 8. Effect of Filter Sterilized Nitrite in the Unneutralized "Induction" Medium on the Specific Activity of β-Galactosidase

Mycelia were grown and induced as described in Methods (Fig. 4). Various amounts of filter sterilized sodium nitrite were added to unneutralized "induction" media as shown. The control was grown on a medium containing glycerol as sole carbon source. After induction the mycelial pads were harvested and extracted, and the extracts assayed for β-galactosidase and glucose-6-phosphate dehydrogenase activities (METHODS).

Media	Specific activity (Units/mg. protein)		Ratio of β-galactosidase to glucose-6-phosphate dehydrogenase (a/b)
	(a) β-galactosidase	(b) Glucose-6-phosphate dehydrogenase	
No induction[1]	15.57	0.447	3.49
Induction[2]	169.7	0.321	528.2
Induction +0.1g/l NO_2^-	16.64	0.389	42.7
Induction +0.05g/l NO_2^-	32.06	0.403	79.6
Induction +0.01g/l NO_2^-	97.66	0.486	200.7

[1] Mycelia were grown on media containing glycerol as sole carbon source.

[2] Mycelia were grown on media containing lactose and glycerol as carbon sources.

Fig. 16. Effect of sodium nitrite sterilized by different procedures on the growth of N. crassa in a casamino-acids medium at pH 5.4 and 7.0. The growth procedure, extraction and drying of mycelial pads was as described in Fig. 14. Nitrite was added to basic medium containing 5 g/l casamino-acids which was neutralized where indicated, by the addition of K_2HPO_4. Filter-sterilized nitrite was passed through a millipore filter unit. Cultures were incubated for 24 hrs. ●— ● , nitrite sterilized by steam; o— o , nitrite sterilized by filtration; ■— ■ , nitrite sterilized by filtration and added to neutralized casamino-acids medium.

