STUDIES SUGGESTING THE PRESENCE OF MORE THAN ONE NITRITE REDUCTASE IN *NEUROSPORA CRASSA*

by

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SCOPE AND CONTENTS:

It is usually assumed that the reduction of nitrite by nitrite reductase results in the formation of ammonia.

The purpose of this investigation was to enquire whether more than one nitrite reductase activity is responsible for *in vivo* nitrite reduction.

An assay system which measures the production of ammonia, as a result of nitrite reductase is described. The reduction of nitrite by nitrite reductase did not result in the formation of stoichiometric amounts of ammonia. Nitrite non-utilizing mutants showed that nitrite reduction could occur *in vitro* with no subsequent formation of ammonia or could result in the formation of essentially stoichiometric amounts of ammonia. Sedimentation velocity gradient centrifugation resulted in the separation of at least two peaks of nitrite reductase activity.

A model is described which accounts for the results in terms of two nitrite reductase activities, necessary for *in vivo* nitrite reduction.

ACKNOWLEDGEMENTS

Anything is possible, some things are probable, but nothing is certain.

I would like to thank my supervisor, Dr. G. J. Sorger for his criticisms and suggestions during the course of this research. Thanks are due also to McMaster University, Department of Biology for financial support.

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INTRODUCTION

Nitrite reductase is usually defined as that enzyme which catalyzes the reduction of nitrite to ammonia. Its presence has been reported in plants, fungi and micro-organisms.

An inorganic pathway, comprised of a series of two electron steps has been suggested for the reduction of nitrate to ammonia (26).

$$NO_3^- \longrightarrow NO_2^- \longrightarrow \underset{H_2N_2O_2}{\overset{(HNO)}{NO_2\text{-}NH_2}} \longrightarrow NH_2OH \longrightarrow NH_3$$

nitrate nitrite nitroxyl nitramide hyponitrous acid hydroxylamine ammonia

The enzymatic mechanism for the reduction of nitrate to nitrite has been studied intensively. The enzyme involved, nitrate reductase, is a soluble molybdo-flavoprotein, which is apparently induced by nitrate. However, the pathway, intermediates, regulation and mechanism of reduction, beyond nitrite, is in need of much clarification.

Nitrite Reductase Assays; The Product of Nitrite Reduction

Yamagata, in 1939, first demonstrated the presence

of nitrite reductase activity in cell free preparations of *Pseudomonas aeruginosa*. Since that time the activity has been demonstrated in such widely varying species as *Neurospora crassa*, *Bacillus pumilus*, *Azotobacter agile*, *Anabaena cylindrica* and *Cucurbita pepo* (12,13,27,38,39).

Reports of nitrite reductase in the literature have proven to be confusing due to the different assays used. Early assays measured the nitrite-dependent disappearance of NADH or NADPH and in many cases, either nitrite disappearance or ammonia production was not shown (27,28,32).

Sanderson and Cocking have pointed out that nitrite reductase assays, dependent on pyridine nucleotides as electron donor, are erroneous when the activity is measured as ammonia production (33). When measuring ammonia production, using the Conway microdiffusion method (2), ammonia is made volatile by the presence of alkali. Sanderson and Cocking found that NAD^+ and NADH were partially degraded by alkali to yield ammonia. NAD^+ is degraded to a greater extent than NADH and thus it is necessary to make a correction for the ammonia coming from the NAD^+, which results from NADH oxidation. Earlier work was cast into doubt due to the lack of this control.

Nitrite reductase in *Spinacea oleracea* and *Zea mays* was assayed by Joy and Hageman using reduced benzyl viologen as electron donor. Eighty-five percent of the nitrite reduced

was recovered as ammonia. This discrepancy from stoichiometry may have been due to technical difficulties; however, a second product cannot be ruled out.

Cook (3,4) developed a new assay for nitrite reductase from *Neurospora crassa*. Specific activities of this reductant and extract-dependent activity, as measured by nitrite disappearance, were approximately two times higher than that obtained by Joy and Hageman (17). However, Cook was unable to show that ammonia was the product of the reaction.

An NADH or NADPH-dependent nitrite reductase present in partially purified extracts of *Glycine max* and of *N. crassa* was shown to catalyze the production of ammonia (27). However, in later work on *Glycine max*, Roussos and Nason found that in the NADH-dependent assay, there was neither nitrite disappearance nor ammonia production (32). In view of Sanderson and Cocking's objection, it is questionable whether these results contribute significantly to the understanding of nitrite reductase.

Apparent good stoichiometry was obtained between nitrite reduced and ammonia produced with an enzyme activity obtained from *Azotobacter agile* (38). The assay utilized NADPH as electron donor. No controls were included in the data to indicate how much ammonia was produced in the absence of added nitrite.

Hageman and Cresswell (12) showed that extracts of *Cucurbita pepo* could reduce nitrite to ammonia, using either reduced benzyl viologen or NADH and catalytic amounts of the dye as electron donor. No reduction occurred with NADH or NADPH in the absence of the dye. Hageman suggested that benzyl viologen was substituting for an unidentified natural cofactor.

Sanderson and Cocking (33), using the benzyl viologen assay of Hageman, and tomato leaf extracts found that ammonia was the product of nitrite reduction.

Zumft et al. (44) studied a partially purified preparation of nitrite reductase from *Chlorella*. They found that the enzyme catalyzed the reduction of nitrite to ammonia, the activity specifically requiring reduced ferredoxin as electron donor. Neither NADH nor NADPH were effective electron donors. Using reduced methyl viologen as electron donor, the enzymatic reduction of nitrite was accompanied by the formation of stoichiometric amounts of ammonia.

Hattori et al. (13), working with the blue-green alga, *Anabaena cylindrica*, purified nitrite reductase forty-fold. A stoichiometry of one mole of ammonia formed per mole of nitrite disappeared was found, using reduced ferredoxin as electron donor.

There are a few published reports which consider the possibility that nitrite might be reduced to the reduction

level of ammonia via an organic pathway, such that free ammonia would not be produced.

Silver and McElroy (34) found that extracts of *N. crassa* could reduce m-dinitrobenzene (DNB) to nitroaniline via nitrophenylhydroxylamine. Extracts of nitrate and nitrite non-utilizing mutants were surveyed for DNB reducing activity by measuring nitrophenylhydroxylamine and nitroaniline formation. Preparations of two of the mutant strains were incapable of reducing DNB. Nitrophenylhydroxylamine was accumulated in extracts of three of the other mutant strains. Preparations of a third class of mutant rapidly reduced DNB to nitroaniline. These findings were observed only when the mutants were grown on ammonium nitrate. When they were grown on ammonium chloride, all of the mutants reduced DNB to nitroaniline. The results obtained with the ammonium nitrate medium were believed to have been due to the formation of reduction products of nitrate which inhibited DNB reduction. No investigations were carried out to determine whether or not this DNB reducing activity was physically or genetically different from the nitrite reducing activity.

The isolation of mutants of Neurospora which required pyridoxine for growth on nitrate but not for growth on ammonia, led McElroy to postulate an organic pathway going from hydroxylamine through pyridoxal oxime

phosphate and pyridoxamine phosphate to amino nitrogen (22).

Another pyridoxine requiring mutant of *N. crassa*, employed by Nicholas (28), required pyridoxine when grown on ammonia. It was reported that the nitrite reductase in crude extracts of this pyridoxine requiring mutant was stimulated by the addition of either pyridoxine, pyridoxal phosphate or pyridoxal. This effect was not observed with partially purified preparations.

Cook (3), using dialyzed extracts of *N. crassa*, was unable to show a response of nitrite reductase activity to pyridoxine.

The role of pyridoxine, then, in nitrite reduction, is not clear; there is no substantial evidence, based on the role of pyridoxine, to invoke an organic pathway.

Wood (42) concluded that free hydroxylamine, which is highly toxic, is unlikely to be present in plants in appreciable quantities. The presence of oximes was regarded as likely and consistent with the supposed production of hydroxylamine as an intermediate in nitrite reduction.

Virtanen (41) held the same opinion regarding the significance of oximes and hydroxylamates produced by *Torulopsis utilis* and rye roots grown in nitrate or nitrite. These products were not detected when ammonia was used as the nitrogen source. The possibility was considered,

however, that hydroxylamine normally remains bound to the nitrite reducing enzyme and does not appear as a free intermediate.

Vaidyanathan and Street suggested oxime formation to explain the fact that only 2 percent of the nitrite reduced by extracts of tomato roots was recovered as ammonia (40).

The available evidence suggests that ammonia rather than an organic amine is a likely product of nitrite reductase activity. However, the suggestions of oxime formation cannot be ignored. Indeed, the somewhat conflicting evidence, concerning the products of nitrite reduction, may be suggestive that more than one enzyme system is involved in nitrite reduction.

Localization and Regulation of Nitrite Reductase

Although it has been quite firmly established, by Ritenour (31), that nitrite reductase in leaf tissue is localized within chloroplasts, there have been few reports of the localization of nitrite reductase in non-photosynthetic tissues. Miflin (23,24), using differential centrifugation, obtained evidence that approximately 40 percent of the nitrite reductase activity in barley roots is associated with a 'mitochondrial' fraction. However, this 'mitochondrial' preparation was not tested to confirm the presence of

mitochondria, and localization in other sub-cellular particles, present in this 'mitochondrial' fraction, was not considered.

Sims proposed that individual enzymes for nitrate reduction in *Candida utilis* are organized in an oligomeric complex referred to as a nitrosome (36). This enzyme complex has a specific requirement for NADPH, and the nitrosome is believed to be capable of carrying out the reduction of nitrate or nitrite to ammonia without the formation of free intermediates. Nitrate and nitrite are assumed to be inducers of the complex, since only minute amounts of enzyme are formed in their absence, including under conditions of nitrogen starvation. When the organism is grown in an ammonia or amino acids medium, containing nitrate also, varying degrees of repression of enzyme production are observed. In the presence of ammonia alone, repression is complete. *In vitro* inhibition can be demonstrated with a wide range of compounds including ammonia, amino acids, nucleotides, pyridoxamine phosphate, and carbamyl phosphate. There is an indication that several different receptor sites are involved in these inhibitions because both competitive and non-competitive kinetics were observed.

Pateman (29) suggests that nitrite reductase from *Aspergillus nidulans* is repressed by ammonia. The specific activity of nitrite reductase in extracts of *Aspergillus* grown on ammonia was lower than that in extracts of mycelia

grown on nitrate. These data cannot be used to invoke any specific regulatory mechanism because nitrite reductase levels in preparations of mycelia exposed to a nitrogen-deficient media were not measured.

Cook (3,4) demonstrated that nitrite reductase in *Neurospora crassa* was only partially repressed when the organism was grown in a medium containing casamino acids as nitrogen source. Evidence was also presented to suggest that nitrite reductase may be derepressible rather than inducible, since maximum specific activities of nitrite reductase were obtained when *Neurospora* was exposed to a nitrogen-deficient media.

Filner (11) suggested that nitrite reductase in cultured tobacco cells is induced by nitrate. From his results, however, it can be seen that a low level of activity is present in extracts of cells grown in the absence of nitrate. The development of nitrite reductase was inhibited by a casein hydrolysate.

Nitrite reductase levels in extracts of *Raphanus sativus* cotyledons grown for five days on various nitrogen sources were compared with nitrite reductase levels in preparations of cotyledons grown for the same time in nitrogen-deficient media. Ingle concluded from these results that nitrite reductase was inducible by nitrate. However, it seems doubtful that after five days, cotyledons

would have sufficient nitrogen reserves for the synthesis of new enzymes (16).

Losada et al. (19) working with *Chlorella*, concluded that ammonia is the nutritional repressor of the entire nitrate reducing system, and that nitrate was not the inducer. Two different effects on the system were observed when nitrate grown cells were transferred to an ammonia medium. Nitrate reductase, but not nitrite reductase, was quickly inactivated. The enzyme could be reactivated, *in vitro*, by removal of ammonia and reincubation in nitrate medium. The other effect was repression of nitrite reductase and nitrate reductase by ammonia. Re-expression of activity, upon removal of ammonia, was dependent on protein synthesis.

Pateman et al. found nitrate reductase mutants at six genetic loci (29,30). The mutants in these loci were constitutive for nitrite reductase, hydroxylamine reductase and all the nitrate-induced NADPH diaphorase activities. Their hypothesis, to explain these findings, was that constitutivity arises because the functional nitrate reductase is a component of the regulatory system of the pathway; a complex between nitrate reductase and nitrate would act as the inducer of the enzymes of the pathway.

It is apparent that studies on the regulation of nitrite reductase from different organisms has resulted in

a very complex and confusing picture. However, from the results that have been obtained, it would seem that ammonia may be a key molecule in the regulation of nitrite reductase.

Genetic Studies of Nitrite Reductase

Silver and McElroy (34) concluded that five genes control nitrite reduction in *N. crassa*, because five of their mutant strains showed positive complementation with each other. Intracistronic complementation was unknown at this time and the interpretation of these results is now open to question.

Pateman et al. (29) isolated mutants of *Aspergillus* at two genetic loci: nii A and nii B. The mutants could not grow on nitrite and extracts lacked nitrite reductase activity, measured as nitrite dependent NADPH oxidation. Later, it was concluded that nii B was a regulatory locus (30). These results suggested that only one structural protein made up nitrite reductase.

Dyer (10) obtained mutants of *N. crassa* which could not grow on nitrate or nitrite and which accumulated nitrite when grown on an ammonium nitrate medium. On the basis of studies with these mutants, it was suggested that three, and possibly four genes control nitrite reduction.

Multiplicity of Enzymes Involved in Nitrite Reduction

The presence of more than one nitrite reductase activity could explain the fact that nitrite non-utilizing mutants have *in vitro* nitrite reductase activity (3,10,22,34). There is some evidence that certain organisms may have more than one nitrite reductase. Czygan (7) has found two species of nitrite reductase activity in extracts of *Ankistrodesmus braunii*. One of the species was associated with large particles. The other species, an assimilatory nitrite reductase that requires high energy phosphates for activity, was associated with smaller particles.

Leinweber et al. (18) and Siegel et al. (35) investigated hydroxylamine reductase and sulfite reductase activities from *N. crassa*. Using gel filtration and sucrose density gradient centrifugation techniques, they were able to separate three peaks of hydroxylamine reductase activity. Peak A had sulfite reductase activity also, and was missing from extracts of a sulfite reductase-less mutant. Peaks B and C had both NADPH and NADH hydroxylamine reductase activity, but peak B was absent from extracts of mycelia grown on an ammonia medium. Little attention was paid to the peak C activity. Thus, there are two peaks which are involved in hydroxylamine reduction, and possibly in nitrite reduction.

Hewitt, Hucklesby and Betts (14,15), using molecular

exclusion chromatography, separated nitrite reductase activity from hydroxylamine reductase activity in extracts of *C. pepo* and of *Spinacea oleracea*. Extracts from either species contained only one nitrite reductase activity, which had a molecular weight of 60,000. Two hydroxylamine reductase activities were found in cell-free preparations of each species. Hydroxylamine I had a molecular weight corresponding to the nitrite reductase activity, while hydroxylamine II had a molecular weight of 32,000. There was, furthermore, an indication of 'sub-peaks' suggesting aggregations of an oligomeric system.

Summary

In conclusion, from the work that has been done, we know that nitrite reductase, in green tissue, is localized in chloroplasts and utilizes ferredoxin as electron donor. The location of nitrite reductase in non-photosynthetic tissues must be examined more critically. Natural electron donors in non-photosynthetic tissues are unknown.

Many other questions concerning nitrite reductase are in need of answers. Among these are:

1. Is nitrite reductase an inducible enzyme or is it derepressible, as suggested by Cook; and does ammonia act as repressor?
2. Is ammonia the only product of nitrite reduction or does

nitrite reduction proceed via an organic pathway in *Neurospora crassa*?

3. Is there a multiplicity of nitrite reductases?
4. From Dyer's work, it is known that there are three and possibly four genes controlling nitrite reductase. Are all of these genes responsible for structural proteins, or do any serve a regulatory function?

Purpose

The present investigation was undertaken in an attempt to answer the following questions:

1. Is nitrite reductase localized in an organelle, such as the mitochondrion?
2. Is there a multiplicity of nitrite reductases? An answer to the latter question was attempted by considering:
   (a) Is ammonia the product of nitrite reduction?
   (b) What role does ammonia play in the regulation of nitrite reductase activity?
   (c) If more than one nitrite reductase exists, can mutants be found which lack one or more of the activities? and,
   (d) If more than one nitrite reductase exists, can they be separated physically?

MATERIALS AND METHODS

Fungal Strains

Neurospora crassa strain, *pan-2-B-36A*, requiring pantothenic acid for growth was used as wild type. Mutants O, Z, t-13 and t-23 were isolated and characterized by Miss C. Dyer (10). *Cys-4* was obtained from the Fungal Genetics Stock Centre at Dartmouth, New Hampshire. All of these strains were in St. Lawrence type background.

Media

The basic medium used was that described by Sorger and Giles (37). Mycelia were pre-grown on a basic medium to which a casamino acids digest (Difco) was added to give a concentration of 5 gm/l. For repressed conditions, ammonium tartrate was added to give a final concentration of 4 gm/l. Potassium nitrate was added at a final concentration of 5 gm/l to give derepressed or induced* conditions. For solid media, 1.5 % agar (Difco) was used. Calcium pantothenate was added to all media at a concentration of 20 mg/l. Filter sterilized DL-methionine was added to give a final

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* Since the mechanism of regulation of nitrite reductase is unclear, both terms are used to describe the appearance of enzyme activity upon removal of repressor.

concentration of 2.4×10^{-4}M and filter sterilized DL-cysteine was added to give a concentration of 10^{-3}M. Sterilization was with steam at 15 p.s.i. and 121°C for twenty minutes. All media will subsequently be 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°C and a shaker setting of two. Cultures on solid media were incubated in a standing incubator (Model 805, Precision Scientific Co., Chicago, Illinois) at 27°C.

Growth of Mycelia

N. crassa was grown, with shaking, from a conidial inoculum, into stationary phase (28-30 hours) in liquid casamino acids medium. After this time, mycelial pads were harvested by filtration and washed with distilled water. The average yield of mycelia from 400 ml of media was five grams, wet weight. 0.3 gm sectors of the mycelial pads were usually added to 400 ml of media containing the appropriate nitrogen source. When mutants unable to grow on a given nitrogen source were used or mycelia were transferred to a nitrogen-deficient medium, 3.0 gm pads

were added. The flasks were then incubated, with shaking, at 27°C for 15-17 hours. Mycelia obtained from such growth were used for enzyme extraction.

Extraction of Enzyme

Mycelial pads, which had been harvested by filtration, were washed with distilled water and blotted between paper towels until no more moisture could be squeezed out by application of manual pressure. Three methods of enzyme extraction were utilized:

A. S5000: This extraction procedure was as described by Cook (3). The blotted mycelia were immersed in liquid nitrogen for ten minutes, and subsequently ground in an ice cold mortar with an equal volume of silica (240 mesh, Fisher Scientific Co.) as abrasive. The resulting paste was suspended in three volumes of cold potassium phosphate buffer (0.1 M, pH 7.0) and the slurry centrifuged at 3020 g for twenty minutes in a refrigerated centrifuge (Sorvall, Model RC-2B, Norwalk, Connecticut).

B. S16000: This extraction procedure was a modification of one described by Munkres (25). The mycelial mat was ground in an ice cold mortar with three volumes of cold 0.1 M phosphate buffer, pH 7.0, containing 0.44 M sucrose and 10^{-3}M EDTA. The homogenate was first centrifuged at 2000 g for ten minutes in a refrigerated centrifuge

to remove whole cells, nuclei, and large cell fragments. The supernatent was further subjected to centrifugation at 16,000 g for twenty minutes, twice. The resulting supernatant, which is essentially mitochondria-free, was used as enzyme source.

C. S500: The procedure used was that described by Luck (20,21). Mycelial pads were ground with five volumes of phosphate buffer, 0.1 M, pH 7.0, containing 0.44 M sucrose and 10^{-3} M EDTA. The resulting homogenate was centrifuged at 500 g for ten minutes in a refrigerated centrifuge, to remove nuclei and cell debris, but leaving intact mitochondria in the supernatant.

The resulting supernatants from each extraction procedure were kept on ice and used as enzyme source within eight hours of their preparation. The three enzyme sources will subsequently be referred to as S5000, S16000 and S500.

Enzyme Assays

1. Nitrite Reductase. The procedure was that of Cook (3,4). The reaction mixture contained, in a final volume of 3 ml: (μmoles); potassium phosphate, pH 7.5, 120; benzyl viologen (Mann Research Labs.) 0.5; sodium nitrite, 3.3. 0.2 to 0.4 ml of extract were added, and the reaction initiated by adding 7.5 μmoles of

sodium dithionite in 0.6 ml of distilled water. The tubes were gently shaken and incubated in a water bath at 30°C for ten minutes. After this time the reaction was terminated by vigorously shaking the tubes on a mechanical mixer (Scientific Products, Evanston, Illinois) for at least five seconds after the violet colour of the reduced benzyl viologen had disappeared. 0.1 ml samples of the reaction mixture were then added to 7.9 ml of distilled water and 1 ml each of sulfanilamide and of N-1-naphthylethylenediamine dihydrochloride reagents were added. After ten minutes, the intensity of the colour was estimated using a Klett-Summerson colourimeter (Klett Manufacturing Co. Inc., New York), equipped with a number 54 filter. Reaction mixtures lacking enzyme and ones lacking nitrite were included as controls, the former being used routinely as the blank. One unit of nitrite reductase activity is defined as the reduction of 1 mμmole of nitrite per minute at 30°C. The specific activity was usually expressed as units per mg protein, or in some cases, as units per mg protein squared. The experimental error in measurement of nitrite reductase was found to be 4.8 units of activity.

2. Ammonia Production. The microdiffusion method of Conway was used (2). 2.0 ml of a test solution, from a

nitrite reductase assay, carried out as described above, was added to the outer well of a plastic Conway dish. The inner well contained 1.5 ml of 2% boric acid. Saturated K_2CO_3 (1.5 ml) was added to the outer well and the dishes were sealed. Volatile ammonia, released from the test solution by addition of K_2CO_3, was allowed to diffuse into the boric acid. After at least five hours, 1.0 ml of the boric acid was removed and reacted with 2.0 ml of commercially prepared Nessler's reagent (Paragon Co., Bronx, New York) and the optical density at 440 mμ was read on a Beckman DBG spectrophotometer. The blank was a reaction mixture lacking added nitrite. The optical densities were compared with a standard curve (Fig. 1) and total ammonia produced in the reaction mixture as a result of nitrite reduction, was estimated as:

$$(\text{m}\mu\text{moles } NH_3)_{\text{test}} - (\text{m}\mu\text{moles } NH_3)_{\text{blank}} \times 9/4^*$$

One unit of ammonia produced is expressed as the appearance of one mμmole of ammonia per minute. The experimental error in measurement of ammonia production was calculated to be 4.0 units.