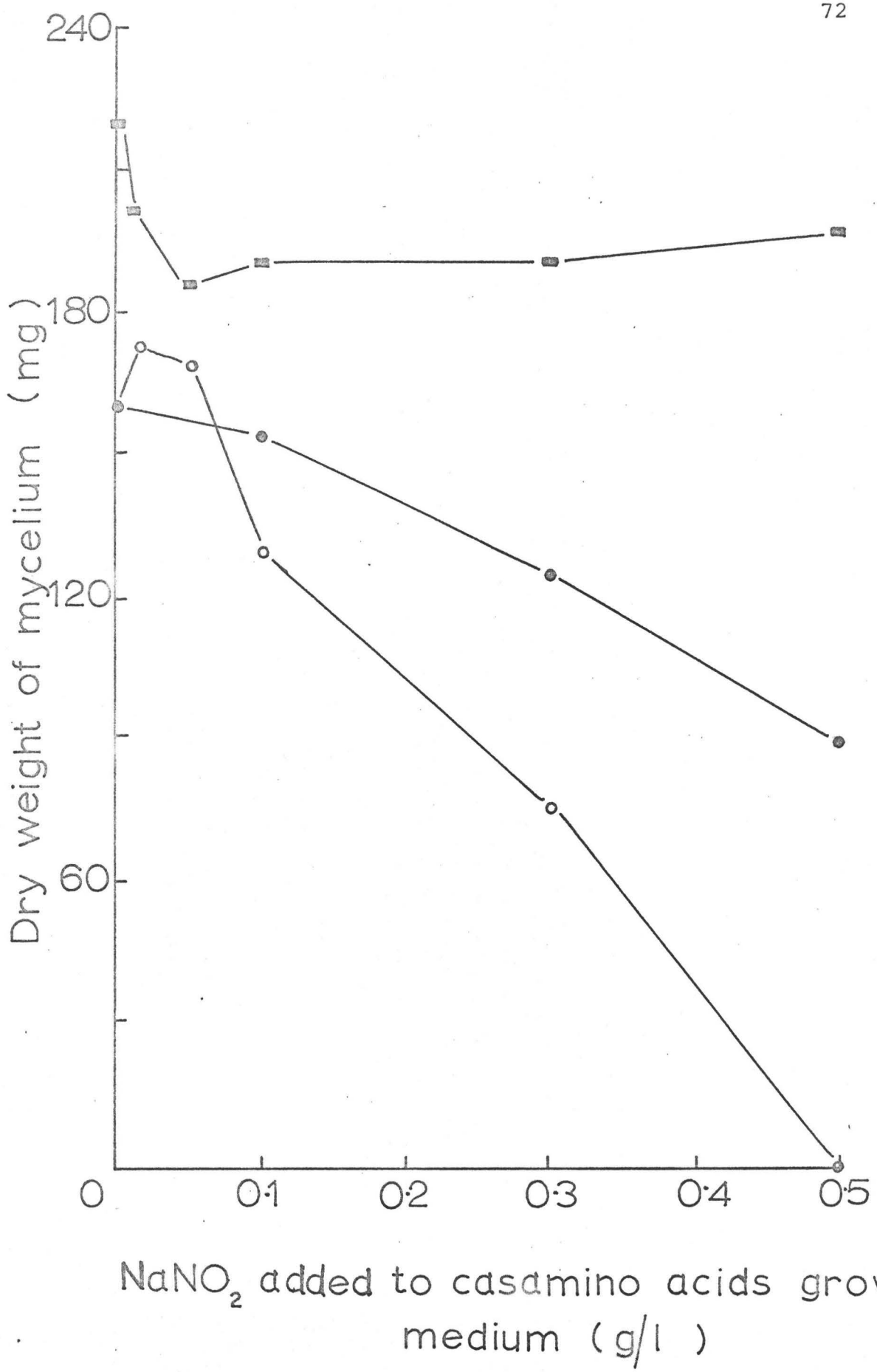

240
180
120
60
0
0·1
0·2
0·3
0·4
0·5
Dry weight of mycelium (mg)
NaNO2 added to casamino acids growth medium (g/l)

Table 9. Effect of Filter Sterilized Nitrite on the Induction of β-Galactosidase at pH 5.4 and 7.0

Preincubation was as described in METHODS (Fig. 4). The resulting mycelia pad was then harvested and cut into sectors which were subsequently transferred to β-galactosidase induction media (Fig. 4) at pH 5.4 or 7.0. Filter sterilized nitrite was added as indicated. After induction, pads were harvested, extracted and assayed for β-galactosidase activity.

Additions to Medium	pH of medium	Specific activity of β-galactosidase (Units/mg. protein)
-Inducer	5.4	2.71
+Inducer	5.4	163.4
+Inducer	7.0	130.9
+Inducer +0.1g/l $NaNO_2$	5.4	9.17
+Inducer +0.1g/l $NaNO_2$	7.0	131.8

Table 10. Effect of Various Nitrogen Sources in the "Induction" Medium on the Ratio of Nitrite Reductase to Glucose-6-Phosphate Dehydrogenase in Mycelial Extracts

Medium	Specific activity (Units/mg of protein)				Ratio of nitrite reductase to glucose-6-phosphate dehydrogenase (a/b)	
	(a) Nitrite reductase		(b) Glucose-6-phosphate dehydrogenase			
No nitrogen source	49.4	54.8	0.398	0.503	124.3	108.9
5g/l KNO_3	29.5	26.8	0.45	0.465	65.64	57.8
0.1g/l $NaNO_2$[1]	50.5	50.6	0.52	0.65	103.4	77.5
5g/l casamino-acids	12.98	8.7	0.48	0.52	27.33	16.81

[1] This was found to be the amount of $NaNO_2$ giving maximum enzyme activity. Nitrite was filter sterilized and added to neutralized medium.

Fig. 17. Effect of time on the ratio of nitrite reductase to glucose-6-phosphate dehydrogenase in mycelialextracts of N. crassa grown in "induction" media containing different nitrogen sources. Mycelia were grown, "induced", extracted and assayed as described in METHODS. The time of "induction" was varied as indicated. ●—● ,■—■ ,□—□ ,○—○ represent the N/G activity ratios found in extracts of mycelia grown on basic media containing 5 g/l casamino-acids, 5 g/l KNO_3, 0.1 g/l $NaNO_2$ and no nitrogen source, respectively. Nitrite was filter sterilized and added to neutralized medium.

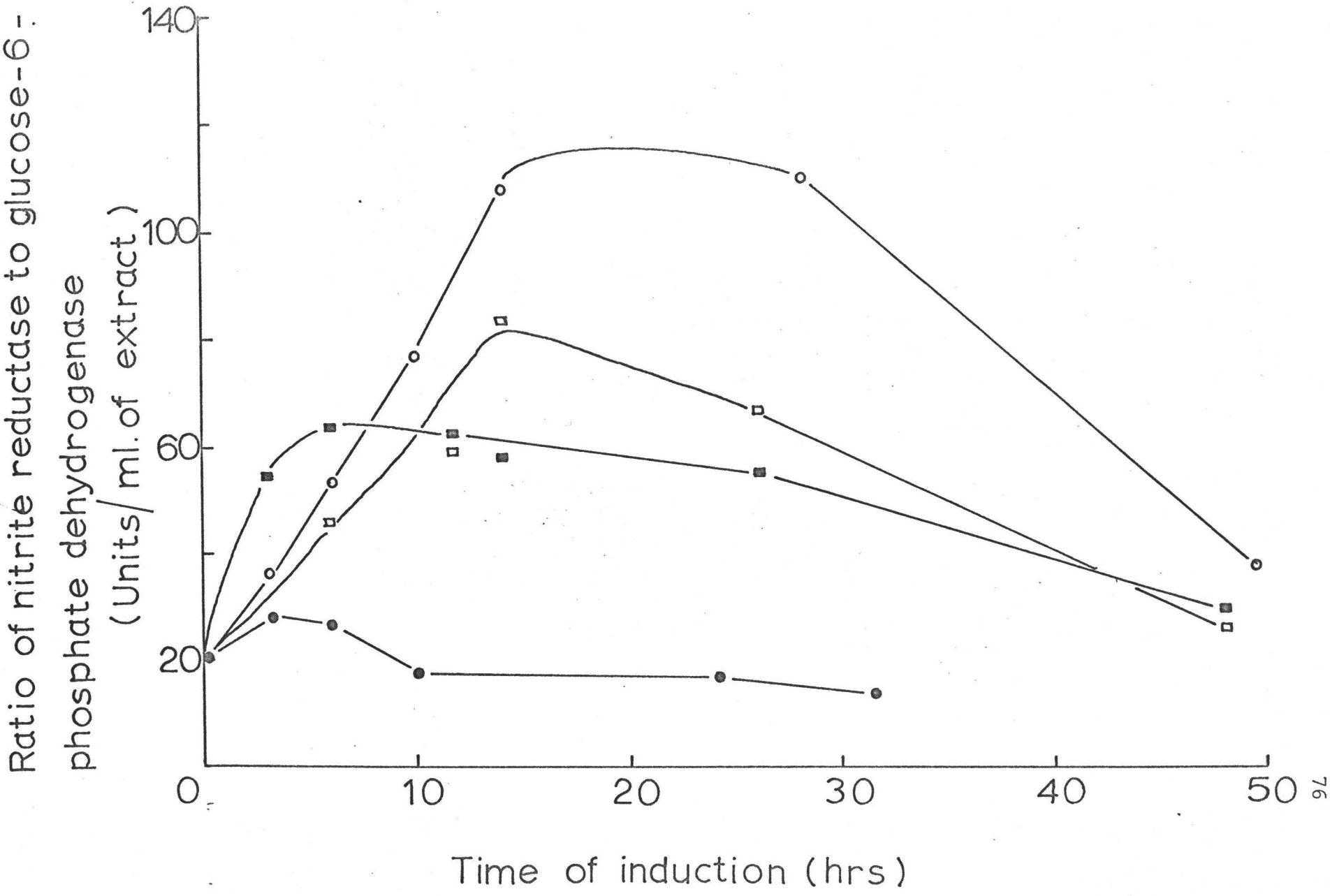

Ratio of nitrite reductase to glucose-6-phosphate dehydrogenase (Units/ml. of extract)
140
100
60
20
0
10
20
30
40
50
Time of induction (hrs)

Table 11. Effect of Known Repressors of Nitrate Reductase and Sulphite Reductase on the Ratio of Nitrite Reductase to Glucose-6-Phosphate Dehydrogenase in Extracts of N. crassa

Mycelia were grown and "induced" as described in METHODS. Extracts were then prepared and assayed for the enzymes shown.