3. Catalase. The enzyme was assayed as described by Beers

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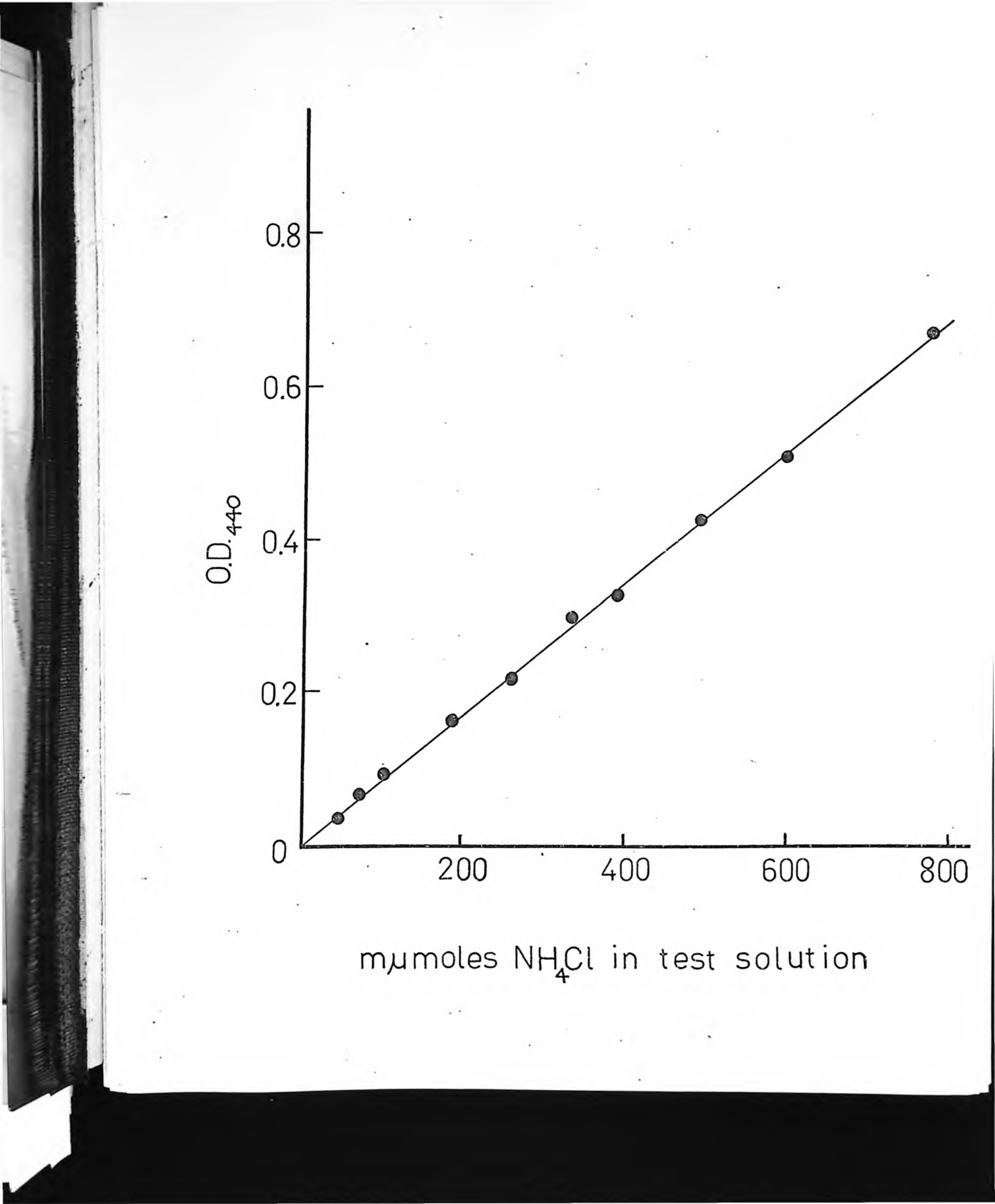
* A correction factor is necessary since only 2/3 of the test mixture is reacted and 2/3 of the boric acid volume is analyzed.

Fig. 1

Reference curve for ammonia as estimated by the Conway microdiffusion Method.

Ammonium chloride solutions in 2% boric acid were made. One ml aliquots of ammonium chloride solutions of known concentration were reacted with Nessler's reagent (see METHODS) and the optical density at 440 mμ estimated. All values were corrected for no NH_4Cl added to test solution.


0.8
0.6
0.4
0.2
0
O.D.440
200
400
600
800
mμmoles NH4Cl in test solution

and Sizer (1). The disappearance of hydrogen peroxide was followed spectrophotometrically at 240 mμ on a Beckman DBG spectrophotometer. One unit of catalase activity is expressed as a change of 0.1 OD units per minute.

Nitrite Estimation

A modification of the method described by Sanderson and Cocking was used (33). 1.0 ml of 1% (w/v) sulfanilamide in 1 N HCl and 1.0 ml of 0.01% (w/v) of N-1 naphthylethylene-diamine dihydrochloride in double distilled water, were added to a test solution of nitrite in 8.0 ml of distilled water. The resulting magenta colour was estimated after ten minutes, using a Klett-Summerson colourimeter equipped with a number 54 filter, and the intensity of the colour related to a standard curve (Fig. 2). There is a linear relationship between Klett units and nitrite concentration up to 300 mμmoles of nitrite in the 10 ml test mixture. In the linear range indicated, one Klett unit is equivalent to 0.4 mμmoles of nitrite. Samples to be estimated for nitrite content were diluted, where necessary, in order to contain less than 300 mμmoles of nitrite.

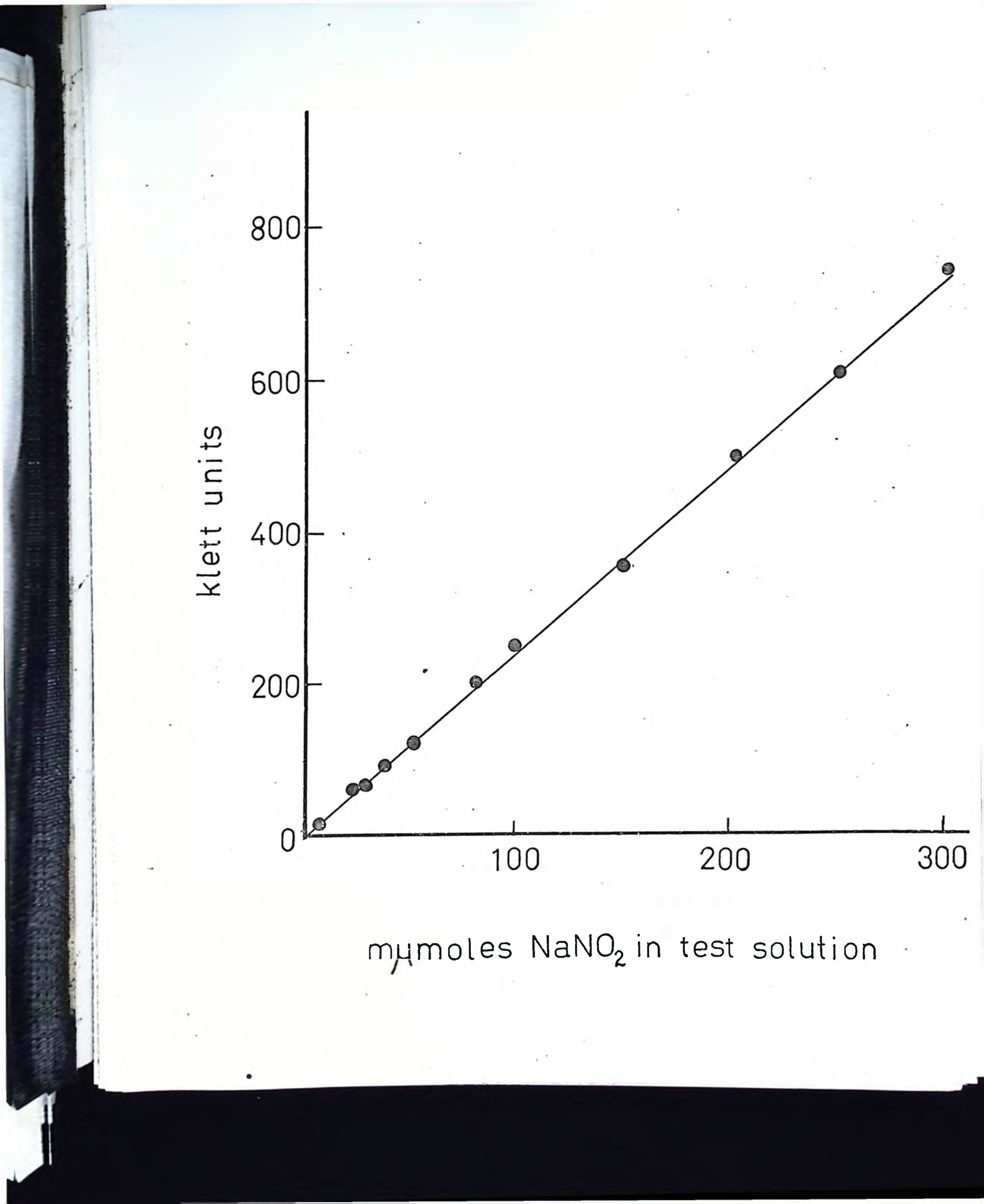
Protein Estimation

This was by the Biuret test as described by Dawson

Fig. 2

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 added to 7.9 ml of glass distilled water. Nitrite was estimated as described in METHODS, to give a test solution volume of 10 ml.


800
600
400
200
0
100
200
300
Klett units
mμmoles NaNO2 in test solution

et al. (8), using bovine serum albumin as standard. In the case of equilibrium sucrose density gradients, protein was monitored by measuring the optical density at 280 mμ. Bovine serum albumin was used as standard.

Dialysis and Desalting

Dialysis was usually carried out for two hours, against three changes of phosphate buffer, 0.1 M, pH 7.0. 3 ml of extract was placed in the sac; the volume surrounding the sac was one litre.

Desalting was carried out on columns of Sephadex G-10 (Pharmacia, Upsalla, Sweden), 20 cm x 1 cm, equilibrated with 0.1 M phosphate, pH 7.0, 0.44 M in sucrose and 10^{-3}M in EDTA. Elution was with the same solution.

Differential Centrifugation

The differential centrifugation was carried out by a modification of the procedure used by Munkres (25) for isolating mitochondria. Mycelia were ground in three volumes of 0.1 M phosphate, pH 7.0 containing 0.44 M sucrose and 10^{-3}M EDTA. The preparation was centrifuged at 2000 g for ten minutes. The supernatant, designated as S2000, was assayed for nitrite reductase activity, and then further subjected to centrifugation at 16000 g for twenty minutes two times. The two pellets, designated as P16000, were

washed with the extraction solution, resuspended in the same solution and combined. Nitrite reductase activity was measured in S16000 and P16000.

Equilibrium Sucrose Density Gradients

The method of Luck was used (20). Linear 4 ml gradients, ranging in density from 1.24 to 1.08 were prepared in cellulose nitrate tubes (Spinco Division of Beckman Instruments, Inc., Palo Alto, California). 0.3 ml of a 3 M sucrose solution was pipetted into the bottom of each tube to provide a cushion for pelleting cellular debris. The gradients were prepared on top of this cushion from solutions of 1.9 M and 0.58 M sucrose. All sucrose solutions were prepared in phosphate buffer, 0.1 M, pH 7.0 containing 10^{-3} M EDTA. Gradients were made 12-18 hours before centrifugation and stored at 4°C.

One ml of S500 extract was layered on top of the preformed gradient and centrifuged at 39,000 RPM (average g value was 121,000 g) for five hours at 4°C, in an SW 50 rotor in a Spinco Model L ultracentrifuge. Equilibrium is reported to be reached within three hours (20). After centrifugation, the bottom of each tube was perforated and approximately 40 fractions of 10 drops each were collected. Each fraction was assayed for nitrite reductase, as previously described, and the OD_{280} was also determined.

Sedimentation Velocity Gradients

The procedure adopted was a modification of that used by Leinweber et al. (18). Linear sucrose gradients of 4.5 ml each were prepared in cellulose nitrate tubes from 20% and 33% (w/v) sucrose solutions in 0.1 M potassium phosphate, pH 7.0, containing 10^{-3}M EDTA. Gradients were kept at 4°C for 12-18 hours before use.

0.1 ml of catalase (Worthington) was added to 2 ml of S16000 extract. 0.5 ml of this mixture was then layered on top of each gradient. Centrifugation at 4°C, was performed at 39,000 RPM (average g value was 121,000 g), for 18 hours, in a Spinco Model L ultracentrifuge, equipped with an SW 50 rotor. Five drop fractions were collected and each fraction assayed for nitrite reductase activity. Catalase activity was also measured. The results are presented in terms of movement of activity (R) through the gradient relative to catalase, which is defined by the equation:

$$R = \frac{(T - x)}{(T - c)}$$

where T ≡ total number of fractions, x ≡ fraction number of peak activity and c ≡ fraction number of maximum catalase activity, counting from the bottom to the top of the centrifuge tube.

Column Chromatography

Sephadex G-100 columns (28 cm x 2 cm) were prepared from Sephadex G-100 beads (Pharmacia) which had been allowed to swell for three days in 0.1 M phosphate buffer, pH 7.0. The void volume of this column was determined to be 34 ml and the flow rate was 17 ml/hour. All operations were carried out in the cold. Elution was with 0.1 M phosphate buffer, pH 7.0. 1.5 ml fractions were collected, and each fraction assayed for nitrite reductase activity. In other experiments, protein coming off immediately following the void volume (referred to as post void volume protein), was collected in bulk and assayed under various conditions, as indicated in RESULTS.

Growth Studies

50 ml of liquid medium containing the appropriate nitrogen source and a conidial inoculum, in 250 ml flasks, were incubated at 27°C on a rotary shaker (New Brunswick Scientific), with an agitation setting of 2. At appropriate times, the mycelia were collected by filtration, dried in a 100°C oven and weighed on a microbalance.

Induction Studies

One gram sectors of mycelial pads, pre-grown on casamino acids were transferred to 250 ml flasks containing

50 ml of media supplemented with the appropriate nitrogen source. At given time intervals, the mycelia were removed, filtered by suction and the enzyme prepared as given for the S500 extraction procedure. The extracts were then assayed for nitrite reductase activity.

Mutagenesis and Selection of Nitrite Non-Utilizing Mutants

Suspensions containing 9×10^7 conidia/ml were incubated, without shaking, in the dark and at room temperature for 100 minutes, in the presence of 20 µg/ml, final concentration, of N-methyl-N'-Nitro-N'-nitrosoguanidine. This treatment resulted in 15% survival. The conidial suspension was centrifuged at 3000 g for thirty minutes and the conidia resuspended in neutralized nitrite medium (0.5 g/l). The treated culture was subsequently incubated on a rotary shaker and subjected to filtration enrichment (43). Wild type spores can germinate in minimal nitrite medium while nitrite non-utilizing spores cannot. When tufts of mycelia were visible, the contents of the incubation flask were filtered through cheesecloth and the filtrate reincubated. When most of the conidia capable of growth on nitrite had been removed by repeated filtration, the remaining conidia in the final filtrate were plated on solid ammonia medium. Colonies from these plates were isolated into tubes of liquid ammonia media, and after

conidiation were tested for the ability to grow on liquid nitrate and nitrite medium. Strains which grew normally on ammonia, but poorly on nitrite, were kept as presumptive mutants.

Materials

Sources were as follows: benzyl viologen was from Mann Research Labs., New York, New York; bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Missouri; catalase was a product of Worthington Enzymes, Freehold, New Jersey; Lubrol wx was from C.I.L. Chemicals, Montreal, P.Q.; Nessler's reagent was obtained from Paragon Co., Bronx, New York; nitrosoguanidine was from Aldrich Chemical Industries, Milwaukee, Wisconsin; sulfanilamide and N-1 naphthylethylenediamine dihydrochloride were products of Eastman Organic Chemicals, Rochester, New York.

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

RESULTS

Section I. Is nitrite reductase localized in mitochondria?

In order to determine whether nitrite reductase is localized in an organelle, such as the mitochondrion, equilibrium sucrose density gradient centrifugation experiments were carried out. S500 extracts from nitrate grown mycelia were layered onto linear gradients as described in the METHODS. Centrifugation was allowed to proceed until equilibrium was reached. Nitrite reductase activity was found only from fractions thirty to forty, indicating little or no movement of enzyme into the preformed gradient (Fig. 3). No activity was found in the region of the gradient where mitochondria are reported to be located (20).

This result does not rule out the possibility that nitrite reductase may be associated with mitochondria *in vivo* but becomes dissociated from the mitochondria during centrifugation. In order to test this possibility, a differential centrifugation was carried out, in which the mitochondria are separated from the rest of the crude extract. The protocol for this differential centrifugation was as given in the METHODS. Nitrite reductase activity and ammonia production were measured for fractions S2000, S16000 and P16000. Table I indicates that no nitrite reductase activity

Fig. 3.

Profile of nitrite reductase activity from wild type extracts of nitrate grown mycelia centrifuged through a sucrose density gradient until equilibrium was reached.

288 units of nitrite reductase were put on the gradient and 214 units were recovered. Percent recovery was 76%. Units of nitrite reductase activity are represented by ●——● and mg protein per fraction by ○——○ .

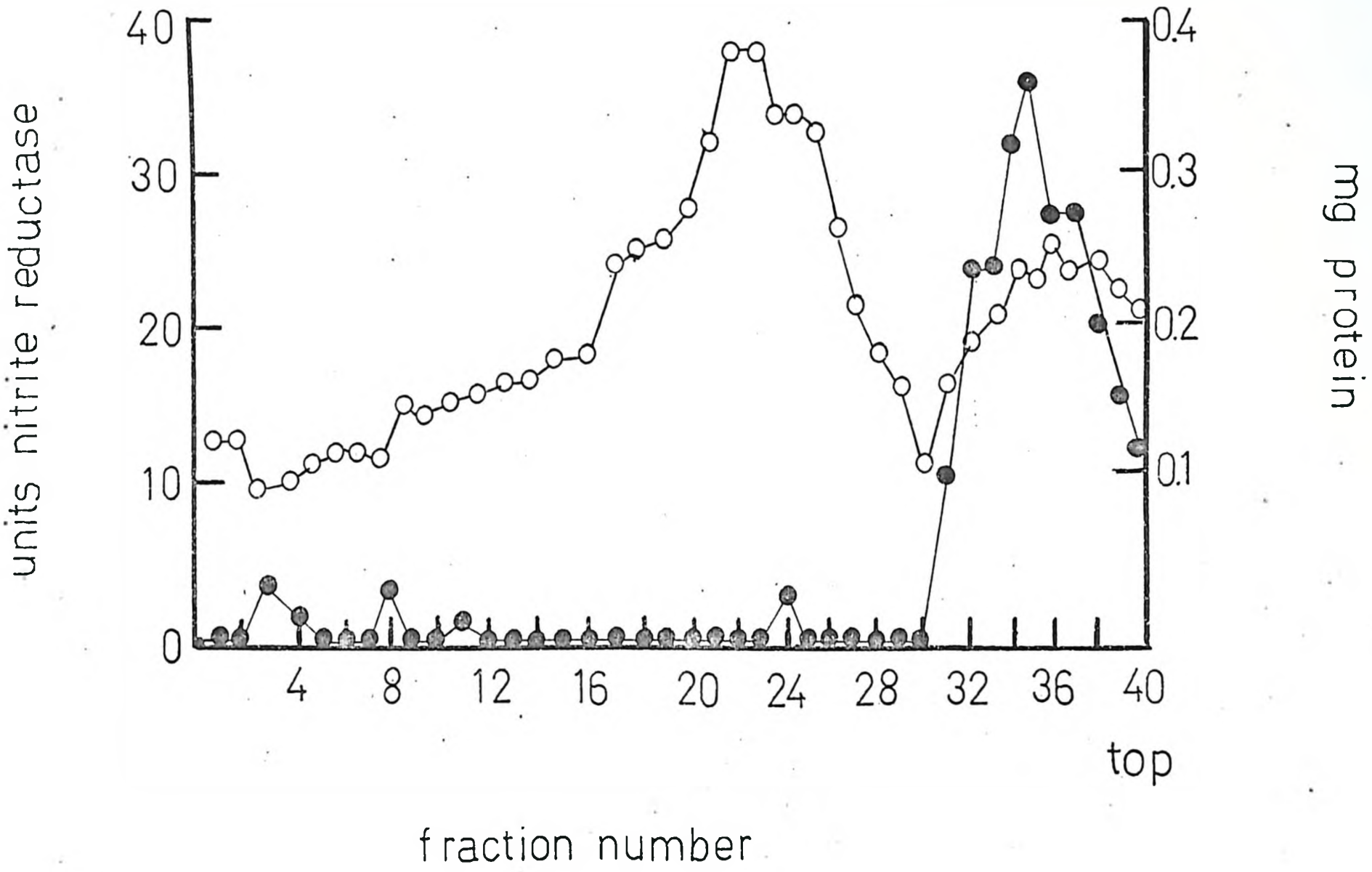

units nitrite reductase
mg protein
40
30
20
10
0
0.4
0.3
0.2
0.1
4
8
12
16
20
24
28
32
36
40
top
fraction number

Table 1

Nitrite Reductase Activity and Ammonia Production of Fractions From a Differential Centrifugation

The extract was made from mycelia grown on nitrate. The protocol for the differential centrifugation is as described in METHODS.

Fraction	Volume (ml)	Total mg Protein	Nitrite Reductase Activity (Total Units)	Ammonia Produced (Total Units)	$\frac{NO_2^-}{NH_4^+}$	% Recovery (Nitrite Reductase)
S2000	50	355	102,000	50,000	2.04	100
S16000	45	268	99,500	85,000	1.17	98
P16000	5	72	0	0	-	0

is associated with P16000, the fraction supposed to contain mitochondria. 98% of the nitrite reductase activity present in fraction S2000 was recovered in fraction S16000. It was noted that the nitrite reduced to ammonia produced ratio approached one in fraction S16000, whereas this ratio was considerably higher in fraction S2000. These differences are probably due to the concentration of protein that was assayed (see later RESULTS).

To test the possibility that a factor may be present in P16000 which could affect S16000 activity, a mixing experiment was carried out. P16000 and S16000 extracts were obtained as for differential centrifugation (see METHODS), from mycelia grown on nitrate. The concentration of S16000 protein was kept constant while that of P16000 protein was varied. The addition of P16000 to S16000 did not affect the nitrite reductase activity from S16000 (Table 2).

Since the foregoing experiment dealt with intact mitochondria, it was possible that a factor or a nitrite reductase activity might be found if the mitochondria were ruptured. Lubrol WX, a non-ionic detergent, was added to fraction P16000. Various concentrations of Lubrol-treated P16000 protein were added to S16000 protein and nitrite reductase activity was measured. There was very little, if any, nitrite reductase activity in the Lubrol treated

Table 2

Effect of Adding Different Concentrations of P16000 Protein to S16000 Protein on Nitrite Reductase Activity

The extract was made from mycelia grown on nitrate. P16000 and S16000 were prepared by the differential centrifugation described in METHODS.

mg P16000 Protein in Assay	mg S16000 Protein in Assay	Units of Nitrite Reductase*
0	0.6	32.4
0.075	0.6	32.4
0.15	0.6	32.4
0.22	0.6	34.8
0.3	0.6	31.2
0.3	0	2.4

* The experimental error in measurement of nitrite reductase is 4.8 units of activity.

P16000 fraction (Table 3). However, addition of Lubrol-treated P16000 to untreated S16000 resulted in an enhancement of nitrite reductase activity.

The results from these experiments suggest then, that nitrite reductase, measured by the method of Cook, is not localized in the mitochondria. The results do not discount the possibility that nitrite reductase may be associated with mitochondria *in vivo* and that the extraction procedure results in a dissociation. The results indicate that there is a factor(s) present in the mitochondria, which is released on being ruptured, and stimulates nitrite reductase activity.

Section II. Is there a multiplicity of nitrite reductases?

(a) Is ammonia the product of nitrite reduction?

Cook was unable to show that ammonia was the product of nitrite reduction in his assay (3). However, benzyl viologen was not included in the reaction mixtures used to determine ammonia production (3). It seemed possible then, that ammonia production was dependent on the presence of benzyl viologen, in reaction mixtures. Table 4 shows that this is indeed the case. The ratio of nitrite reduced to ammonia produced (subsequently referred to as N/A) in the presence of benzyl viologen was higher with S5000 extracts than with S16000 extracts, indicating that the S5000

Table 3

Effect of Adding Lubrol wx Treated P16000 Protein to S16000 Protein, on Nitrite Reductase Activity

P16000 or S16000 was mixed with Lubrol (final concentration, 1 mg/mg protein) and reacted for 30 minutes. Fractions were obtained as for differential centrifugation (see METHODS) from mycelia grown on nitrate.

mg P16000 Lubrol Treated Protein In Assay	mg S16000 Untreated Protein In Assay	mg S16000 Lubrol Treated Protein In Assay	Units* Nitrite Reductase
0	0.61	-	28.8
0.87	0.61	-	38.4
1.74	0.61	-	40.8
0	1.0	-	46.8
0.87	1.0	-	67.2
1.74	1.0	-	66.0
0.87	0	-	2.4
1.74	0	-	4.8
0	0.5	-	21.6
0	-	0.5	19.2

* The limit of accuracy in measuring nitrite reductase is 4.8 units.

Table 4

The Effect of the Presence of Benzyl Viologen (BV.) in Reaction Mixtures, on Ammonia Production

Nitrate grown mycelia were extracted by two methods; S5000 and S16000. Nitrite reductase activity and ammonia production were determined on the same samples.

BV.	Extract	mg Protein In Assay	Units Nitrite Reductase	Units Ammonia Produced	$\frac{NO_2^-}{NH_4^+}$
+	S5000	3.4	55.2	19.5	2.8
-	S5000	3.4	32.4	0	-
+	S16000	1.0	52.8	31.5	1.7
-	S16000	1.0	21.6	7.5	2.9

extraction procedure used by Cook is not optimum for ammonia production.

If ammonia is the product of nitrite reduction *in vivo* then nitrite should be reduced stoichiometrically to ammonia. The results shown in Table 4 indicate that nitrite loss does not equal ammonia production in reaction mixtures containing crude extracts.

Fig. 4 shows the effect of S16000 protein concentration on nitrite reductase activity and ammonia production. Nitrite reduction and ammonia production are not stoichiometric. A dilution effect is noted for both nitrite reductase activity and ammonia production.

When units of activity are plotted as a function of mg extract protein squared (Fig. 4A), a linear relationship is found. At concentrations of extract protein greater than 0.65 mg^2 nitrite reductase activity continues to increase, although at a decreased rate, while the level of ammonia produced remains essentially constant. This result could be interpreted in three ways; first, that there are at least two nitrite reductase activities. One yields ammonia as product, and another, which exhibits a dilution effect and whose activity is expressed at protein concentrations greater than 0.8 mg does not yield ammonia as product of the reduction. The second interpretation would be that at protein concentrations greater than 0.8 mg a

Fig. 4

Effect of S16000 protein concentration on nitrite reductase activity and ammonia production.

S16000 extracts were made from nitrate grown mycelia. Nitrite reductase activity is represented by ●——● and ammonia production by ○——○ . Ammonia production and nitrite reductase activity were determined on the same samples.

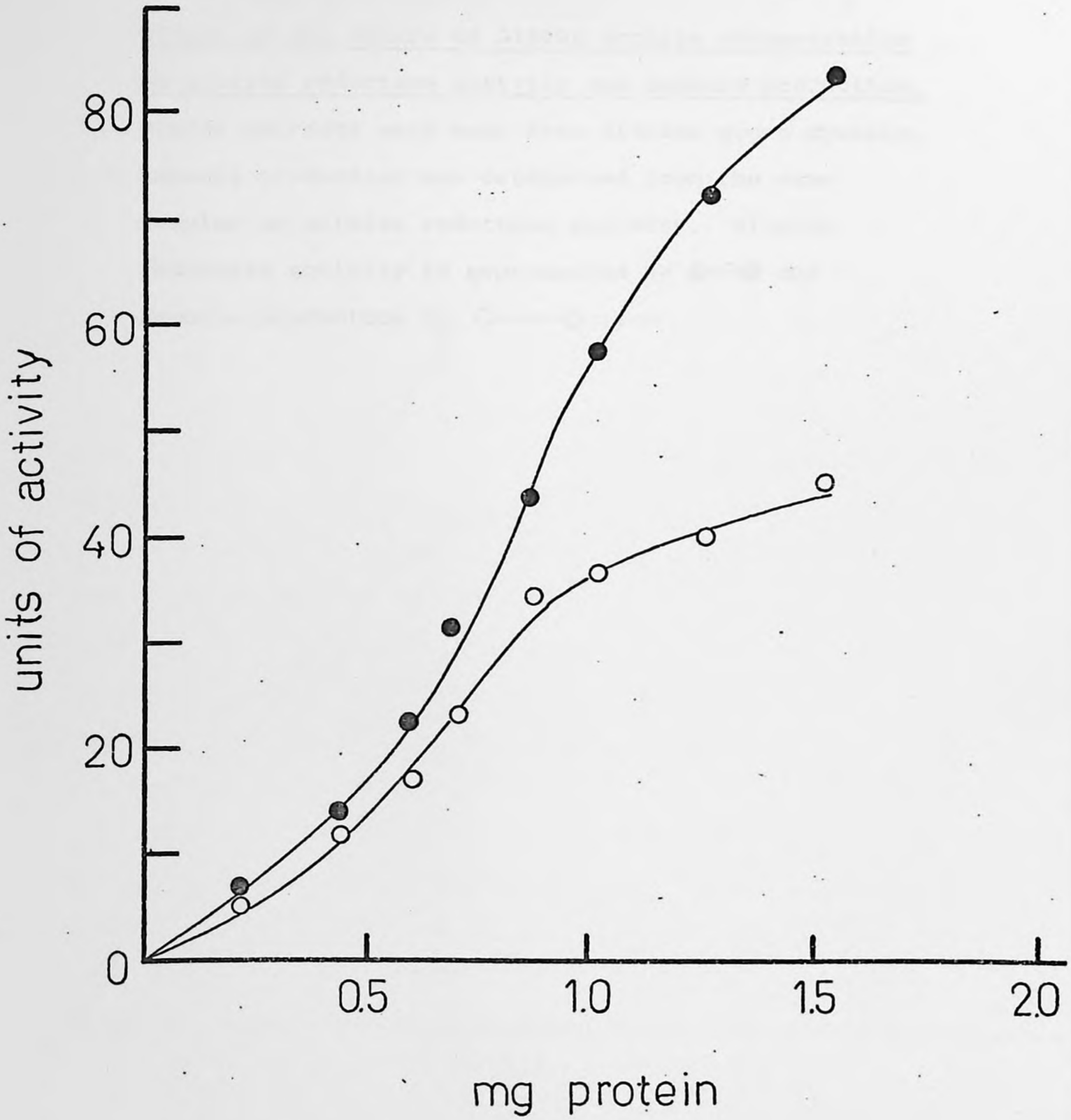
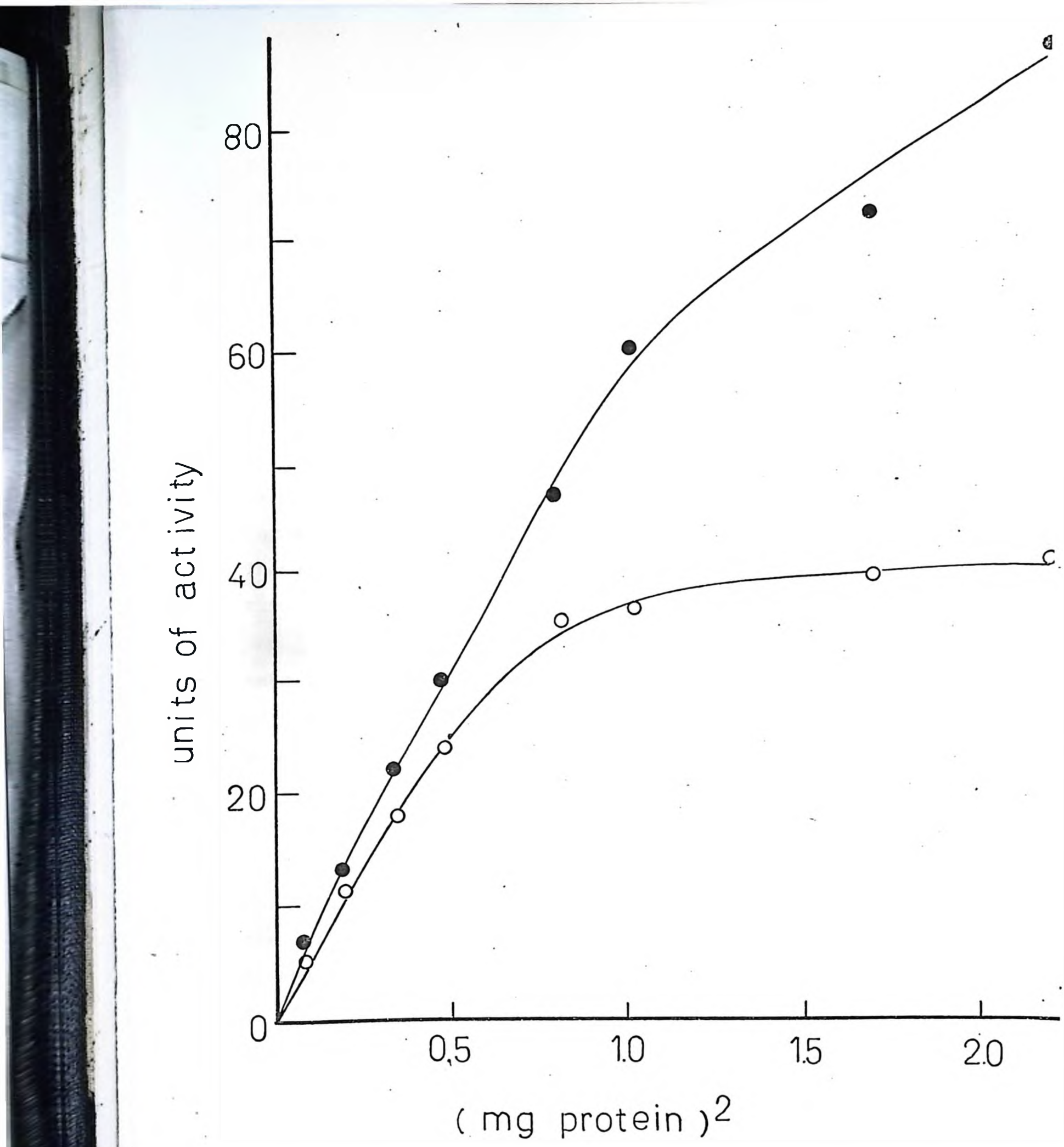

units of activity
0
20
40
60
80
0.5
1.0
1.5
2.0
mg protein

Fig. 4A.

Effect of the square of S16000 protein concentration on nitrite reductase activity and ammonia production. S16000 extracts were made from nitrate grown mycelia. Ammonia production was determined from the same samples as nitrite reductase activity. Nitrite reductase activity is represented by ●——● and ammonia production by ○——○ .


80
60
40
20
0
units of activity
0,5
1.0
1.5
2.0
(mg protein)2

portion of the ammonia produced from nitrite is incorporated into a molecule such as gultamate *in vitro*. A third possibility is that the effect of protein concentration on nitrite reductase activity and ammonia production is a result of protein aggregation. At concentrations of protein less than 0.8 mg, nitrite reductase would exist mainly in a form of aggregation, a_x. Ammonia would be the product of the reaction catalyzed by a_x. At protein concentrations greater than 0.8 mg, a second form a_y, as well as a_x, would exist. This a_y form of protein would not catalyze the formation of ammonia as a result of nitrite reduction. These three possibilities will be considered elsewhere in RESULTS, as well as in the DISCUSSION.

In contrast to the results obtained with S16000 extracts, a very different picture, similar to that obtained by Cook (3,4) is found with S5000 extracts. Ammonia production appeared to follow the same course as nitrite reduction; however, the N/A ratio was much higher than that obtained with S16000 extracts (Figure 5).

Two types of experiments were carried out to test for *in vitro* disappearance of ammonia during incubation of the reaction mixture. In the first experiment, S5000 and S16000 extracts were dialyzed or desalted, before assaying. In this way, small molecules which would be necessary for the disappearance of ammonia, would be removed.

Fig. 5.

Effect of S5000 protein concentration on nitrite reductase activity and ammonia production.

S5000 extracts were made from nitrate grown mycelia. Units of nitrite reductase are represented by ●—● and ammonia production by O—O . Ammonia production was determined on the same samples as nitrite reductase activity.

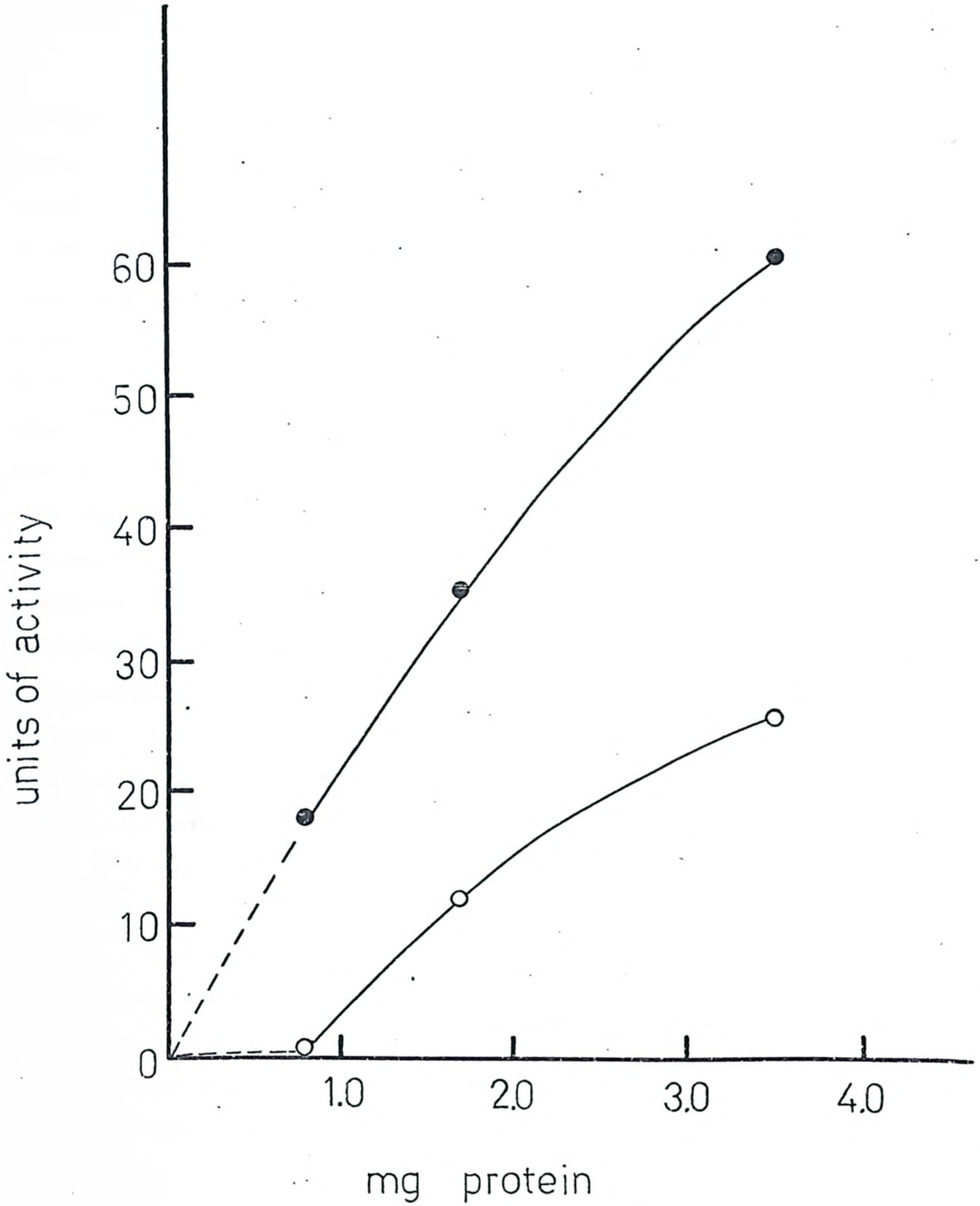

units of activity
60
50
40
30
20
10
0
1.0
2.0
3.0
4.0
mg protein

Table 5 shows the results of such an experiment. Dialysis or desalting of S16000 extracts did not appreciably affect the N/A ratio, indicating that non-stoichiometric nitrite reduction and ammonia production is probably not a result of *in vitro* ammonia incorporation into a molecule such as glutamate. Dialysis of S5000 extracts results in a great loss of nitrite reductase activity. The N/A ratio is not appreciably affected. Desalting of S5000 extracts resulted in an approach to stoichiometric nitrite reduction and ammonia production. This result suggests the possibility that the S5000 extraction procedure results in a change in the conformation of the enzyme which affects the ammonia producing activity. In the presence of the sucrose-EDTA solution (see METHODS), the conformation of the enzyme would be restored and the enzyme would then be able to produce ammonia. It is also possible that this result is indicative that *in vitro* ammonia disappearance takes place when S5000 protein is assayed.

To test this possibility directly, ammonium chloride was added to nitrite reductase reaction mixtures lacking nitrite. The assays were carried out otherwise as given in the METHODS, and the ammonia remaining in the reaction mixture was measured. It was found that ammonia, added at two different concentrations, did not disappear under the described assay conditions. Thus, the possibility that

Table 5

Effect of Dialysis and Desalting of S5000 and S16000 Extracts on Nitrite Reductase Activity and Ammonia Production

Extracts were made from nitrate grown mycelia. Dialysis and desalting were carried out as given in METHODS. Specific activities are expressed as units per mg protein squared for S16000 and units per mg protein for S5000. Nitrite reductase activity and ammonia production were determined on the same samples.

mg Protein	Extract	Treatment	S.A. Nitrite Reductase	S.A. Ammonia Production	$\frac{NO_2^-}{NH_4^+}$
0.9	S16000	-	60.0	38.0	1.6
1.0	S16000	Desalted	45.6	27.0	1.7
0.96	S16000	Dialyzed	52.0	28.0	1.8
3.0	S5000	-	24.8	10.0	2.5
4.5	S5000	Desalted	16.2	12.4	1.3
2.0	S5000	Dialyzed	8.4	4.0	2.1

discrepancies in values between nitrite reduction and ammonia production are due to *in vitro* ammonia disappearance, during the course of the reaction, is eliminated (Table 6).

A few of the parameters of the nitrite reductase assay, as described by Cook (3), were investigated, to insure that optimum conditions were being used to measure ammonia production.

Figure 6 shows the effect of varying the concentration of benzyl viologen, in assay mixtures, on nitrite reductase activity and on ammonia production. The results indicate that the same conditions are optimum for nitrite reductase activity and ammonia production with both S5000 and S16000 extracts; optimum nitrite reduction and ammonia production occur when 0.5 μmoles of benzyl viologen are present in reaction mixtures.

S16000 extracted protein was much more reliable a catalyst of nitrite reduction and ammonia production than S5000 extracted protein. Hence, all further work concerned with ammonia production was with S16000 extracts.

A series of controls were examined to insure that the production of ammonia was actually enzyme dependent. Table 7 shows that ammonia is produced only when the reaction mixture is complete; although, as was shown previously in Table 4 and Figure 6, a small amount of ammonia was produced as a result of nitrite reduction in the absence of benzyl

Table 6

Recovery of Added Ammonia in Nitrite Reductase Assays

Ammonium chloride was added, at two different concentrations, to nitrite reductase reaction mixtures, which lacked added nitrite. Assays were then carried out as given in METHODS and the ammonia present in reaction mixtures determined as outlined in METHODS. S5000 and S16000 extracts were made from nitrate grown mycelia.

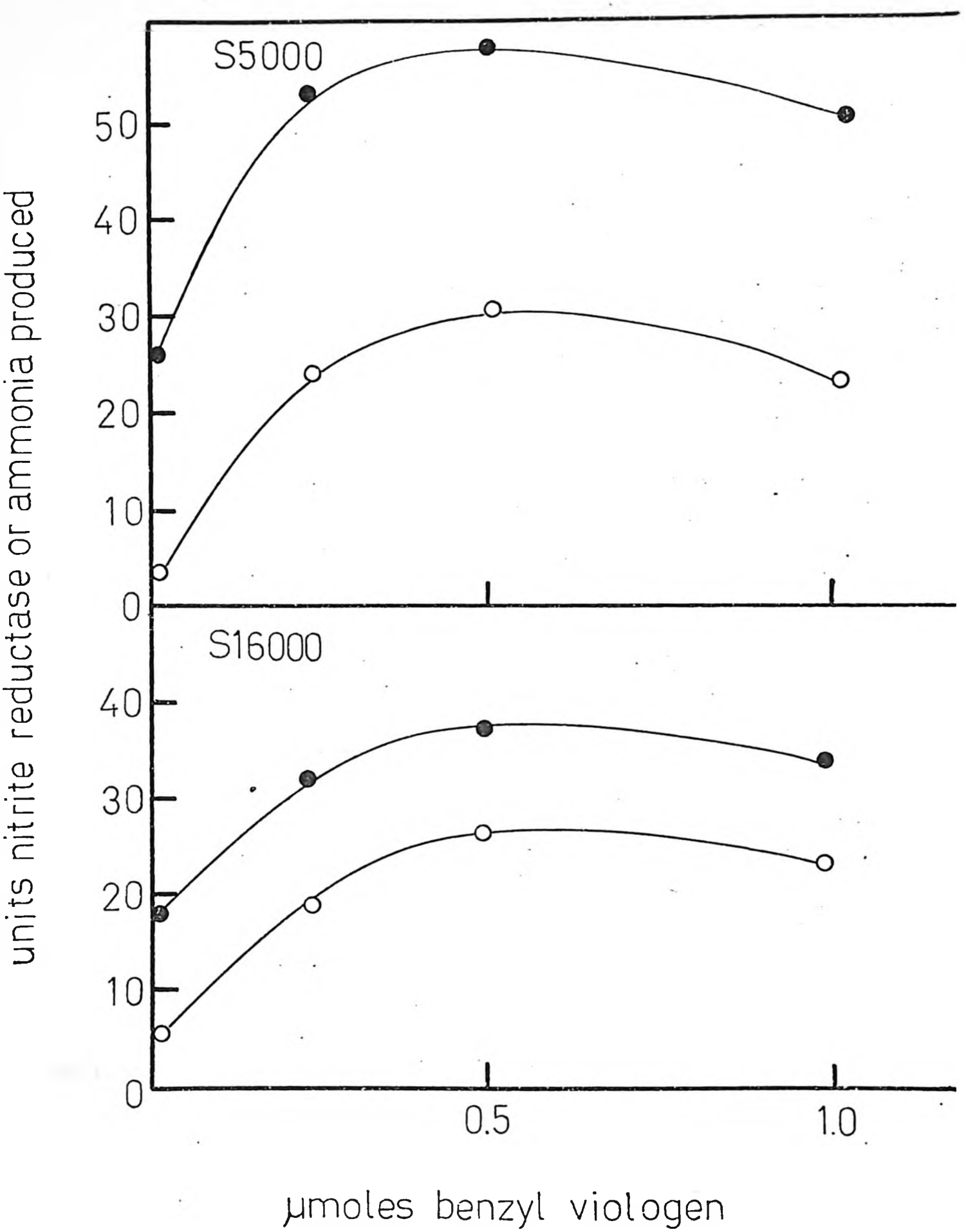
Extract	mg Protein in Assay	mμmoles Ammonia Added	mμmoles Ammonia Recovered*
-	0	835	855
S5000	2.25	835	880
S16000	1.56	835	820
S16000	0.62	835	855
-	0	305	305
S5000	2.25	305	335
S16000	1.56	305	340
S16000	0.62	305	305

* The limit of accuracy in measuring ammonia production is 4.0 units, or 40 mμmoles of ammonia.

Fig. 6.

Effect of benzyl viologen concentration in reaction mixtures on nitrite reductase activity and ammonia production.

Nitrate grown mycelia were extracted as for the S16000 and S5000 procedures. 2 mg of S5000 protein and 0.8 mg of S16000 protein were assayed. Units of nitrite reductase are represented by ●——● and ammonia production by ○——○. Nitrite reductase activity and ammonia production were determined on the same samples.


S5000
S16000
50
40
30
20
10
0
40
30
20
10
0
0.5
1.0
units nitrite reductase or ammonia produced
μmoles benzyl viologen

viologen. Heat denatured protein, added to assays, results in no ammonia production, although a small amount of nitrite is reduced. This loss of nitrite may possibly be the result of adsorption of nitrite to protein.

An experiment was conducted to be certain that ammonia production was linear with time. Ammonia production, as well as nitrite reduction, was found to be linear until ten minutes of incubation. After thirty minutes of incubation, the N/A ratio was the same as after ten minutes incubation (Figure 7).

It is concluded that ammonia is a product of nitrite reduction by nitrite reductase. The parameters of the nitrite reductase assay, as described by Cook (3) were also found to be optimum for ammonia production.

It is possible that S16000 and S5000 extracted protein catalyze the formation of some product other than ammonia. It is also possible that this product is a result of a nitrite reductase activity, different from that which catalyzes the production of ammonia.