N source in "induction" medium	Additions to "induction" medium	Specific activities (Units/mg. protein)		Ratio of a/b
		Nitrite reductase (a)	Glucose-6-phosphate dehydrogenase (b)	
5g/l KNO_3	-	33.1	0.458	72.19
5g/l KNO_3	1g/l methylalanine	16.8	0.545	30.84
5g/l casamino-acids	-	15.96	0.425	37.59
5g/l casamino-acids	0.85 mM cysteine	13.17	0.499	26.39

Table 12. Effect of Nitrate and Casamino Acids in the "Induction" Medium on the Ratio of Nitrite Reductase to G-6-P Dehydrogenase in Mycelial Extracts

Mycelial were grown, "induced", extracted, and assayed as described in METHODS. The figures shown represent the results of two independent experiments.

N source in "induction" medium	Enzyme activities				Ratio of nitrite reductase to glucose-6-phosphate dehydrogenase (a/b)	
	(a) Nitrite reductase (Units/ml. of extract)		(b) Glucose-6-phosphate dehydrogenase (Units/ml. of extract)			
5g/l KNO_3	246.0	150.0	2.75	1.722	89.64	87.08
5g/l casamino-acids	150.0	144.0	4.588	4.347	33.99	33.12
5g/l KNO_3 plus 5g/l casamino-acids	144.0	60.0	5.246	2.048	27.45	29.22

Table 13. Effect of Various Nitrogen Sources in the "Induction" Medium on the Ratio of Nitrite Reductase to G-6-P Dehydrogenase in Extracts of a Nitrate Non-Utilizing Strain of *N. crassa*.

Mycelia of nitrate non-utilizing strain nit-1-8A were grown, "induced", extracted, and assayed as described in METHODS.

N source in "induction" medium	Enzyme activities		Ratio of nitrite reductase to glucose-6-phosphate dehydrogenase (a/b)
	(a) Nitrite reductase (Units/ml. of extract)	(b) Glucose-6-phosphate dehydrogenase (Units/ml. of extract)	
5 g/l KNO_3	96.0	0.7914	124.2
0.1 g/l $NaNO_2$	99.0	0.7246	136.7
No nitrogen source	111.0	0.7729	143.6
5 g/l casamino-acids	273.0	6.956	39.24

Table 14. Effect of Various Nitrogen Sources in the "Induction" Medium on the Ratio of Nitrite Reductase to G-6-P Dehydrogenase in Extracts of Mycelia "Preinduced" on Ammonium Chloride Medium.

Mycelia were grown, "induced", extracted and assayed as described in METHODS using 3.9 g/l NH_4Cl as the sole nitrogen source in the "preincubation" medium.

N source in "induction" medium	Enzyme activities		Ratio of nitrite reductase to glucose-6-phosphate dehydrogenase (a/b)
	(a) Nitrite reductase (Units/ml. of extract)	(b) Glucose-6-phosphate dehydrogenase (Units/ml. of extract)	
No nitrogen source	180.0	1.288	139.7
5 g/l KNO_3	117.0	1.4	83.58
0.1 g/l $NaNO_2$	156.0	1.5	104.0
5 g/l casamino-acids	126.0	4.21	29.96

Table 15. Effects of Nitrate, Ammonium Ions and Casamino-Acids on Nitrite Reductase Activity *in vitro*

Mycelia were grown, "induced", extracted, and assayed as described in METHODS. Nitrite reductase activity was measured in the presence of KNO_3, NH_4Cl and casamino-acids. The volume of water in the reaction mixture was reduced to compensate for the additions. The figures given are results obtained from two independent experiments.

Expt.	Addition to assay mixture	Specific activity of nitrite reductase (Units/mg. protein)	
1	None	37.49	28.57
	3.9 g/l NH_4Cl	33.66	30.95
	5 g/l casamino-acids	36.24	40.95
2	None	30.0	
	5 g/l KNO_3	31.0[+]	

[+] Corrected for nitrite accumulation due to nitrate reductase (RESULTS p. 36).

Table 16. Nitrite Reductase in Wild Type and Mutant 0

Mycelia were grown, "induced", extracted and assayed as described in METHODS. 0 is a nitrate and nitrite non-utilizing mutant isolated in this laboratory by Miss C. Dyer.