A difference was found between the N/A ratios in S16000 extracts and S5000 extracts when each were assayed. The high N/A ratio obtained with S5000 extracts could be substantially lowered by desalting such extracts on Sephadex G-10 columns in the presence of sucrose-EDTA. The high N/A ratio was not due to the incorporation of ammonia into a

Table 7

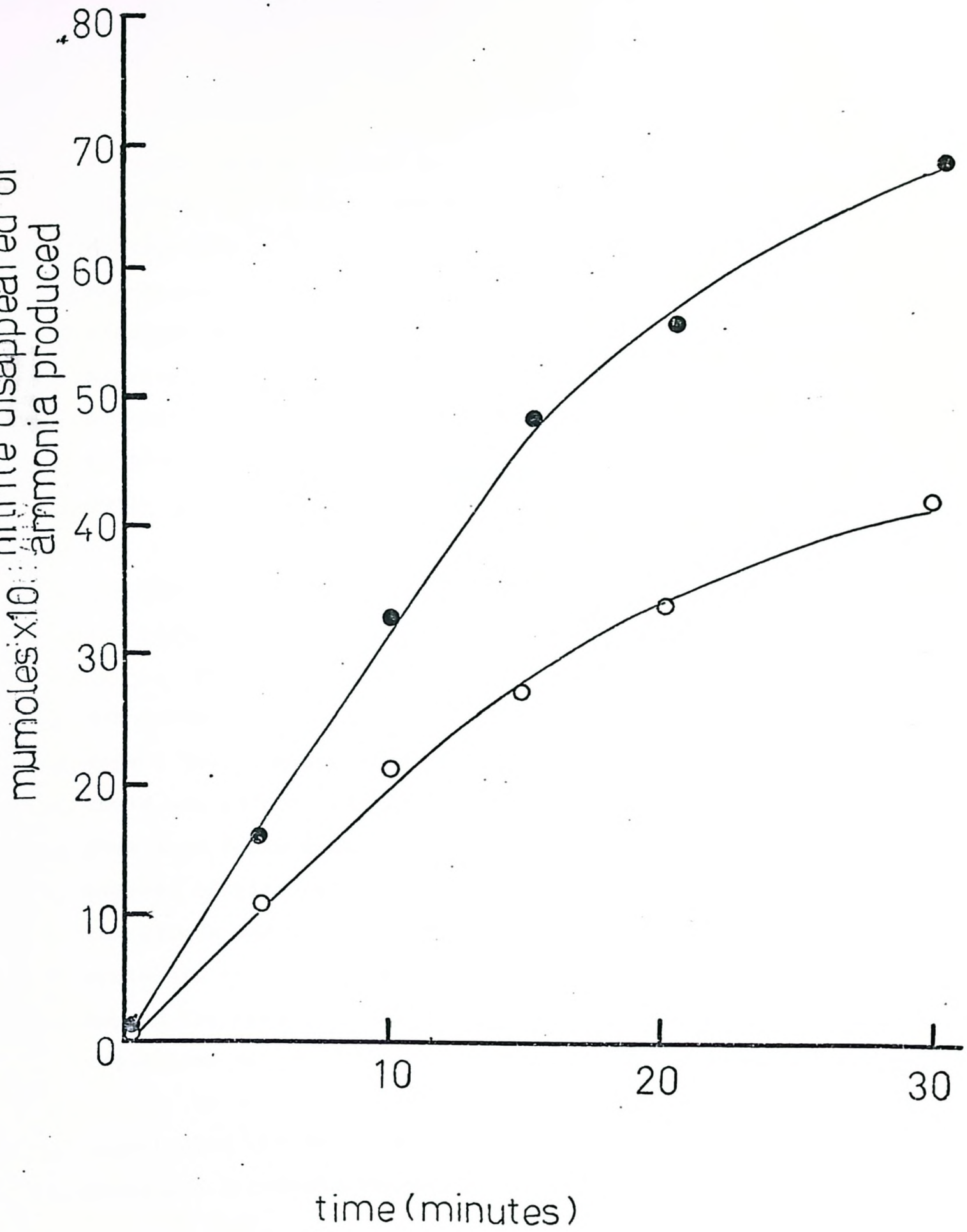
Controls for Ammonia Production

Nitrite reductase assays were set up so that they were complete; lacked enzyme; contained heat denatured enzyme; lacked benzyl viologen or lacked added sodium dithionite. The assays were carried out and ammonia measured as described in METHODS, using reaction mixtures lacking added nitrite as blank. 0.7 mg of S16000 protein, extracted from nitrate grown mycelia, was the enzyme source. Nitrite reductase activity and ammonia production were determined from the same samples.

Assay	Units Nitrite Reductase	Units Ammonia Produced
Complete	33.6	24.0
- Enzyme	0	0
+ Heat denatured enzyme	7.2	0
- Benzyl viologen	14.4	6.5
- Dithionite	4.8	0

Fig. 7.

Effect of incubation time of reaction mixtures on nitrite disappearance and ammonia production. Nitrite reductase assays were carried out as given in METHODS except that tubes were incubated in a 27° water bath for the times indicated. Ammonia was assayed from these reactions as given in METHODS. The S16000 extract was made from nitrate grown mycelia, and each assay contained 0.8 mg of protein. Nitrite disappearance is represented by ●—● and ammonia production by ○——○ . Nitrite reductase activity and ammonia production were determined on the same sample.


mumoles x10⁻¹ : nitrite disappeared or ammonia produced
0
10
20
30
40
50
60
70
80
10
20
30
time (minutes)

molecule such as glutamate, *in vitro*. It is possible that the S5000 extraction procedure results in a partial destruction of the conformation of the enzyme. This change would result in the inability of the enzyme to catalyze the production of maximum amounts of ammonia from nitrite. Desalting in the presence of the sucrose-EDTA solution restores, at least partially, the configuration of the enzyme, thus making the ammonia producing moiety fully active.

(b) What role does ammonia play in the regulation of nitrite reductase?

Previously, Cook postulated that nitrite reductase was derepressible (3,4). If this is the case, one would expect that the presence of nitrate in the growth medium would not affect the 'induction' of nitrite reductase. Cook also found that a casamino acids digest did not completely repress nitrite reductase. The presence of repressors in the growth medium, such as ammonium ions, should not allow synthesis of nitrite reductase; consequently, one would expect the specific activity of extracts to remain constant, regardless of incubation time in ammonia.

An 'induction' study was carried out in order to investigate the effect of time of incubation of mycelia in different nitrogen sources, on the level of nitrite reductase

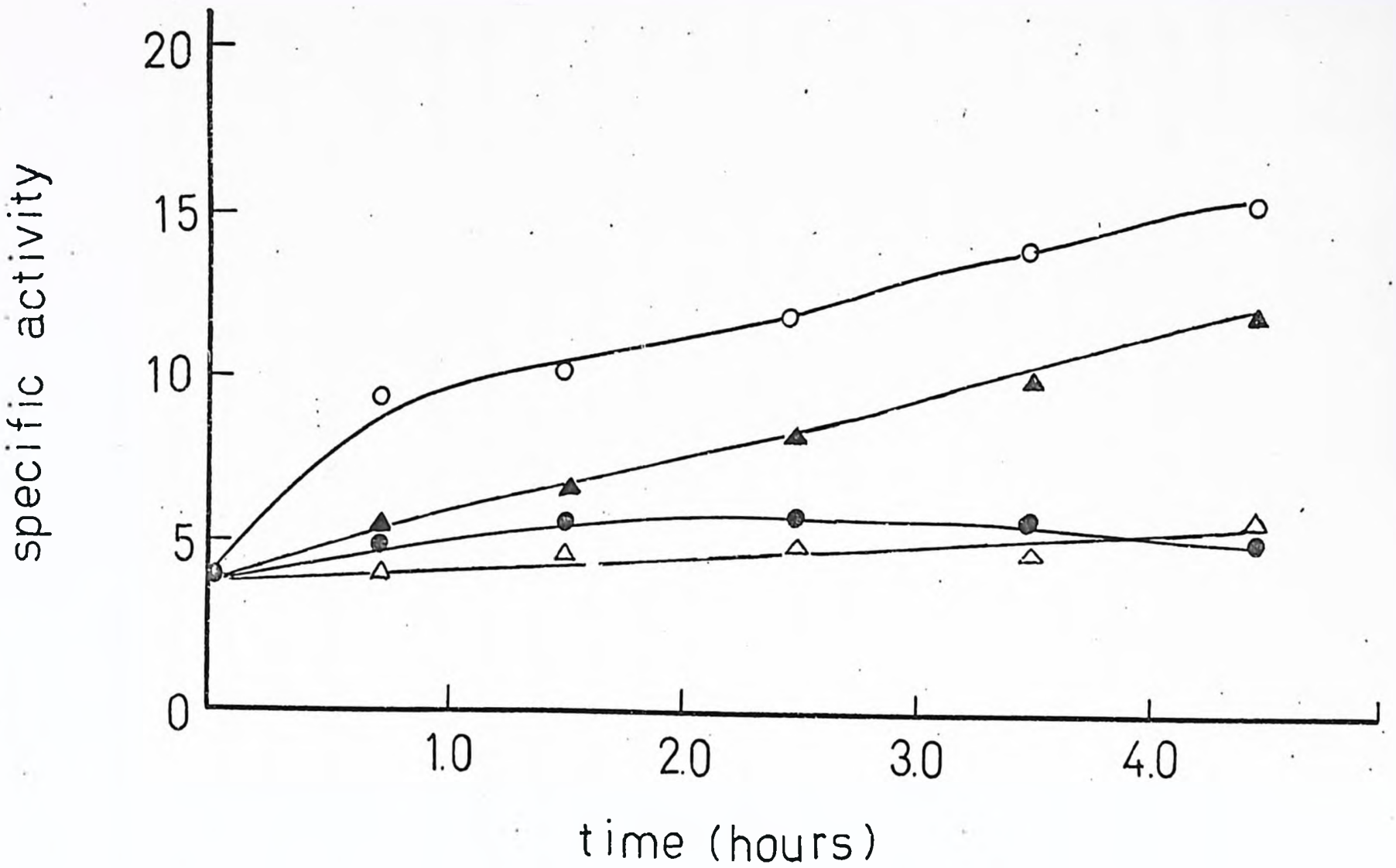
in cell-free extracts. Mycelia, pre-grown on casamino acids, were transferred to various nitrogen sources. At different time intervals, flasks were removed from a rotary shaker and S500 extracts were made of the mycelia. Figure 8 shows the variation with time of the specific activity of nitrite reductase in such extracts. The specific activity of nitrite reductase from mycelia exposed to a nitrogen-deficient medium increases steadily with time. Although the appearance of nitrite reductase activity from mycelia 'induced on a nitrate medium is similar, there is initially a greater rate of increase of activity. This observation suggests that nitrate does play a role in the 'induction' of nitrite reductase and therefore that the enzyme(s) may not be entirely derepressible. The specific activity of nitrite reductase from ammonia-grown mycelia remains essentially constant with time. Addition of 3 mM ammonium chloride to reaction mixtures containing extracts of mycelia exposed to a nitrogen-deficient medium resulted in a marked inhibition of nitrite reductase activity. The resultant specific activity was comparable to the specific activity of extracts from ammonia-grown mycelia (Figure 8).

If ammonia is acting as an inhibitor of nitrite reductase, it is possible that ammonium grown mycelia contain an inactive nitrite reductase. If this were so, removal of ammonia from such extracts might result in an increase in

Fig. 8.

'Induction' of nitrite reductase on different nitrogen sources.

Mycelial pads, pre-grown on a casamino acids digest, were cut into sectors. 1.0 gm portions were transferred to media containing nitrate ○—○; ammonia ●——● ; or no nitrogen-source ▲——▲ . The cultures were incubated on a rotary shaker and mycelia were harvested at the indicated intervals. S500 extracts were prepared and assayed for nitrite reductase activity. △——△ represents extracts of mycelia exposed to a nitrogen-deficient media which were assayed in the presence of 3 mM NH_4Cl. Specific activity is expressed as units per mg protein in the assay.


specific activity
20
15
10
5
0
1.0
2.0
3.0
4.0
time (hours)

nitrite reductase activity. S16000 extracts of ammonia-grown mycelia were passed through a Sephadex G-10 column, as described in METHODS, and subsequently assayed for nitrite reductase activity in the presence or absence of 3 mM ammonium chloride. For comparison, S16000 extracts of nitrate-grown mycelia were treated similarily. The results of this experiment are shown in Table 8. Desalting of the ammonia-grown extracts resulted in an increase in the specific activity of nitrite reductase. Activity in the desalted extract was very sensitive to the presence of ammonia in the assay. This result confirms the expectation that an inactive nitrite reductase may be present in ammonia-grown mycelial extracts. Nitrite reductase from untreated nitrate grown extracts is slightly sensitive to the presence of ammonium ions in reaction mixtures; desalted extracts from the same source are insensitive to ammonium ions.

Maximum inhibition of nitrite reductase from desalted extracts of mycelia grown on ammonia occurred when 3 mM ammonium chloride was present in reaction mixtures (Fig. 9a). An ammonium ion concentration of 3×10^{-9} M had no effect on nitrite reductase activity.

Desalted extracts were incubated at 27°C for various times with ammonia concentrations known to cause less than 50% inhibition. Although increasing the time

Table 8

In Vitro Effect of Ammonia on Nitrite Reductase Activity from Extracts of Mycelia grown on Nitrate or Ammonia

S16000 extracts were prepared and then assayed before or after desalting on a Sephadex G-10 column, in the presence or absence of 3 mM NH_4Cl. The assays contained the following amounts of protein: NO_3^- untreated, 1.08 mg; NO_3^- desalted, 0.8 mg; NH_4^+ untreated, 1.02 mg; NH_4^+ desalted, 0.74 mg.

Nitrogen Source in Growth Medium	Units Nitrite Reductase			
	Untreated		Desalted	
	$-NH_4Cl$	+3 mM NH_4Cl	$-NH_4Cl$	+3 mM NH_4Cl
NO_3^-	55.2	48.0	21.6	21.6
NH_4^+	14.4	12.0	26.4	4.8

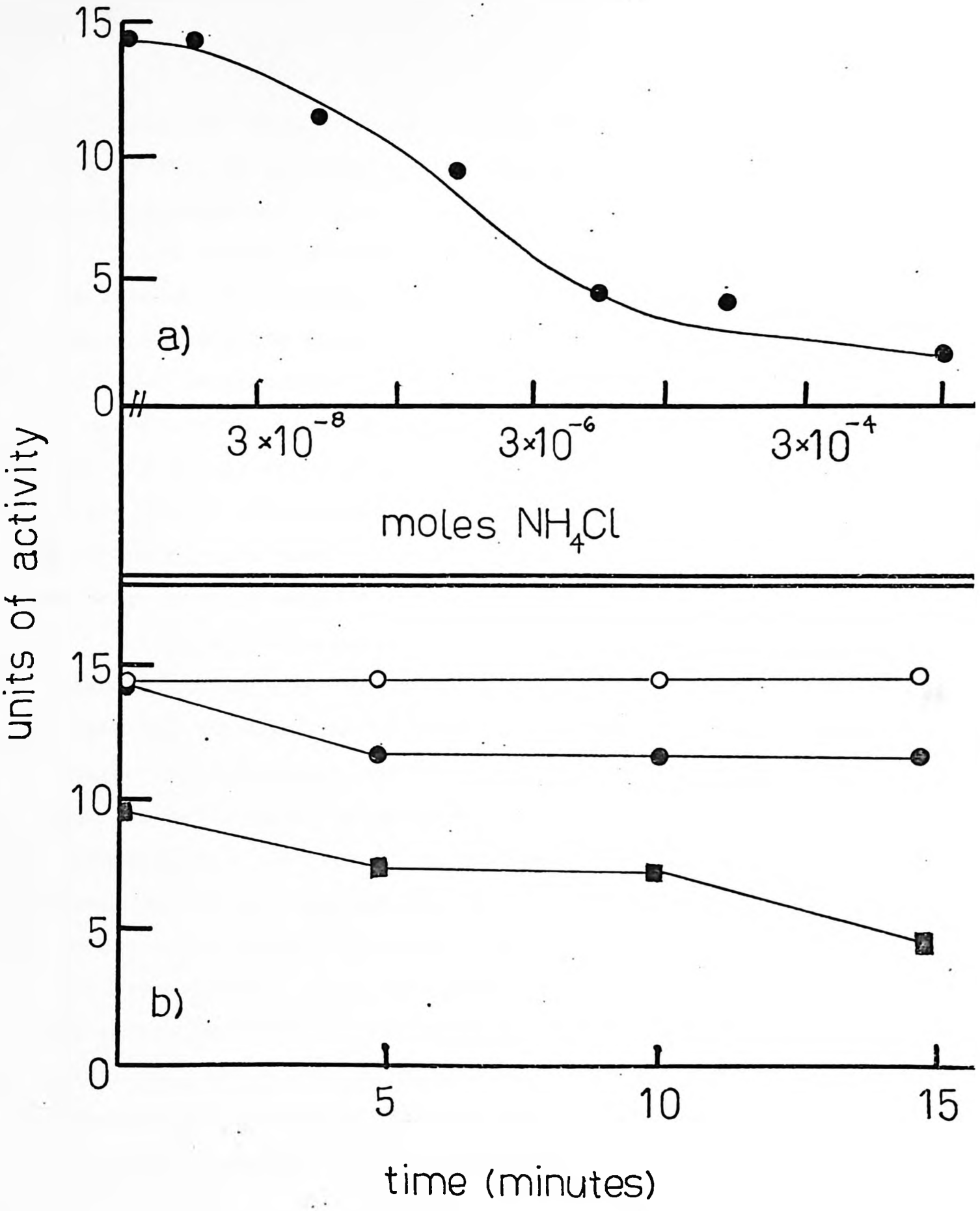
Fig. 9.

(a) Effect of ammonium concentration on nitrite reductase activity.

S16000 extracts of ammonia grown mycelia were desalted on a Sephadex G-10 column as described in METHODS. 0.8 mg of the treated extract were added to reaction mixtures and the given concentration of ammonia added, prior to adding the reductant.

(b) Effect of incubation time on ammonia inhibition.

Complete assays, including ammonia, were incubated at 20°C for the times indicated. Reductant was then added and the assay continued as given in METHODS. O—O represents assays to which no ammonia was added; ●—● 3×10^{-8}M ammonia added; ■—■ 3×10^{-7}M ammonia added. All assays contained 0.8 mg protein, treated as described above.


15
10
5
0
a)
3 ×10⁻⁸
3×10⁻⁶
3×10⁻⁴
moles NH₄Cl
units of activity
15
10
5
0
b)
5
10
15
time (minutes)

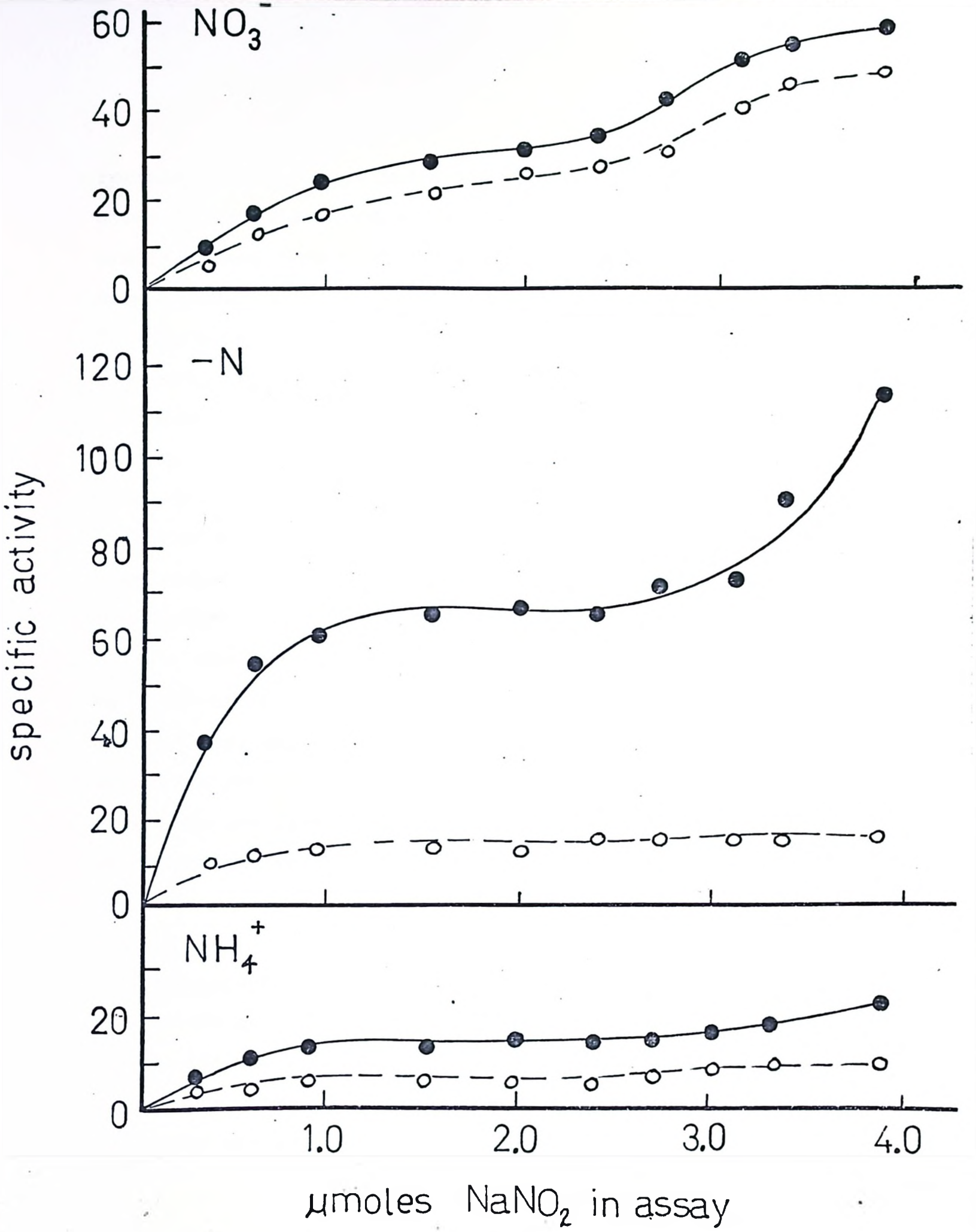
of incubation results in an increase in inhibition (Figure 9b), it proceeds at too slow a rate to influence previous results.

To summarize these results: Mycelia grown in the absence of a nitrogen source possess a nitrite reductase activity which would appear to be derepressible, and which is inhibited by ammonium ions *in vitro*. Extracts of mycelia grown on ammonia contain a nitrite reductase activity which, after desalting on Sephadex G-10, is more active and is ammonia-sensitive. Extracts of mycelia grown on nitrate have a nitrite reductase activity which is only slightly sensitive to ammonia *in vitro*.

The evidence presented so far suggests that the enzyme(s) present in extracts of ammonia grown mycelia may be similar to that (those) present in extracts of mycelia exposed to a nitrogen-deficient medium, and different from that (those) present in cell-free preparations of nitrate grown mycelia. If this is so, similar substrate saturation curves should be expected with the enzyme extracted from ammonia grown mycelia or mycelia exposed to a nitrogen-deficient medium. Also, the substrate saturation curve of the enzyme prepared from nitrate grown mycelia might be different. The substrate saturation curve for nitrite reductase and ammonia production from nitrate grown mycelia is biphasic (Figure 10). The substrate saturation curves

Fig. 10.

Substrate saturation curves of S16000 extracts obtained from mycelia exposed to different nitrogen sources.
The concentration of $NaNO_2$ in the assays was varied as indicated. S16000 extracts were prepared as given in METHODS. 1.0 mg of 'NO_3-extract', 0.4 mg of '-N extract' and 1.76 mg of 'NH_4^+ extract' were assayed. Nitrite reductase activity is represented by ●—● and ammonia production by ○—○ . Specific activity is defined as units of activity per mg protein. Nitrite reductase activity and ammonia production were determined on the same samples.


60
40
20
0
NO_3^-
120
100
80
60
40
20
0
−N
20
0
NH_4^+
specific activity
1.0
2.0
3.0
4.0
μmoles $NaNO_2$ in assay

for nitrite reductase and ammonia production from mycelia grown on ammonia or exposed to a nitrogen-deficient medium are different from those observed with extracts of nitrate-grown mycelia but are similar with respect to each other. Similar concentrations of nitrite (0.33 mM) yield plateaus, in the case of ammonium grown mycelial extracts or extracts of mycelia exposed to a nitrogen-deficient medium. However, at concentrations of nitrite above 1 mM, the curves appear to diverge. Ammonia is produced as a result of nitrite reduction, in both extracts. The significance of this result will be considered in the DISCUSSION.

The biphasic curve obtained with nitrate-grown mycelial extracts could be indicative of an allosteric interaction, as was found by Hewitt and Hucklesby (15), or of the presence of more than one nitrite reductase, or more than one form of nitrite reductase, with different Km's for nitrite.

(c) <u>If there are more than one form of nitrite reductase, can mutants be found which lack one of the activities?</u>

The evidence presented so far is suggestive of either (a) the presence of more than one nitrite reductase in nitrate grown mycelial extracts, <u>or</u> (b) the presence of more than one aggregation form of a nitrite reductase in

nitrate grown mycelial extracts.

If either of the above is true, mutants should be found which are defective in one or both of the enzymes, or one or both of the aggregation forms of the enzyme.

Five mutants which were defective in growth on nitrate and nitrite were chosen for study. Mutant O does not grow on nitrate or nitrite and accumulates significant amounts of nitrite when grown on an ammonium nitrate medium (10). Mutant Z grows poorly on nitrate and nitrite and accumulates nitrite during later stages of growth on an ammonium nitrate medium (10). Mutant t-13 grows on nitrate and nitrite at 27°C, but not at 37°C and accumulates nitrite when grown on an ammonium nitrate medium at 37°C (10). Mutant t-23 grows on nitrate and nitrite at 27°C and 37°C, but accumulates nitrite when grown on an ammonium nitrate medium at 37°C (10). Mutant 25 grows poorly on nitrate and nitrite (see APPENDIX).

Mutant Z has an active nitrite reductase which produces ammonia as product. Nitrite reduction and ammonia production are essentially stoichiometric (Figs. 11, 11A). The shape of the curves in Figure 11 are very similar to that of the curve depicting ammonia production by extracts of the wild type strain. If there are more than one nitrite reductase, it would seem reasonable that mutant Z lacks the activity which does not yield ammonia as product of

Fig. 11.

Effect of S1600 extract protein concentration from Z grown at 27°C on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●——● and units of ammonia produced by O——O . Nitrite reductase activity and ammonia production were determined on the same samples.

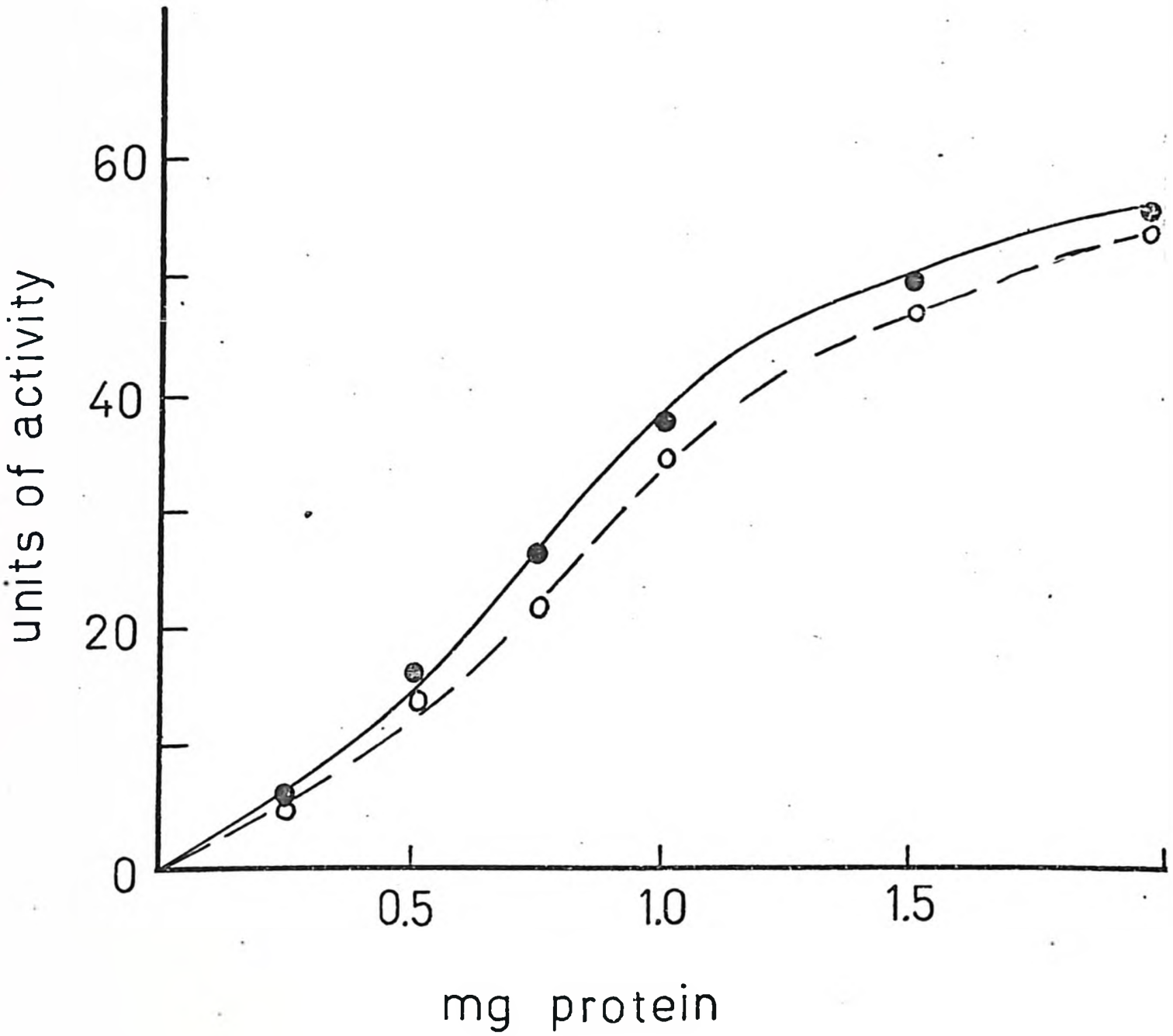
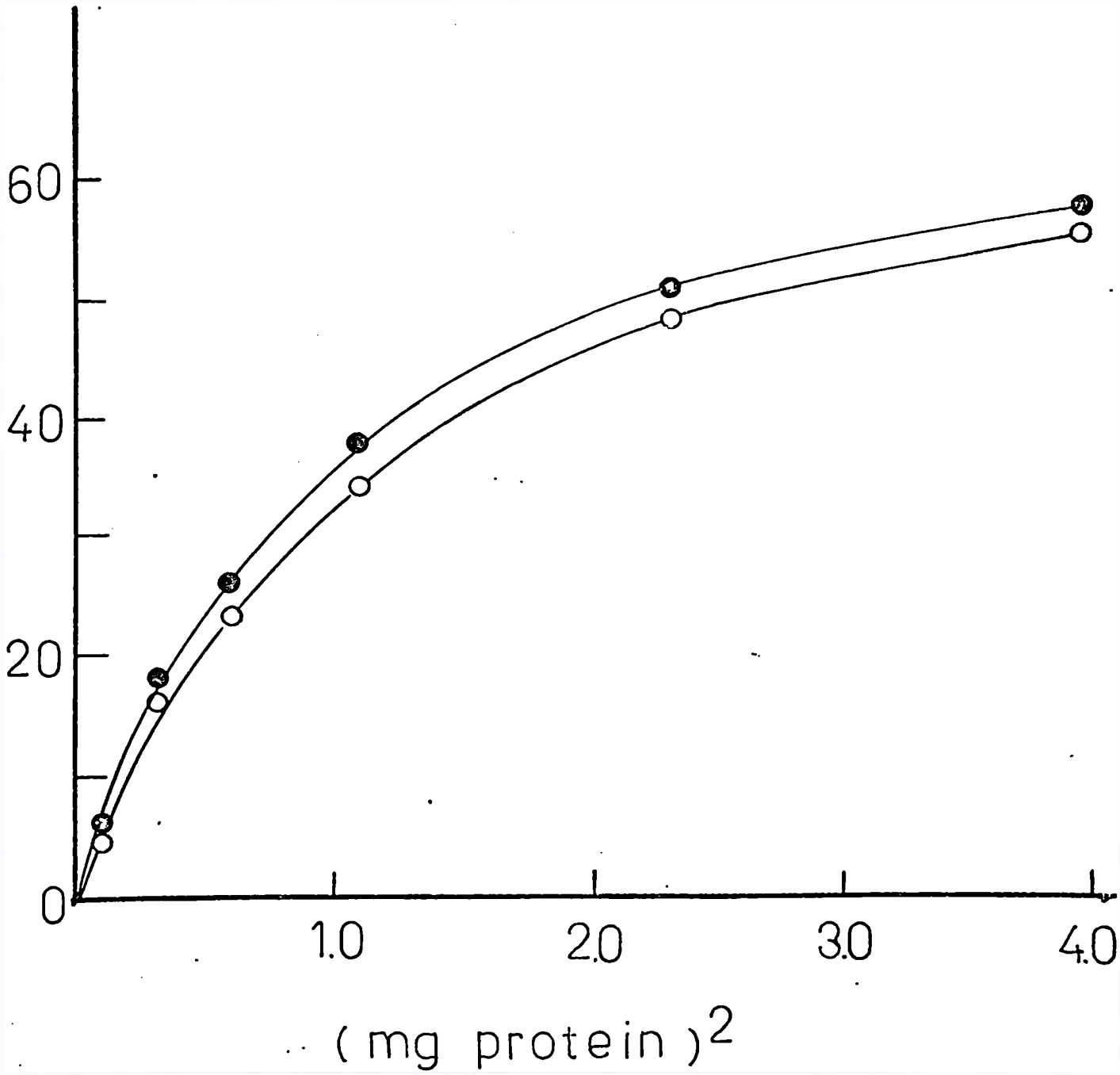

units of activity
60
40
20
0
0.5
1.0
1.5
mg protein

Fig. 11A.

Effect of the square of S16000 extract protein concentration from mutant Z on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●——● and units of ammonia produced by O——O . Nitrite reductase activity and ammonia production were determined on the same samples.


units of activity
60
40
20
0
1.0
2.0
3.0
4.0
(mg protein)^2

reduction. It is also possible, by the same reasoning, that nitrite reductase from mutant Z exists only in that aggregation form which allows ammonia to be formed as product of nitrite reduction. Since Z grows poorly on nitrate and nitrite, it follows that the loss of the enzyme or conformation of enzyme responsible for the reduction of nitrite to a product other than ammonia, is detrimental to growth on nitrate or nitrite.

Extracts of mutant O contain a low level of nitrite reductase activity. No ammonia is produced as a result of nitrite reduction (Figs. 12, 12A). A dilution effect, similar to that of one of the postulated wild type activities, is found. At concentrations of wild type protein greater than 0.8 mg, increased nitrite reduction does not appear to result in ammonia production (see Fig. 4). Similarly, with mutant O, nitrite reductase activity is not expressed until 0.8 mg of protein are present in the assay and no ammonia is produced as a result of reduction. It is possible that this activity represents either that nitrite reductase which does not yield ammonia as product, or that form of aggregation of nitrite reductase which is incapable of catalyzing the production of ammonia. The presence of cysteine in the growth medium which is known to repress sulfite reductase, did not appear to affect nitrite reductase activity, indicating that the residual activity in mutant O

Fig. 12.

Effect of S16000 extract protein concentration from mutant O on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●——● and units of ammonia produced by O——O . ■——■ represents nitrite reductase from S16000 extracts of mycelia grown on nitrate plus 3 mM cysteine. Nitrite reductase and ammonia production were determined on the same samples.

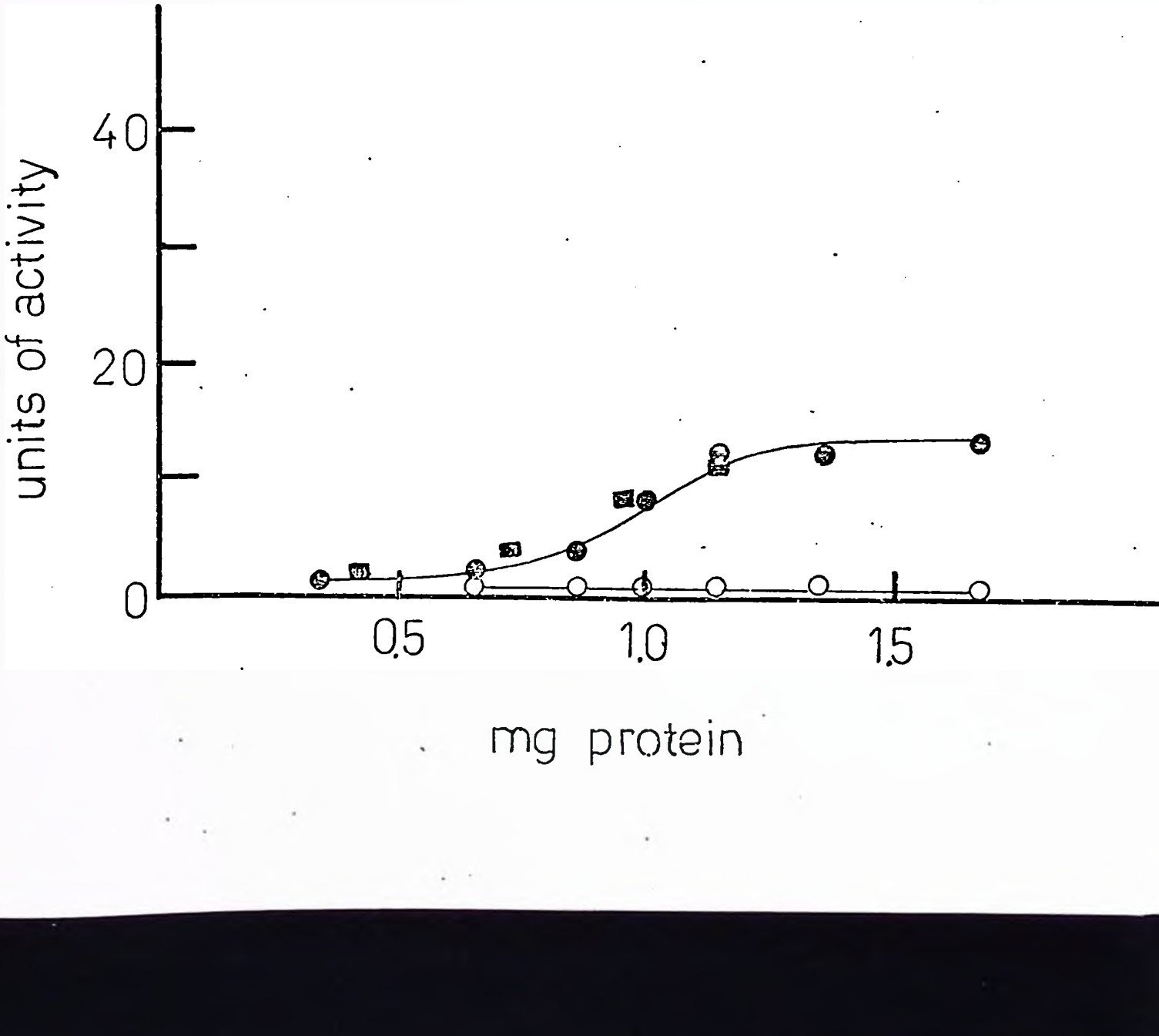
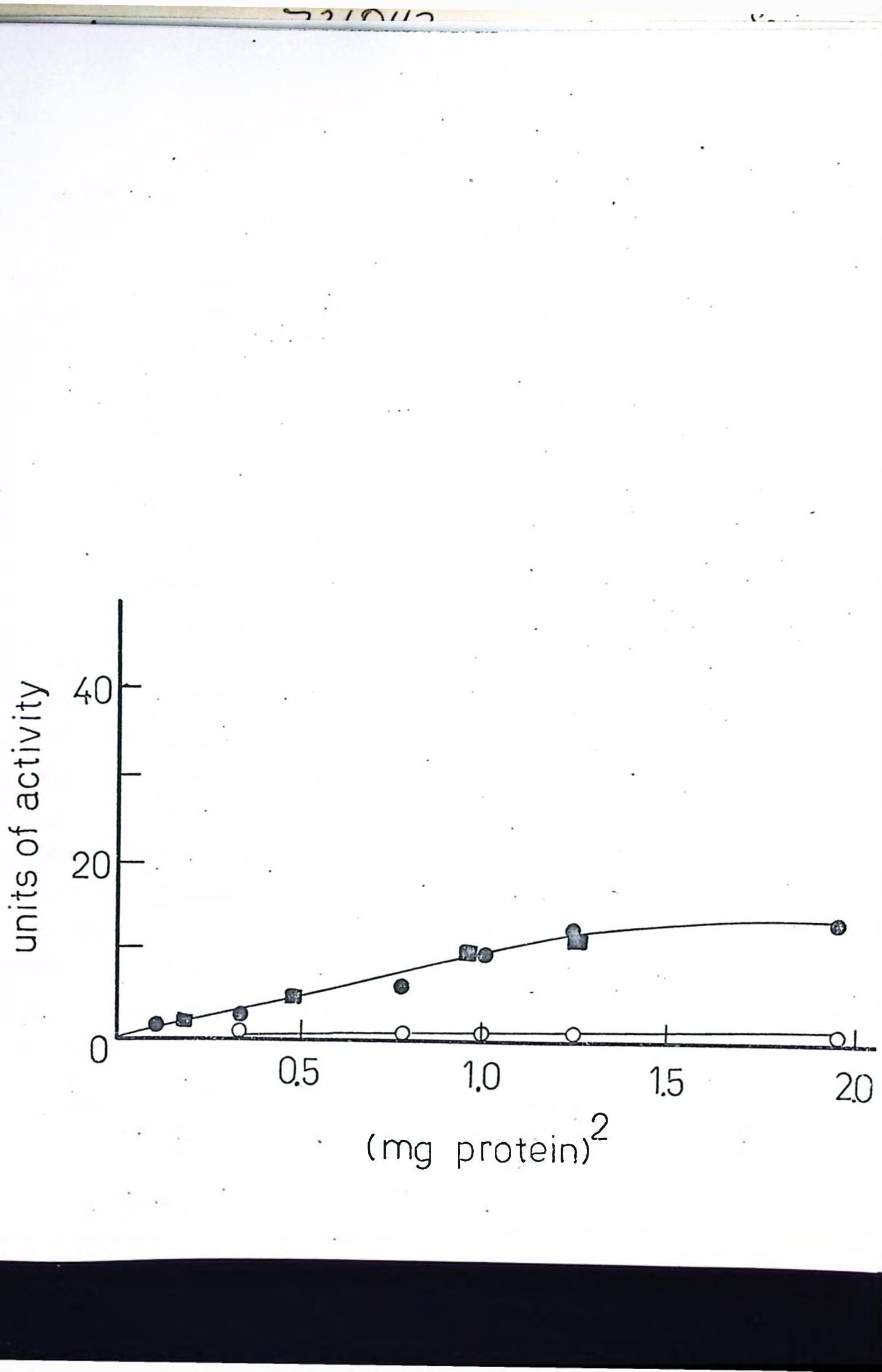

40
20
0
units of activity
0.5
1.0
1.5
mg protein

Fig. 12A.

Effect of the square of S16000 extract protein concentration from mutant O on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●—● and units of ammonia produced by ○—○ . ■—■ represents nitrite reductase activity from S16000 extracts of mycelia grown on nitrate plus 3 mM cysteine. Nitrite reductase and ammonia production were determined on the same samples.


units of activity
40
20
0
0.5
1.0
1.5
2.0
(mg protein)^2

is not concerned with sulfur metabolism (Figs. 12, 12A).

No significant differences were found between the nitrite reductase activity and ammonia producing activity from extracts of t-13 grown at 27°C and at 37°C (Figs. 13, 13A). This result was not expected since the growth rate of t-13 on nitrate or nitrite at 27°C is normal, but at 37°C is negligible. The shape of the curves showing the relationship between nitrite reductase and ammonia producing activities and protein concentration in assays of extracts of t-13 mycelia grown at 27°C and 37°C were different from those of the comparable relationship with extracts of the wild type strain. It is possible that the nitrite reductase(s) of t-13 is (are) different from the wild type enzyme(s) at 27°C, but that the mutation is only lethal when the organism is grown at 37°C; however, this difference cannot be ascertained *in vitro*.

Nitrite reductase and ammonia production activities in extracts of t-23 grown at 27°C are somewhat lower than that at comparable protein concentrations of wild type extract. Preparations of t-23 grown at 37°C exhibit an increased dilution effect for nitrite reductase and ammonia production activities over that obtained for wild type (Figs. 14, 14A). The N/A ratio of extracts of mycelia grown at 27°C appears to be similar to the comparable ratio obtained from extracts of wild type mycelia. However, the

Fig. 13.

Effect of S16000 extract protein concentration from mutant t-13 grown at 27°C or 37°C on nitrate reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase activity are represented by ●—● or ■—■ and ammonia production by ○—○ and □—□ ; ●, ■, ○, □ represent results from two separate experiments. Nitrite reductase activity and ammonia production were determined on the same samples.

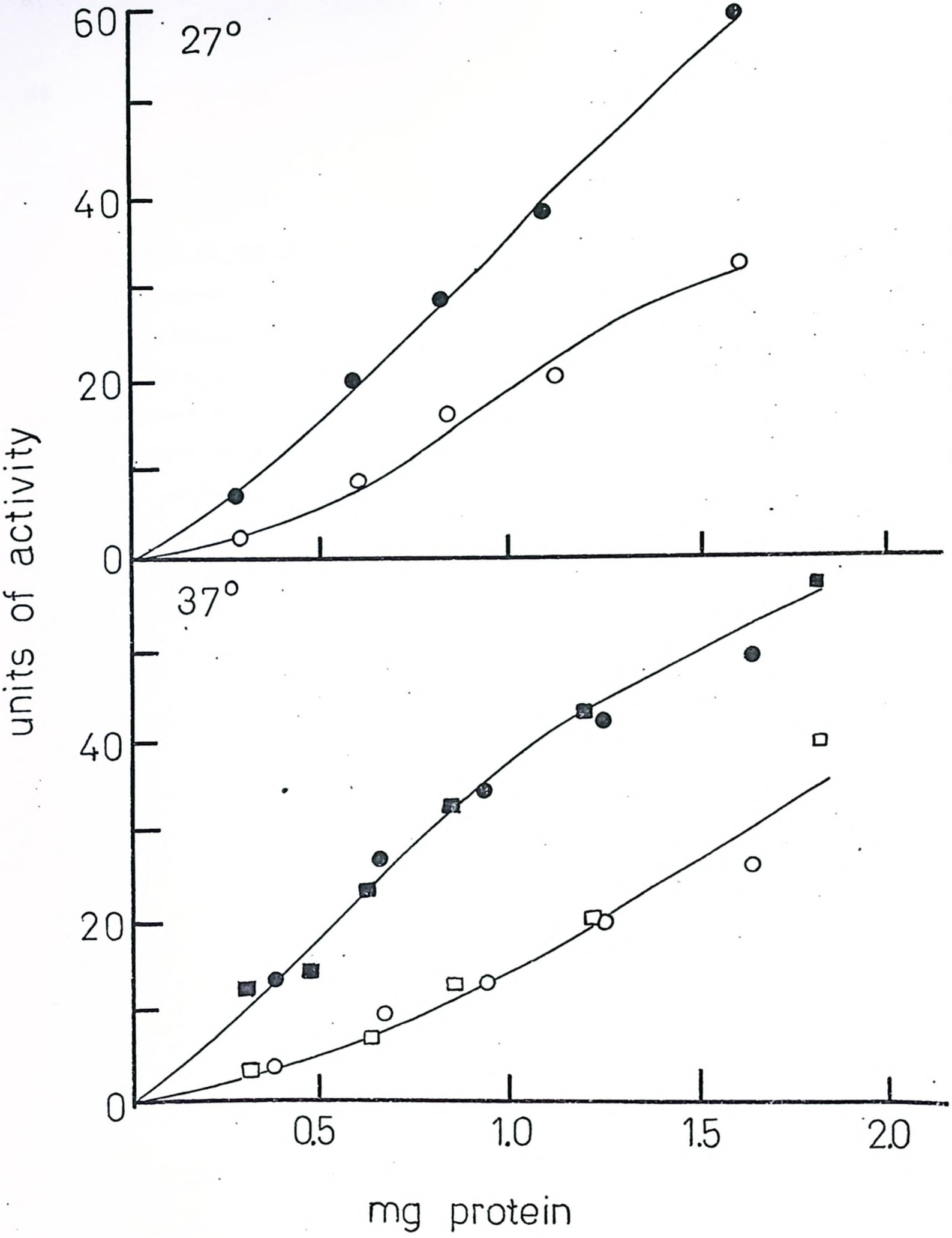

60
40
20
0
27°
37°
units of activity
40
20
0
0.5
1.0
1.5
2.0
mg protein

Fig. 13A.