Strain	N source in "induction" medium	Enzyme activities		Ratio of nitrite reductase to glucose-6-phosphate dehydrogenase (a/b)
		(a) Nitrite reductase (Units/ml. of extract)	(b) Glucose-6-phosphate dehydrogenase (Units/ml. of extract)	
Wild type	5 g/l KNO_3	348.0	5.797	60.03
	Casamino-acids	48.0	3.639	13.19
Mutant 0	5 g/l KNO_3	66.0	1.003	65.8
	Casamino-acids	42.0	4.033	10.4

DISCUSSION

The results presented in Section 1 describe a new assay system for nitrite reductase in N. crassa. This system has the advantage of being both fast and simple to use, thus making it suitable for routine use. A similar system has been used by Joy (16) to study nitrite reductase in S. oleracea and Z. mays extracts, but the assay described here differs from that of Joy in the following ways:

1. The concentrations of nitrite and BV used are different.
2. The rate of nitrite disappearance is constant for 10 mins. as opposed to 20 mins. in Joy's system.
3. The reaction can be carried out in open test tubes and gives reproducible results.

The specific activity of nitrite reductase measured by this assay is considerably higher than that measured in most previous systems described (Table 17).

The product of nitrite reduction in this case was not identified. This product is thought to be ammonia (6,12,16,38) and the inability to demonstrate ammonia production in this work could have been due to the technical difficulties involved, i.e., ammonia was present in appreciable quantities in the extracts used, and tended to obscure the results obtained. An attempt was made to remove ammonia from these extracts by dialysis. However, the enzyme appeared

Table 17. A Comparison of the Specific Activities of Nitrite Reductase Reported by Various Investigators

Extract	Specific activity of nitrite reductase (mμ moles of nitrite disappearing /min/mg of protein)	Organisms	Workers	Reference
Purified	1.5	*N. crassa*	Nicholas	30
Crude	2.85*	*N. crassa*	Medina & Nicholas	23
Purified	52.3	*N. crassa*	Nicholas *et al.*	31
Crude	4.26	*A. agile*	Spencer *et al.*	44
Purified	0.25	*C. pepo* *Z. mays*	Hageman *et al.*	12
Purified	4.0	*L. esculentum*	Sanderson & Corking	38
Crude	27.3	*S. oleracea*	Joy *et al.*	16
Crude	43.0	*N. crassa*	Cook	Section 1 in the RESULTS section in this thesis

* In this case ammonia production was measured.

to lose most of its activity on dialysis and attempts to restore this activity by additions to the dialysed extract were unsuccessful. The possibility that the product of the reaction was not ammonia cannot be ignored, but resolution of the problems of technique mentioned will be required before any definite conclusions can be drawn.

Earlier reports of nitrite reduction by NADPH in N. crassa (23,28,37) were not confirmed in this work. These findings are in agreement with those of Hageman et al. (12) and Sanderson and Cocking (38) who were also unable to demonstrate nitrite reduction when NADPH was used as an electron donor. It is therefore likely that the earlier reports of enzymic nitrite reduction by NADPH are best explained in other ways (38 and INTRODUCTION).

In the course of the investigation on the metabolic control of nitrite reductase it was found that nitrite was degraded to some extent under the conditions of autoclaving, and was therefore sterilized by filtration in subsequent experiments. Nitrite is toxic to the growth of N. crassa and also to protein synthesis. These toxic effects could be overcome by neutralizing media to which nitrite was added. It was necessary to overcome the toxic effects of nitrite as it could not be used in "induction" experiments while it was inhibitory to protein synthesis. The relief of this toxicity of nitrite by neutralization of the medium suggests that it was perhaps nitrous acid, which is present at low pH values, which was responsible for the observed toxicity.

In most of the studies on metabolic control reported here, the activity of nitrite reductase was presented relative to that of glucose-6-phosphate dehydrogenase, which is a constitutive enzyme in N. crassa. In this way fluctuations in nitrite reductase in mycelial extracts are considered with respect to an amount of active protein in the extracts. This is perhaps a more useful criteria than specific activity in which enzyme activity is considered relative to total protein, some of which may be inactive.