Effect of the square of S16000 extract protein concentration from t-13 grown at 27°C and 37°C on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●—● and units of ammonia produced by ○—○ . Nitrite reductase activity and ammonia production were determined on the same samples.

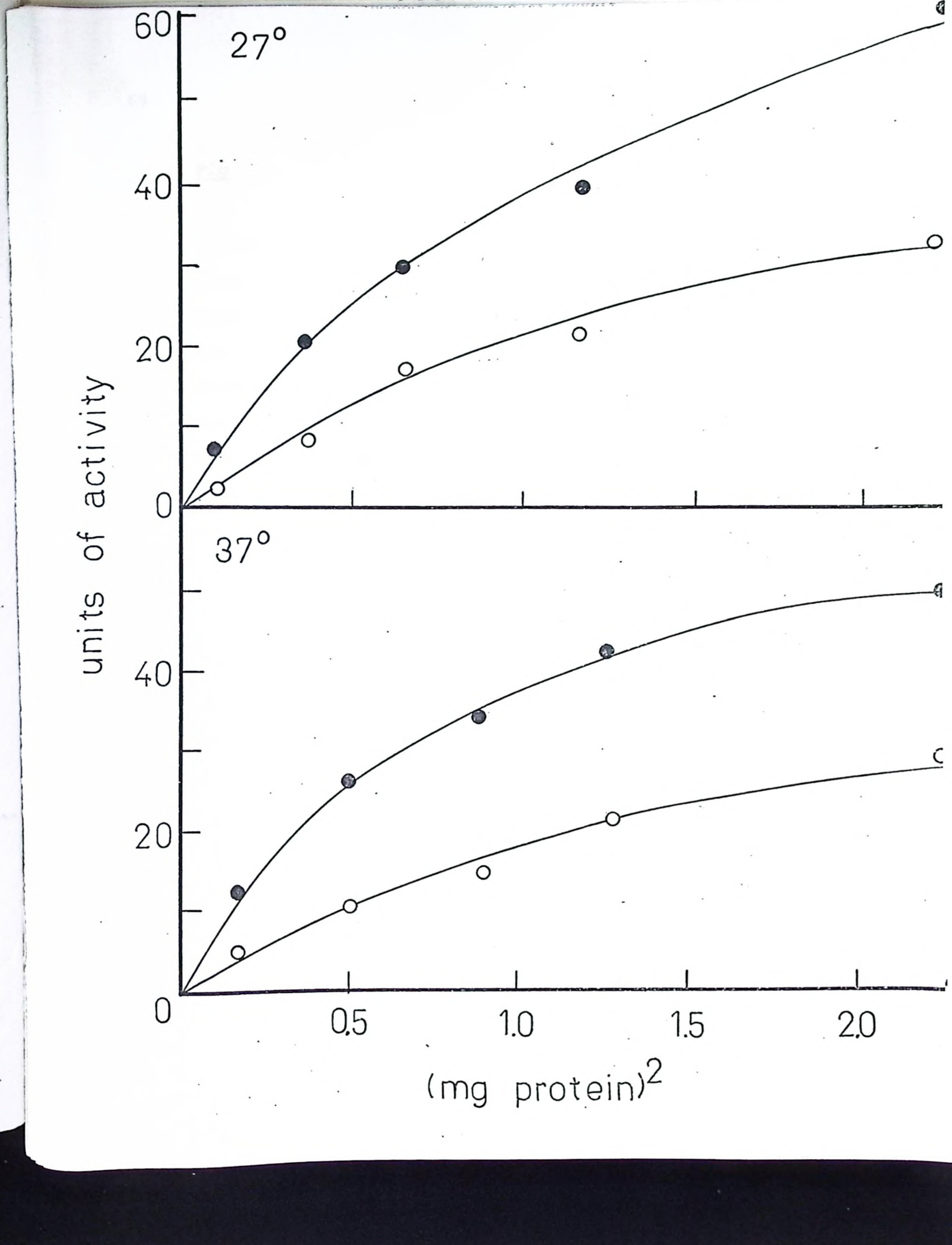

60
40
20
0
27°
37°
units of activity
40
20
0
0.5
1.0
1.5
2.0
(mg protein)2

Fig. 14.

Effect of S16000 extract protein concentration from t-23 grown at 27°C and 37°C on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●—● and units of ammonia produced by O—O . Nitrite reductase activity and ammonia production were measured on the same samples.

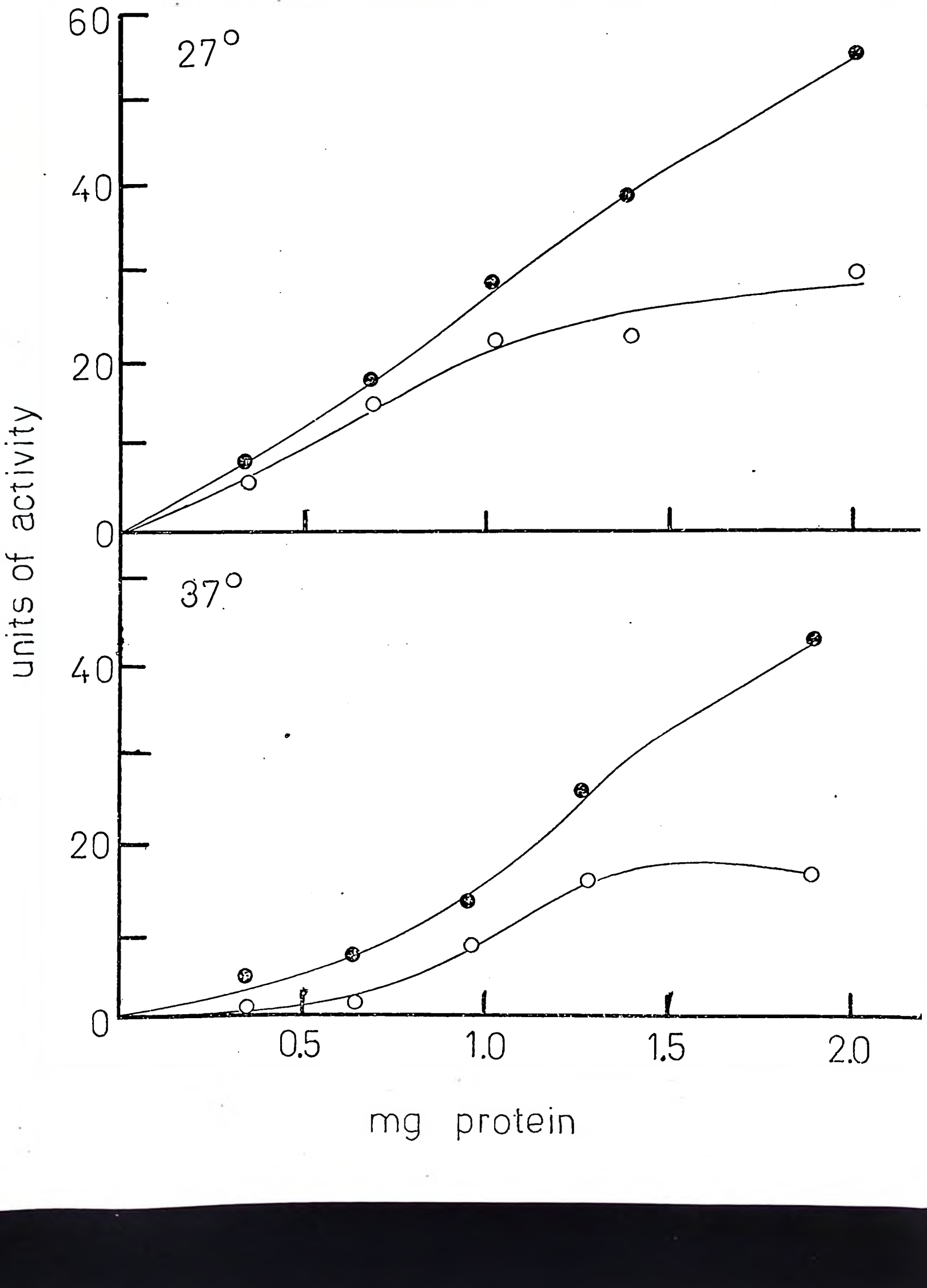
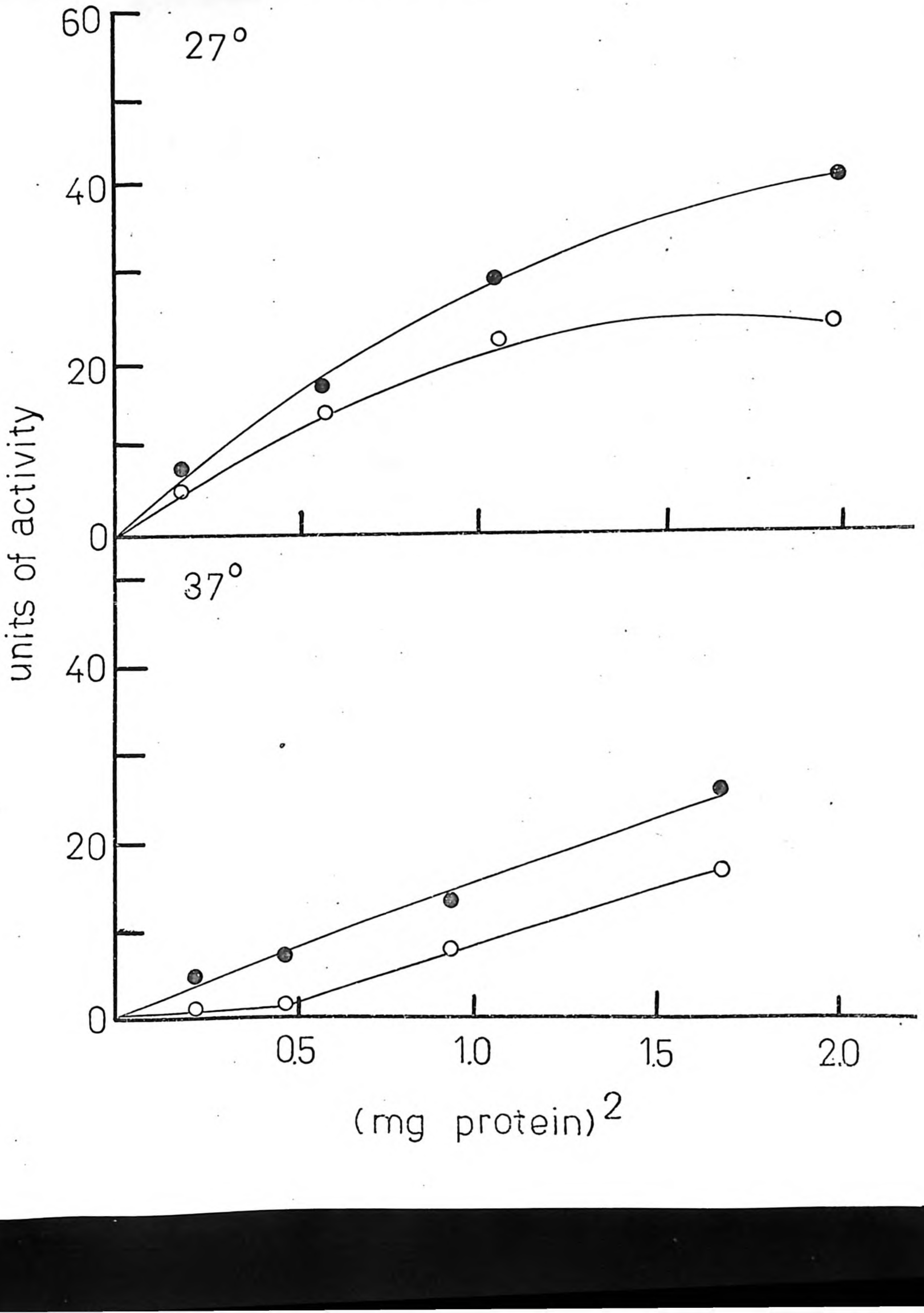

60
40
20
0
27°
37°
units of activity
40
20
0
0.5
1.0
1.5
2.0
mg protein

Fig. 14A.

Effect of the square of S16000 extract protein concentration from t-23 grown at 27°C and 37°C on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●——● and units of ammonia produced by O——O . Nitrite reductase activity and ammonia produced were measured on the same sample.


60
40
20
0
27°
37°
units of activity
40
20
0
0.5
1.0
1.5
2.0
(mg protein)2

same ratio in extracts of t-23 mycelia grown at 37°C is much different than that of wild type extracts. If nitrite reductase in t-23 is temperature sensitive at 37°C, incubation of extracts of t-23 mycelia, previously grown at 27°C, in a 37°C water bath, should result in a reduction of nitrite reductase activity. The specific activity should approach that found in extracts of mycelia grown at 37°C. The results in Figure 15 indicate that after sixty minutes incubation at 37°C, the log of the specific activity of nitrite reductase in extracts of t-23 grown at 27°C is reduced so that it is similar to the specific activity of nitrite reductase in t-23 grown at 37°C.

The nitrite reductase activity from mutant 25 appears to be similar to that from mutant Z, although in the case of 25, the N/A ratio is slightly greater (Fig. 16,16A). This mutant may be of the same class as Z; that is, either the enzyme or form of aggregation of enzyme which catalyzes the formation of a product other than ammonia, is affected.

From these results it would appear that there are at least two distinct enzymes, or two distinct forms of enzyme aggregation, concerned with nitrite reduction. One enzyme, or form of aggregation, as typified by the activity present in extracts of mutant Z, yields ammonia as product of reduction. The other enzyme or form of enzyme aggregation, which is the activity present in mutant O, does not yield

Fig. 15.

Effect of exposure of nitrite reductase from extracts of t-23 grown at 27°C or 37°C and of wild type grown at 27°C, to 37°C.

S16000 extracts of mycelia, grown on nitrate, were prepared and aliquots of similar protein concentration were incubated at 37°C in a water bath. At the times indicated, samples were removed and assayed for nitrite reductase activity. 1.1 mg of *pan-2B-36A* ●—● ; 1.0 mg of t-23 grown at 27°C ■—■ ; and 1.2 mg of t-23 grown at 37°C ▲—▲, were assayed. Specific activity is expressed as units per mg protein.

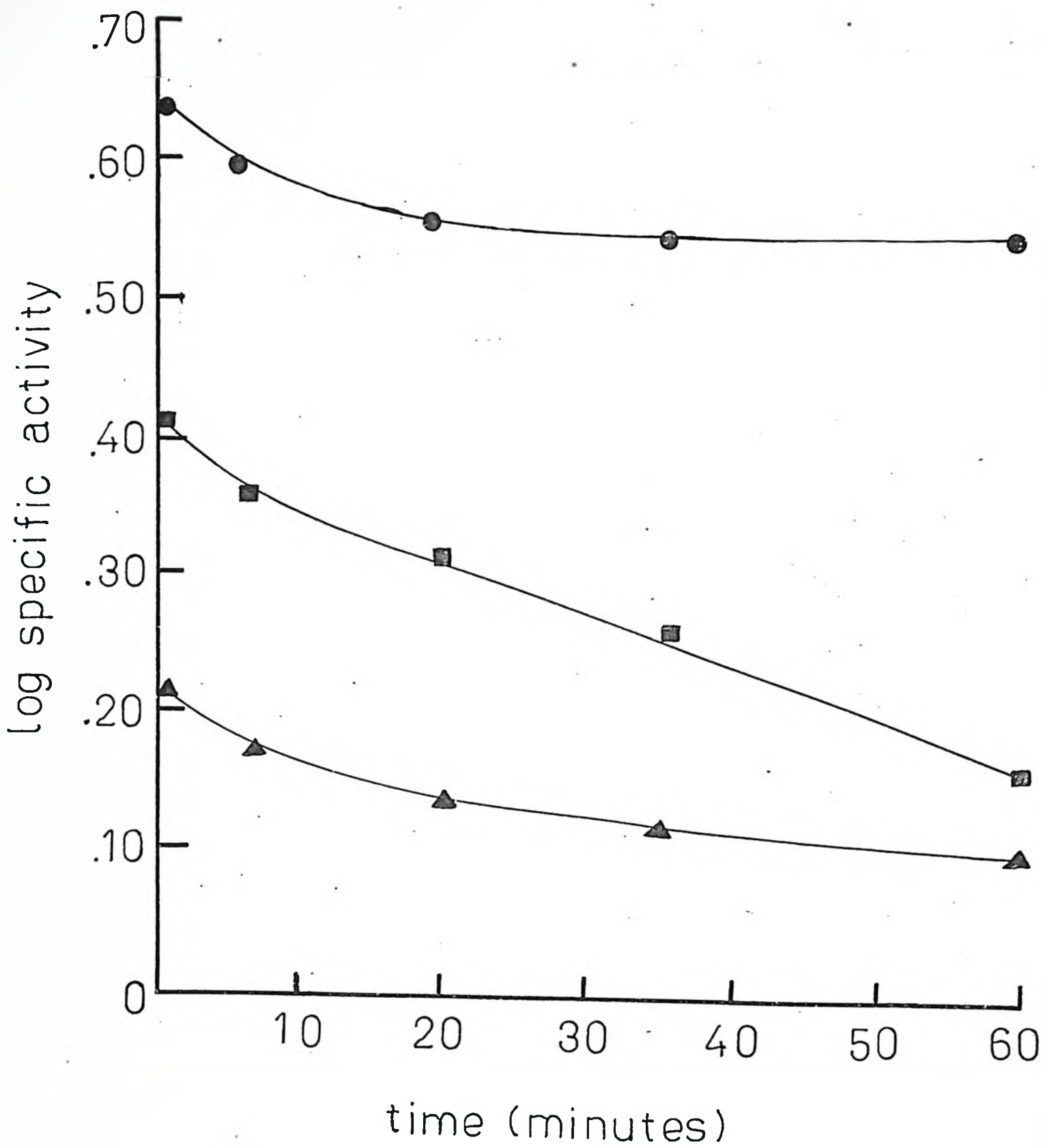

.70
.60
.50
.40
.30
.20
.10
0
Log specific activity
10
20
30
40
50
60
time (minutes)

Fig. 16.

Effect of S16000 extract protein concentration from mutant 25 on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●——● and units of ammonia produced by ○——○ . Nitrite reductase activity and ammonia production were determined on the same sample.

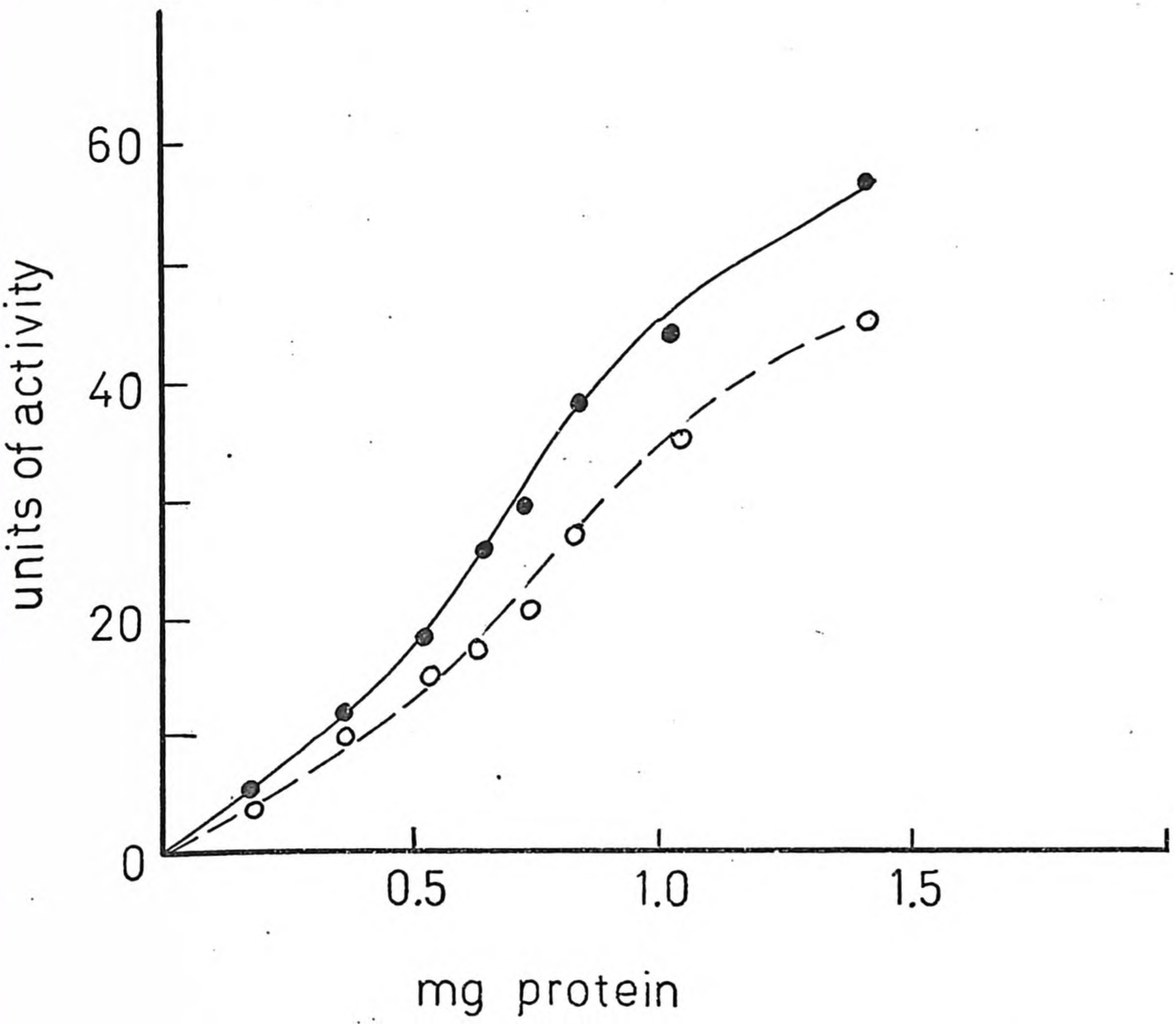
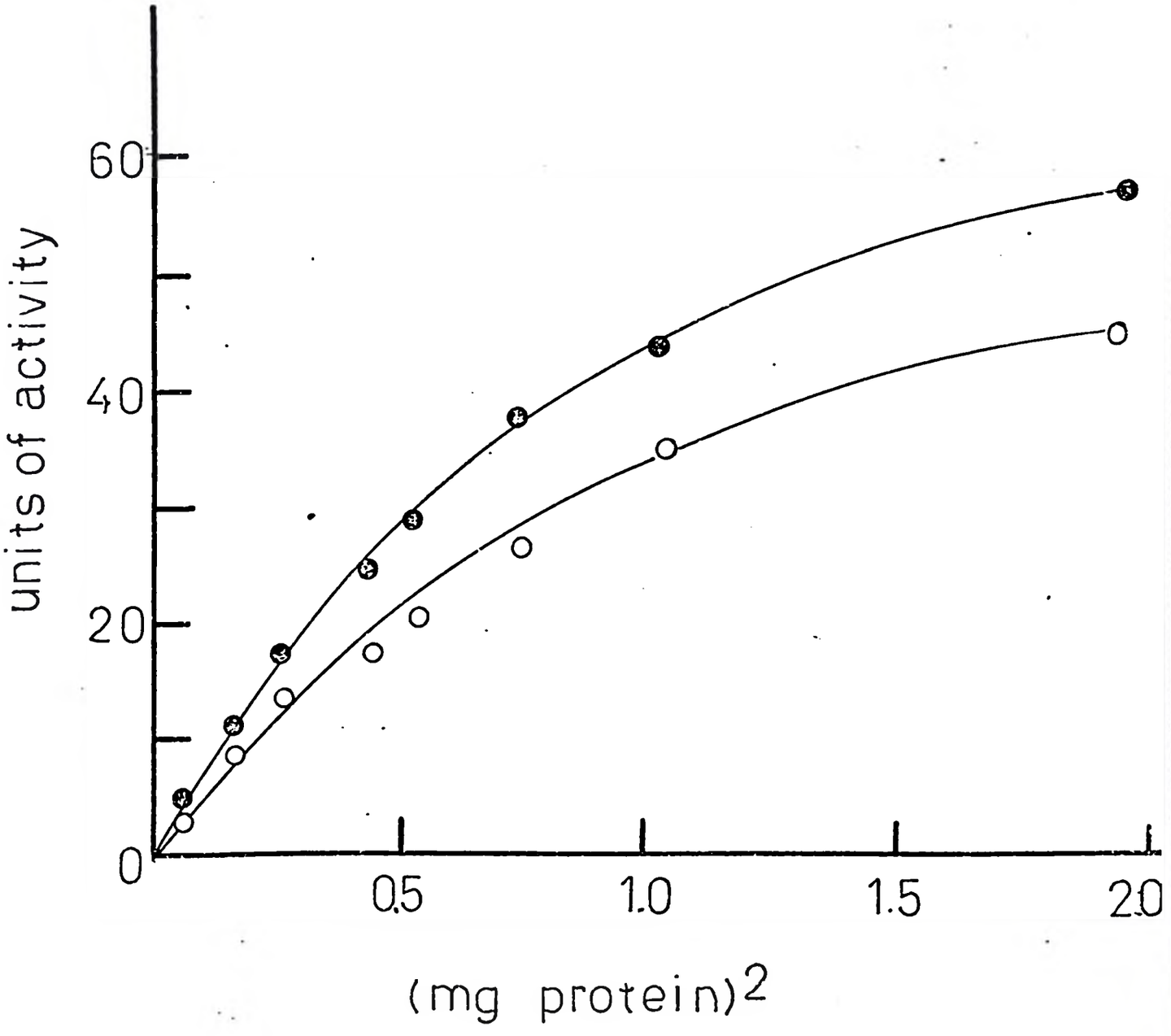

60
40
20
0
units of activity
0.5
1.0
1.5
mg protein

Fig. 16A.