Two conclusions may be drawn from the results on metabolic control:

1. A mixture of casamino-acids, or a number of other possible repressors, do not repress nitrite reductase completely, although a partial repression is observed.
2. Nitrite reductase appears to be a derepressible enzyme.

The low ratio of nitrite reductase to glucose-6-phosphate dehydrogenase found in extracts of mycelia grown on casamino-acids could be due to a number of reasons. First, it could represent activity due to another enzyme, not necessarily involved in nitrogen metabolism, but able to reduce nitrite. This appears to be the case with sulphite reductase in a number of systems (17,18,22). Second, it may be due to the presence of a constitutive nitrite reductase in N. crassa extracts. This possibility could be examined by subjecting extracts to column chromatography to separate different activities, and then to observe which activities are present in extracts of mycelia induced on different N sources. Finally, it could simply

be due to the fact that none of the compounds tested were capable of totally repressing nitrite reductase.

In a true induction process the presence of a specific inducing molecule is required to promote enzyme synthesis. However, in a process of derepression de novo synthesis of an enzyme requires only the absence of repressing molecules. That nitrite reductase is derepressed, rather than induced, is supported by the following evidence:

1. The high ratio of nitrite reductase to glucose-6-phosphate dehydrogenase found in extracts of mycelia grown with no nitrogen source.
2. The inability of nitrate to relieve the repression caused by casamino-acids, thus making it improbable that either nitrate or nitrite are true inducers of the enzyme.

If a derepression process is in fact operative in N. crassa then one would expect that equivalent amounts of enzyme would be formed in mycelia grown on media containing no repressing molecules. The N/G activity ratios observed in extracts of mycelia grown on nitrite, nitrate and no nitrogen source however, were found to differ. It was suggested that these differences in N/G activity ratios were due to the repression of the enzyme by metabolic end products. This hypothesis was tested using a mutant which could not metabolize nitrate. The N/G activity ratios observed in extracts from mycelia of this mutant grown on different nitrogen sources were in fact similar in magnitude. This supports the hypothesis suggested.

Little work has been previously carried out on the metabolic control of nitrite reductase, and few investigators have examined enzyme activity in extracts of cells grown with no nitrogen source. Ingle *et al.* (15) found a negligible level of nitrite reductase in extracts of radish cells grown for 5 days with no nitrogen source. However it is possible that, after 5 days, the cells contained insufficient nitrogenous reserves to be able to synthesize new enzymes.

None of the nitrogen sources used in the repression studies described here inhibited nitrite reductase activity *in vitro*, thus excluding the possibility that the different N/G activity ratios were due to inhibition of nitrite reductase activity *per se*.

The studies on nitrite reductase in extracts of a nitrite non-utilizing mutant produced some results which are difficult to explain. Mycelia which would not grow on nitrate or nitrite nevertheless possessed an active nitrite reductase as measured *in vitro*. Similar results with mutants were obtained in earlier work with *N. crassa* (42). There is evidence that nitrite reductase is localized in an organelle system in green leaf tissue and Ritenour *et al.* have reported its localization in chloroplasts in *Z. mays* and *Setaria faberii*. A possible explanation for these results with mutants can be proposed if we assume that nitrite reductase in *N. crassa* is also localized in some organelle e.g. mitochondria. Nitrate reductase is localized in the cytoplasm in *N. crassa*, in soluble cell fractions. Thus nitrate entering the cells

is converted to nitrite. If nitrite reductase is situated in mitochondria then this nitrite must be transported into the mitochondria before it can be metabolized, a process possibly requiring a specific permease enzyme. If the mutations being studied affected this permease, then nitrite would accumulate in the cytoplasm and no growth would be observed. However on extraction the enzyme would probably be released as the extraction procedure used in this work would tend to break up any mitochondria present. Thus the enzyme would be free to reduce nitrite *in vitro*. No data is presented here to suggest that this is the case, and studies on the localization of nitrite reductase in *N. crassa* are required before it will be known if this is a feasible explanation.

Preliminary studies with other nitrate and nitrite non-utilizing mutants have shown that these will grow quite well on nitrate after preinduction on casamino-acids medium. These results await more careful study, but if they are confirmed then the possibility that a number of different nitrite reductases exist, associated with different parts of the organism, e.g. mycelia and conidiospores, must be considered.

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