Effect of the square of S16000 extract protein concentration from mutant 25 on nitrite reductase activity and ammonia production.

Mycelia were grown on nitrate. Units of nitrite reductase are represented by ●—● and units of ammonia produced ○—○ . Nitrite reductase activity and ammonia production were determined on the same sample.


60
40
20
0
units of activity
0.5
1.0
1.5
2.0
(mg protein)2

ammonia as product of reduction and exhibits a large dilution effect. Both enzymes or enzyme aggregates appear to be necessary for growth on nitrate or nitrite. Dyer (10) has shown that mutants Z and O are mutated at separate genetic loci; indicative that two distinct proteins are affected. This fact does not differentiate between the two possibilities; that is, that these mutations affect two different enzymes or that these mutations affect two forms of enzyme aggregation.

Dyer found that the mutation in t-13 mapped at a genetic locus different from that of O and Z. It was not clear whether mutant t-23 was mutant at a fourth genetic locus (10).

Since the ratio of nitrite reduced to ammonia produced in extracts of t-13 appear to be intermediate between that obtained for Z and O, and since t-13 is mutated at a different genetic locus than Z and O, it is reasonable to assume that t-13 represents a third protein. If it is assumed that there are two enzymes concerned with nitrite reduction, t-13 may represent a protein which is common to both enzymes. That is, because of this affected protein, both postulated activities, the one that catalyzes the formation of ammonia as product of reduction, and the one which catalyzes the formation of a product other than ammonia as a result of nitrite reduction, are affected.

If nitrite reductase exists in two aggregation forms, the protein affected in t-13 may act as a regulatory component. This component could regulate which form of aggregate would be active under specified conditions. The loss of this regulating component would result in equal amounts of both aggregates being formed and thus when extracts are assayed, would result in N/A ratios of approximately 2 (see Figure 18).

It is difficult to interpret the results obtained for extracts of t-23 since it is not known for certain whether it represents a distinct genetic locus.

It is possible that the assay described by Cook (3,4) measures more than one enzyme activity, at least one of which is not concerned with nitrite metabolism. A possible candidate would be sulfite reductase (18,35). *Cys-4*, which lacks sulfite reductase, has normal nitrite reductase activity (Fig. 17). Furthermore, the presence of cysteine or methionine in growth media,which are known repressors of sulfite reductase, did not affect the level of nitrite reductase activity in wild type extracts (Fig. 16). Although it is unlikely that Cook's assay measures sulfite reductase, the results do not rule out the possibility that other enzyme activities (e.g., oxidases) are being measured.

Fig. 17.

Effect of S16000 extract protein concentration on nitrite reductase activity in mutant *cys-4*.

S16000 extracts of *cys-4* were made from mycelia grown on nitrate plus 3 mM cysteine ▲—▲ or nitrate plus 2.4×10^{-4}M methionine ●—● . Wild type extracts, from similarily grown mycelia were used as control; △—△ grown on nitrate plus 3 mM cysteine; ○—○ grown on nitrate plus methionine; ■----■ grown on nitrate alone.

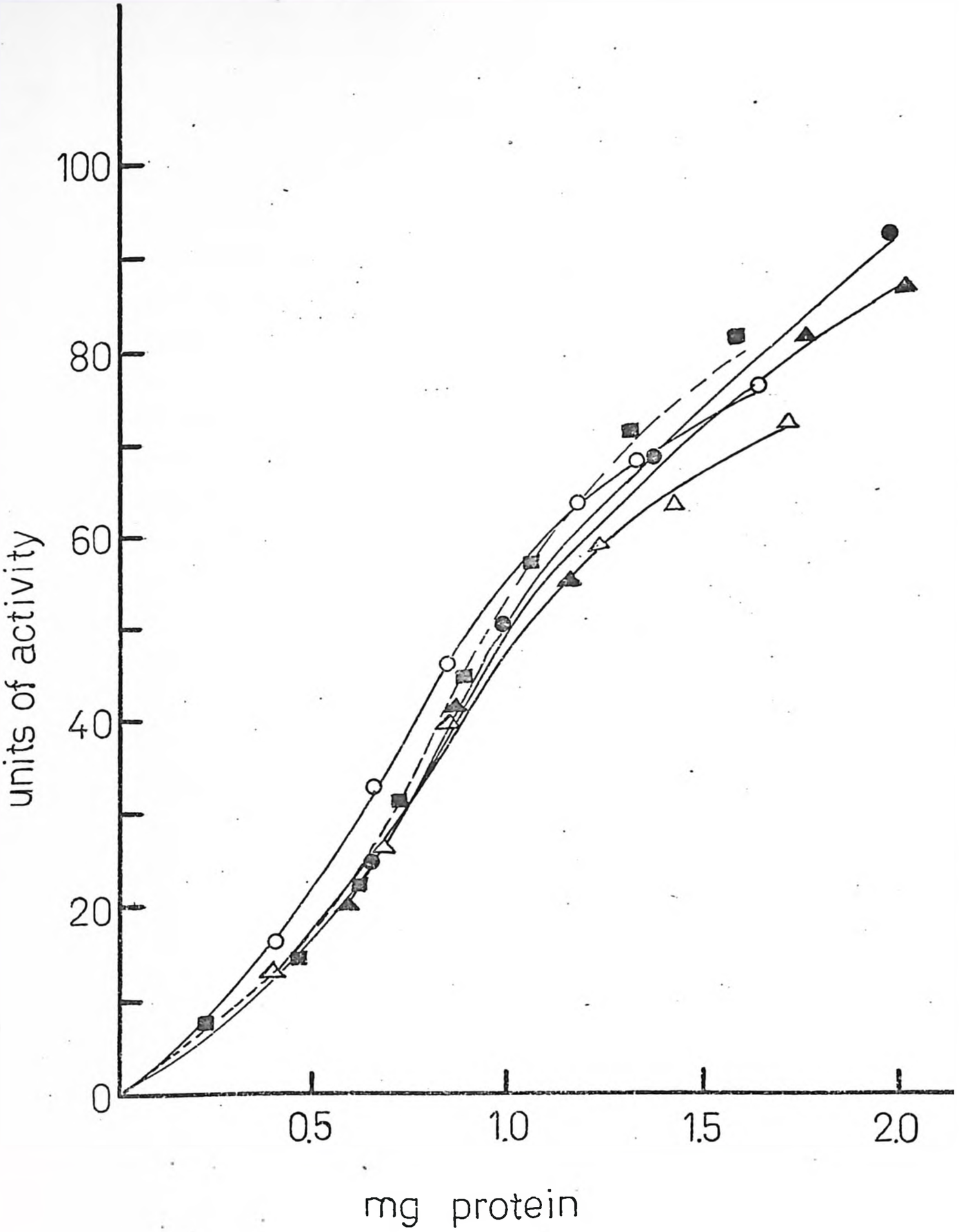
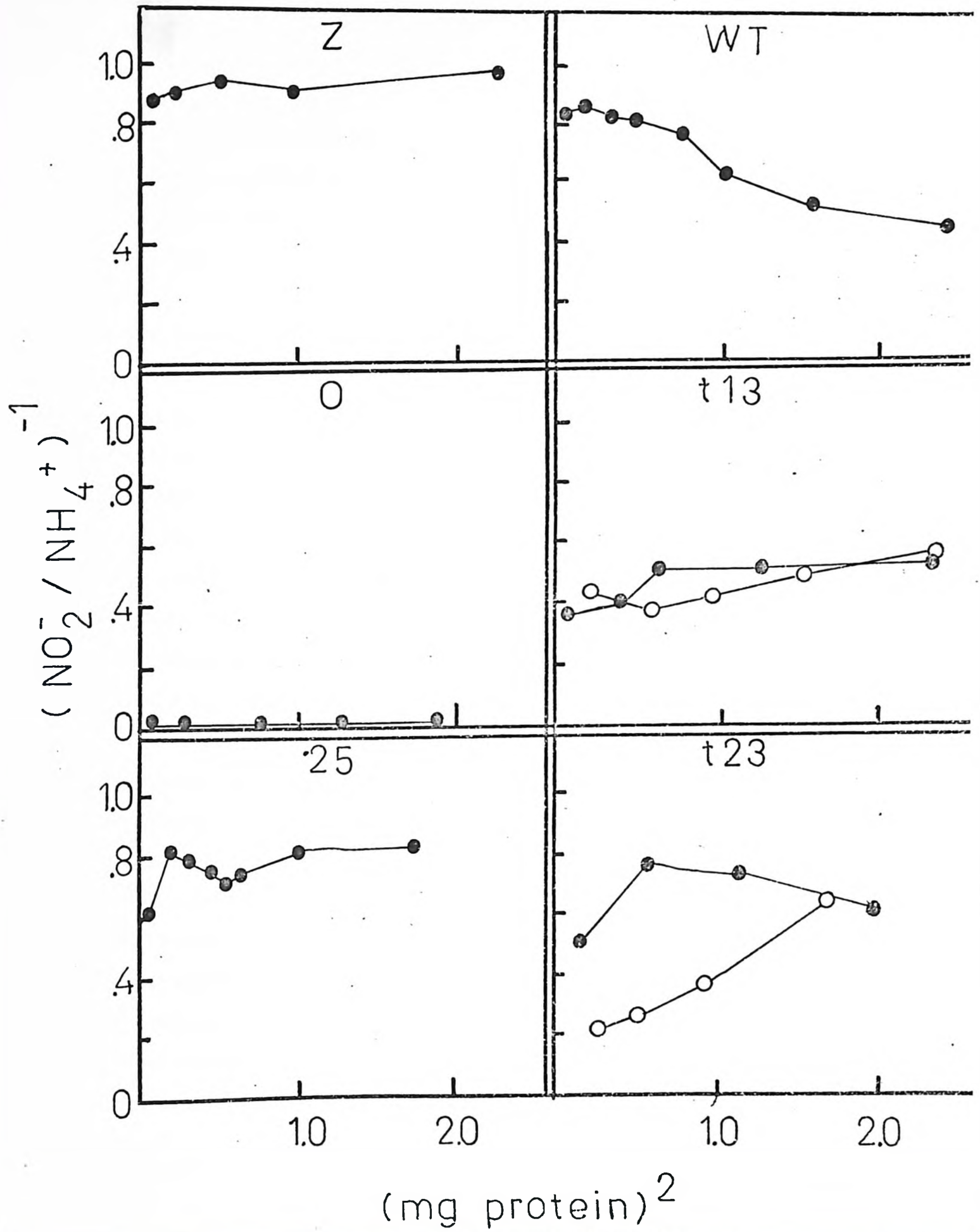

100
80
60
40
20
0
units of activity
0.5
1.0
1.5
2.0
mg protein

Fig. 18.

Summary table of the effect of protein concentration squared on the reciprocal of the ratio of N/A obtained from extracts of mutants Z, O, 25, t-13 and t-23.

Wild type results are included for comparison. ●—● represents results with t-13 and t-23 grown at 27°C, and O—O represents results with t-13 and t-23 grown at 37°C.


Z
WT
O
t13
25
t23
1.0
.8
.4
0
1.0
2.0
$(NO_2^- / NH_4^+)^{-1}$
$(mg\ protein)^2$

(d) If there are more than one nitrite reductase, can they be separated physically?

If there are more than one nitrite reductase, or more than one form of aggregation of nitrite reductase, these might be separable physically. Sedimentation velocity experiments were conducted to look for a multiplicity of nitrite reductase activities. This method was not very satisfactory, due to the limitation of the amount of extract which could be layered onto the gradient. Attempts to concentrate extracts by suction dialysis resulted in a considerable loss of activity after centrifugation through the gradient. The results that were obtained indicated that at least two, and possibly three peaks of nitrite reductase activity could be separated (Figure 19). This result will be further considered in the DISCUSSION.

Column chromatography, using Sephadex Gel was employed, because relatively large quantities of extract could be fractionated.

Column chromatography of S5000 extracts of nitrate-grown mycelia, on Sephadex G-100, resulted in a complete loss of nitrite reductase activity. Addition of bovine serum albumen to fractions collected from the column did not affect this loss in activity. However, the addition of crude S5000 extract to fractions coming off the column following the void volume (post void volume protein)

Fig. 19.

Nitrite reductase activity profile from a sedimentation velocity gradient experiment.

0.5 ml of an S16000 preparation from mycelia grown on a nitrate medium were layered on a gradient as described in METHODS. Centrifugation was allowed to proceed for 18 hours at 39000 RPM and fractions were then collected and assayed. 210 units of nitrite reductase were layered on the gradient and 163 units recovered. The percent recovery was 78%.

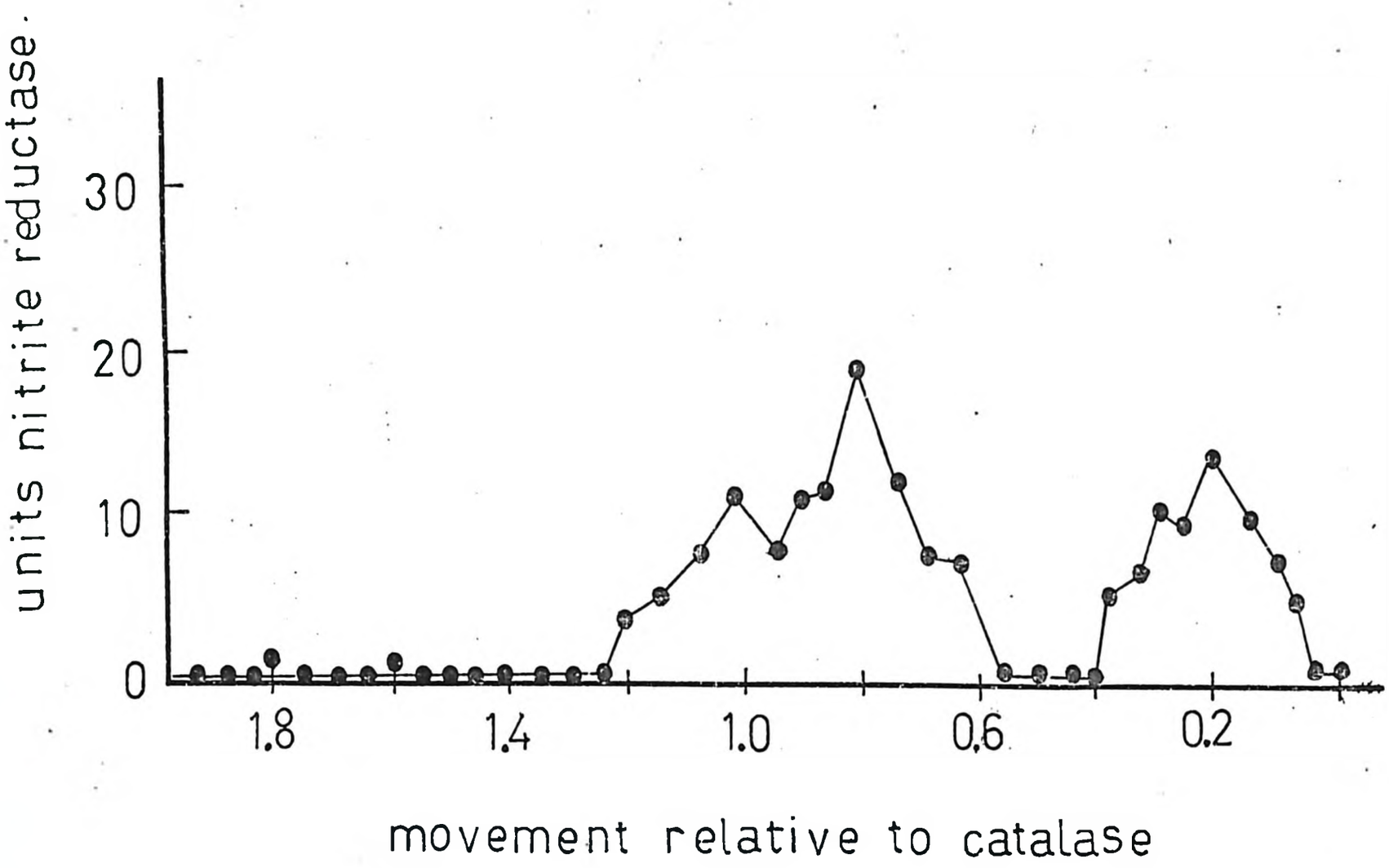

units nitrite reductase.
30
20
10
0
1.8
1.4
1.0
0.6
0.2
movement relative to catalase

Fig. 20.

Nitrite reductase activity of fractions obtained from a Sephadex G-100 column.

3 ml of a S5000 extract from nitrate grown mycelia was placed on a column and eluted as given in METHODS. 34 ml of eluate was collected in bulk as the voil of volume, then fractions of 1.5 ml were collected. Aliquots of the fractions were assayed for nitrite reductase activity O—O ; in the presence of 1.7 mg of S5000 extract protein ●—● ; in the presence of 0.5 mg bovine serum albumin ■—■ . Values obtained for fractions assayed in the presence of crude S5000 extract are corrected for activity present in the crude S5000 extract alone. Cytochrome c, added as marker, was eluted at 83.5 ml. The error in measurement of nitrite reductase activity is 4.8 units.

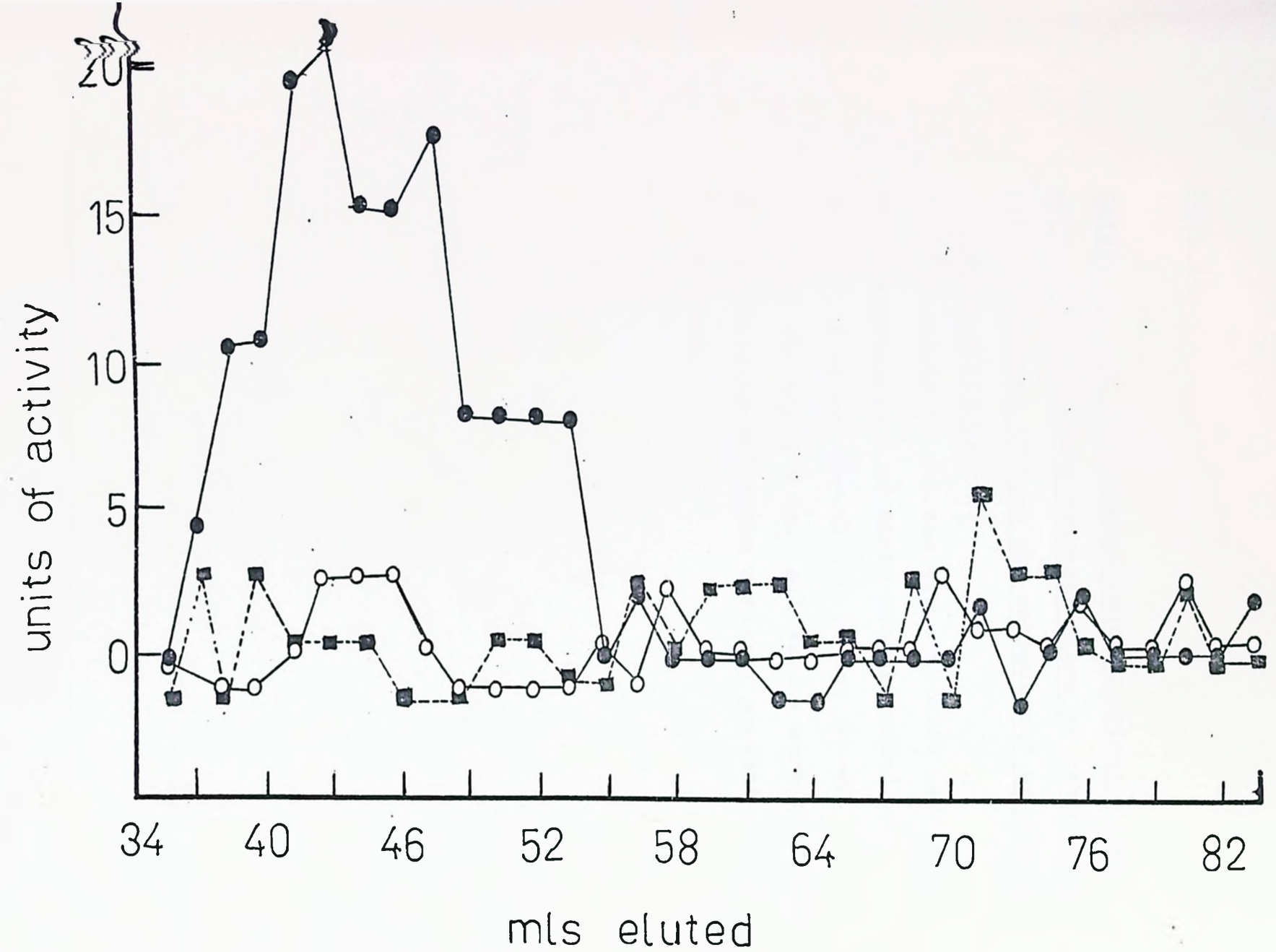

20
15
10
5
0
units of activity
34
40
46
52
58
64
70
76
82
mls eluted

Fig. 21.

Effect of adding different concentrations of PV protein to different concentrations of crude S5000 extract protein (SE) on the enhancement of nitrite reductase activity.

S5000 extracts of nitrate grown mycelia were used. The concentrations of S5000 protein added were: ●—● , 1.74 mg; ○—○ , 0.87 mg; ▲—▲ , 3.48 mg; △—△ , 0 mg. All values were corrected for the nitrite reductase activity present in the S5000 alone.

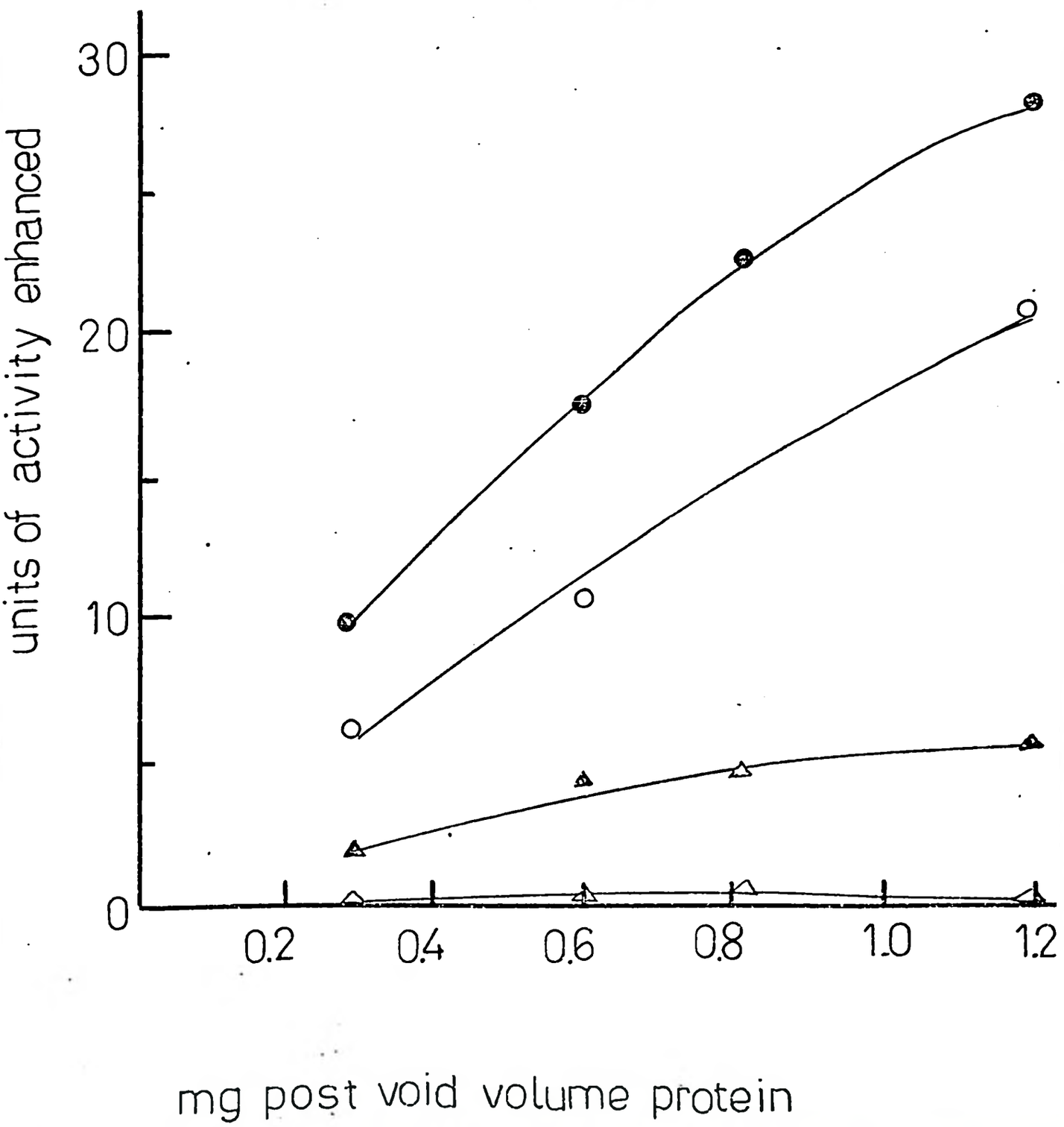

30
20
10
0
units of activity enhanced
0.2
0.4
0.6
0.8
1.0
1.2
mg post void volume protein

Fig. 22.

Effect of the concentration of S5000 extract protein (SE), and VP from extracts of nitrate grown mycelia, on the enhancement of nitrite reductase activity.
SE was obtained from S5000 extracts of mycelia exposed to a nitrate, ammonia or nitrogen-deficient medium. PV was obtained as given in METHODS. 0.9 mg of PV was used in the assays. ●—● represents nitrite reductase activity of PV plus SE and ○—○ represents nitrite reductase activity in the crude S5000 extract alone.

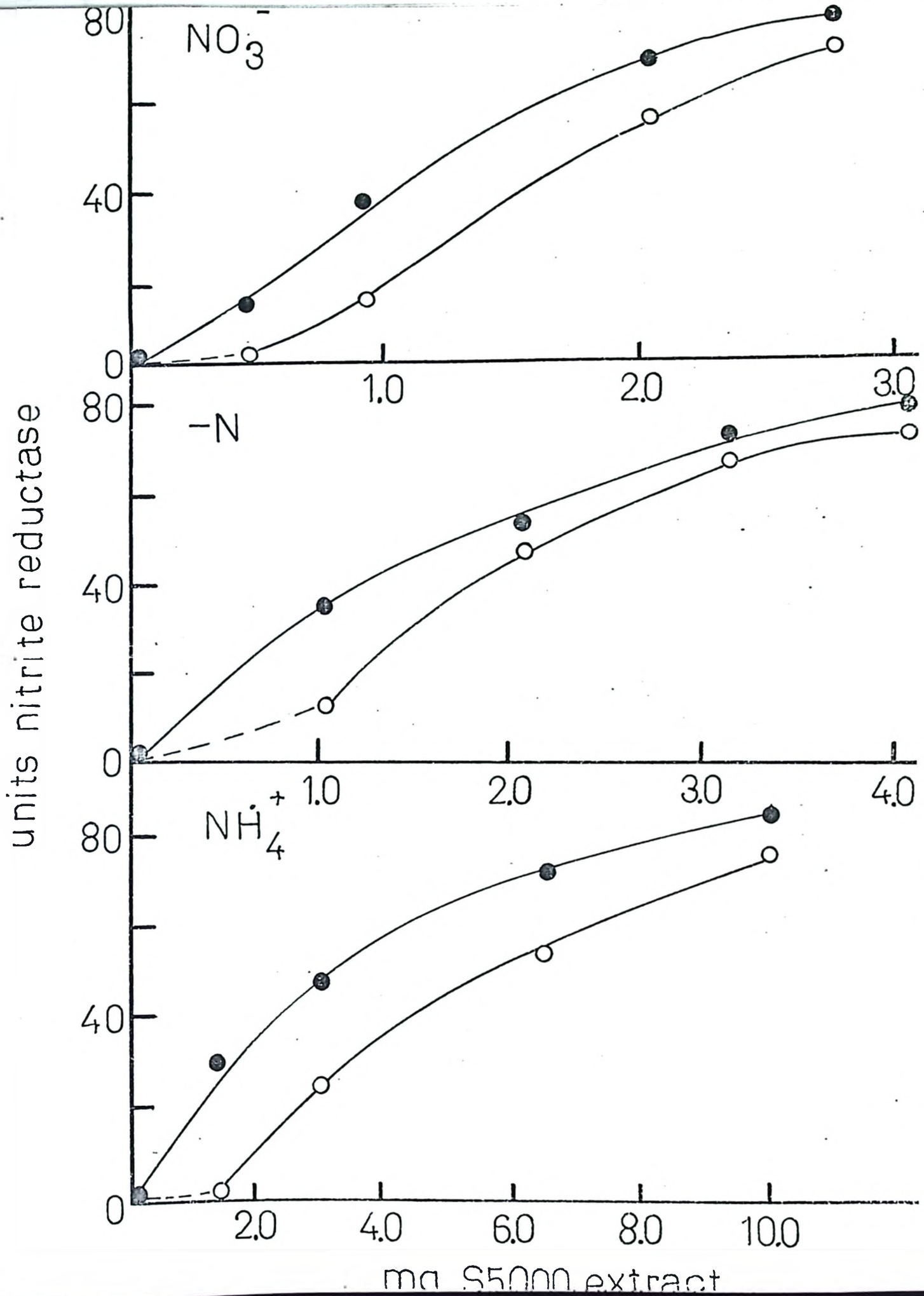

NO_3^-
-N
NH_4^+
units nitrite reductase
mg S5000 extract
0
40
80
1.0
2.0
3.0
4.0
6.0
8.0
10.0

Fig. 23.

Effect of the concentration of S5000 extract protein (SE), and VP from extracts of mycelia exposed to a nitrogen-deficient medium, on the enhancement of nitrite reductase activity.

SE was obtained from S5000 extracts of mycelia exposed to a nitrate, ammonia or nitrogen-deficient medium. PV was obtained as given in METHODS. 1.2 mg of PV was used in the assays. ●——● represents nitrite reductase activity of PV plus SE and O——O represents nitrite reductase activity in the crude S5000 extract alone.

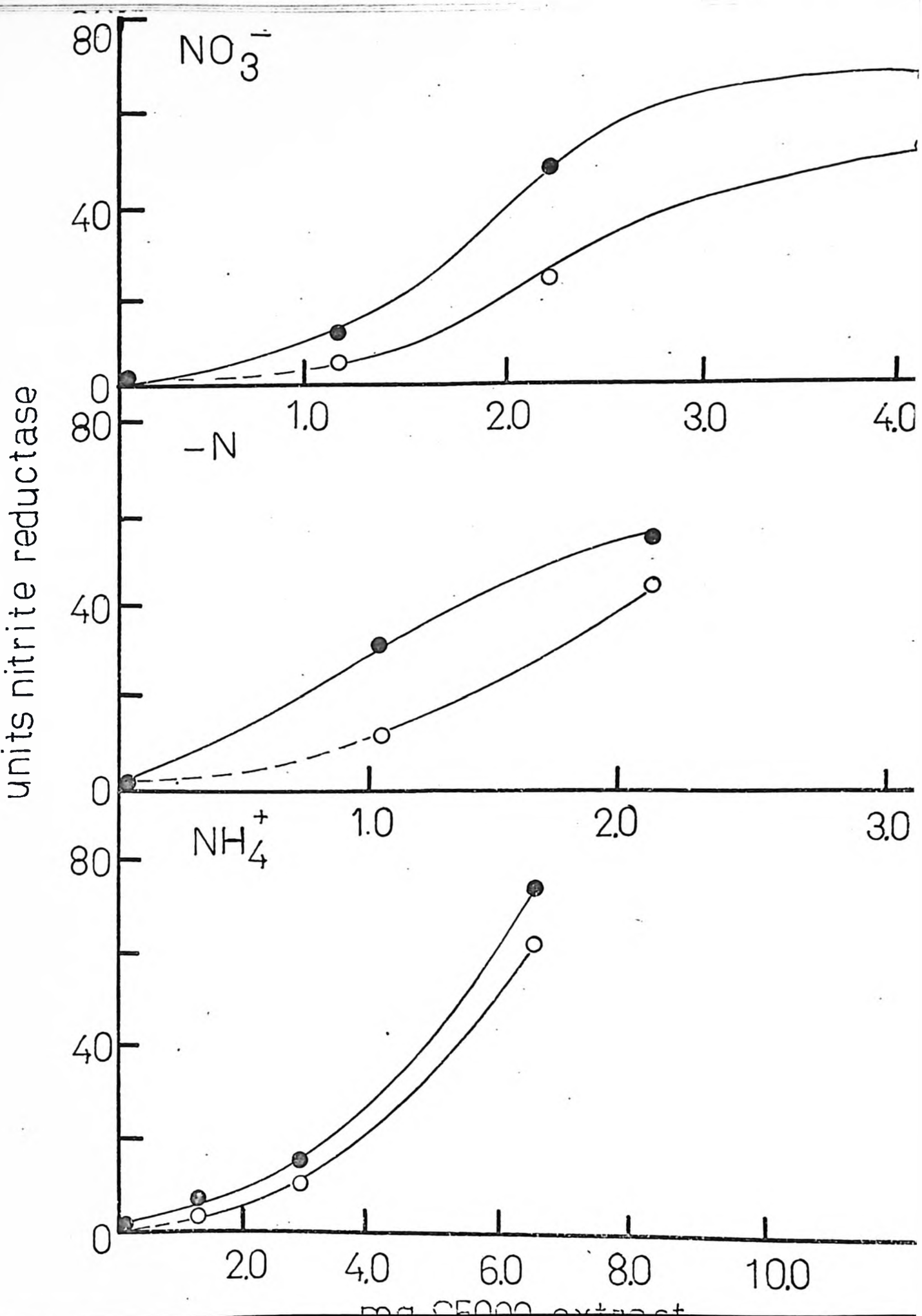
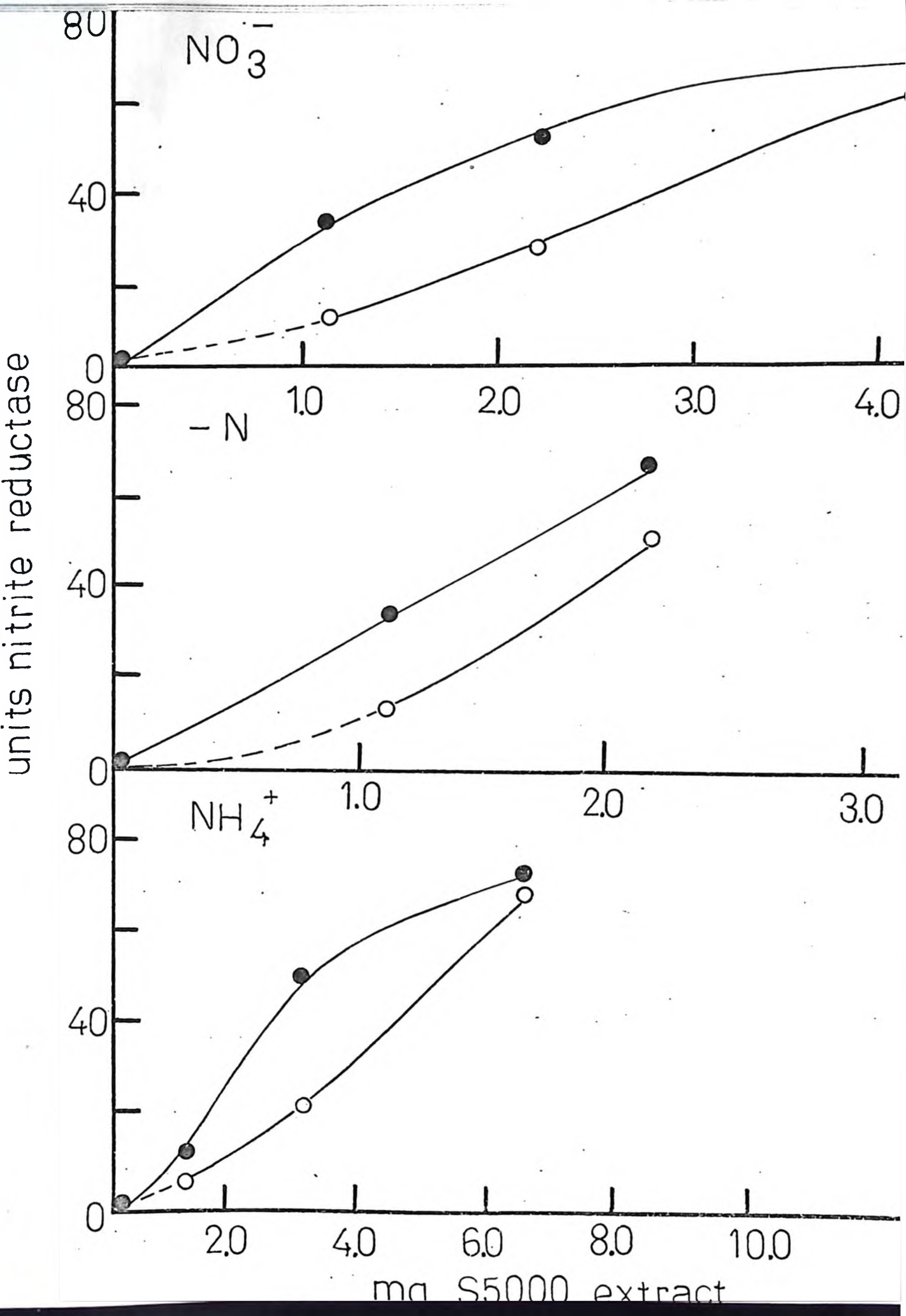

NO_3^-
80
40
0
1.0
2.0
3.0
4.0
-N
80
40
0
1.0
2.0
3.0
NH_4^+
80
40
0
2.0
4.0
6.0
8.0
10.0
units nitrite reductase

Fig. 24.

Effect of the concentration of S5000 extract protein (SE), and VP from extracts of ammonia - grown mycelia, on the enhancement of nitrite reductase activity.
SE was obtained from S5000 extracts of mycelia exposed to a nitrate, ammonia or nitrogen-deficient medium. PV was obtained as given in the METHODS. 2.5 mg of PV was used in the assays. ●—● represents nitrite reductase activity of PV plus SE and ○—○ represents nitrite reductase activity in the crude S5000 extract alone.


80
40
0
NO_3^-
1.0
2.0
3.0
4.0
80
40
0
– N
1.0
2.0
3.0
80
40
0
NH_4^+
2.0
4.0
6.0
8.0
10.0
units nitrite reductase
mg S5000 extract

resulted in an enhancement of the nitrite reductase activity present in S5000 (Figure 20).

Two questions were asked as a result of this finding: (1) What is the nature of the protein(s) present in the post void volume protein fraction (PV) which enhances the nitrite reductase activity in crude S5000 extracts, and (2) What is the nature of the molecule(s) in crude S5000 extracts which is enhanced (SE)?

Enhancement of nitrite reductase is dependent on the concentration of PV, up to 0.8 mg of PV protein (Figure 21). The enhancement is also dependent on the concentration of protein in the S5000 extract, and thus, by inference, on the concentration of SE (Figure 21). The enhancement is less pronounced when relatively high concentrations of SE are used (e.g., 3.48 mg) than when 1.74 mg of SE are employed. This result reflects the fact that at 3.48 mg, activity is being measured at an S5000 extract protein concentration, beyond linear range (see Figure 5). PV appears to overcome a dilution effect exhibited by the activity in the S5000 extract.

It was of interest to know if SE and PV were present in extracts of mycelia grown on a nitrate, ammonia, or nitrogen-deficient medium. This was found to be the case (Figures 22, 23, and 24). The results suggest that there may not be similar concentrations of PV from the

different nitrogen sources.

The question was whether the molecule(s) present in SE, which is (are) enhanced, is (are) protein. SE was stable after two hours of dialysis (Table 9) and sensitive to heat-treatment (Table 10). These results suggest that SE is a heat-sensitive macromolecule which may be protein in nature.

It is difficult to conclude anything concrete from these results. However, they do suggest that nitrite reductase contains at least one protein, which dissociates during fractionation and is necessary for nitrite reductase activity. The presence of PV as well as SE in extracts of mycelia exposed to a nitrate, ammonia or nitrogen-deficient medium suggests that there is at least one component in common to the nitrite reductase(s) from all three sources.

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Table 9

Effect of Dialysis of Crude S5000 Extracts, from which SE is Obtained, on the Enhancement of Nitrite Reductase Activity

PV was obtained from S5000 extracts of nitrate-grown mycelia subjected to column-chromatography. SE was from S5000 crude extracts of nitrate-grown mycelia. Dialysis was carried out as given in METHODS.

Treatment	mg Crude S5000 Extract Protein In Assay (SE)	mg Post Void Volume Protein In Assay (PV)	Units Nitrite Reductase (Total)	Units Nitrite Reductase Enhanced
Untreated	2.8	1.2	62.4	9.6
	1.4	1.2	36.0	26.4
	0	1.2	0	
	2.8	0	52.8	
	1.4	0	9.6	
Dialyzed	2.4	1.2	36.0	4.8
	1.2	1.2	26.4	26.4
	0	1.2	0	
	2.4	0	31.2	
	1.2	0	0	

Table 10

Effect of Heat-Treatment of Crude S5000 Extracts from Which SE is Obtained on the enhancement of Nitrite Reductase Activity

PV was obtained from S5000 extracts of nitrate-grown mycelia subjected to column chromatography. SE was from S5000 crude extracts of nitrate-grown mycelia. The S5000 crude extract (SE) was heated at 100°C for five minutes.

Treatment of SE	mg S5000 Crude Extract Protein In Assay (SE)	mg Post Void Volume Protein In Assay (PV)	Units Nitrite Reductase (Total)	Units Nitrite Reductase Enhanced
Untreated	2.4	1.8	42.0	13.2
	0	1.8	0	
	2.4	0	28.8	
Heated	2.4	1.8	4.8	0
	0	1.8	0	
	2.4	0	4.8	

DISCUSSION

The results presented in Section I demonstrate that nitrite reductase is not localized in mitochondria. They do not rule out the possibility that nitrite reductase might be associated with mitochondria, *in vivo*, and that the extraction procedure used, results in a dissociation. A factor was found to be present in the Lubrol wx treated mitochondrial fraction (P16000) which enhances the nitrite reductase activity present in the supernatant (S16000) fraction. What relationship this factor has to *in vivo* nitrite reduction is not clear.

Ammonia was identified as a product of nitrite reduction. Ammonia production depended on the presence of benzyl viologen in the reaction mixture, although some ammonia production did occur in the absence of the dye.

The nitrite reduced to ammonia produced ratios (N/A) differed in reactions catalyzed by S5000 and S16000 extracts. Although the reason for this difference was not uncovered, it is possible that under the conditions of the S5000 extraction procedure the conformation of the enzyme is disrupted in such a way that maximum ammonia production from nitrite does not take place. The enzyme in S5000 extracts

desalted in the presence of sucrose-EDTA, would have a restored configuration, a configuration which may exist when the enzyme is extracted by S16000. These observations explain why Cook was unable to measure ammonia production in his assay.

The S16000 extraction procedure was found to be the preferred method for the preparation of nitrite reductase. The N/A ratio with S16000 was lower than with S5000 and the specific activity of nitrite reductase was higher in S16000 than in S5000. The relationship between nitrite reduction or ammonia production and S16000 protein concentration exhibited a dilution effect. The data could be replotted to obtain a straight line relationship by expressing the S16000 protein concentration as mg protein squared. The dilution effect may be suggestive of an aggregation of protein necessary for nitrite reduction and ammonia production, and the correction by squaring the protein concentration may suggest a 2x polymer.

The N/A ratio increased with increasing S16000 protein concentration, the effect being most marked at protein concentrations greater than 0.8 mg. This result could be interpreted in two manners:

(1) that nitrite reductase is a polymeric protein that can exist in two states, one of the forms (a_x) catalyzes the production of ammonia from nitrite; the other form (a_y) reduces

nitrite to another unidentified product.

(2) a second interpretation is that the relatively high N/A ratio, obtained at S16000 protein concentrations of greater than 0.8 mg, is the result of at least two separate enzymes catalyzing the reduction of nitrite. One enzyme would catalyze the reduction of nitrite to ammonia and the other, which exhibits a dilution effect of 0.8 mg protein, would catalyze the production of a product other than ammonia.

Both enzymes or aggregates must be necessary for normal growth on nitrate and on nitrite. The mutations in O and Z each affect one of the enzyme activities, and both are adversely affected in their growth on nitrate and on nitrite.

t-13 is mutated at a different genetic locus from Z or O. This fact suggests that a third protein must be involved in nitrite reduction. The nitrite reductase activities, each represented by the activity present in extracts of Z or O, both appear to be affected in t-13 since the N/A ratio is intermediate between that observed with Z and O. The stoichiometry of nitrite reduction catalyzed by extracts of t-13 suggests two molecules of nitrite yield one molecule of ammonia.

The affected protein in t-13 may be common to both enzymes or it may be a regulatory protein which controls

the equilibrium between aggregates a_x and a_y.

Cook previously postulated that nitrite reductase was a derepressible enzyme (3,4); that is, the absence of a repressor molecule, rather than the presence of an inducer molecule, was necessary for the synthesis of the enzyme. He also found that a casamino acids digest did not completely repress nitrite reductase (3,4).

The rates of increase of the specific activity of nitrite reductase in extracts of mycelia exposed to a nitrate or a nitrogen-deficient medium were essentially the same; the initial rate was greater in the case of mycelia grown on nitrate. This result may indicate that nitrate or nitrite play a role in the appearance of active nitrite reductase. It was found, confirming Cook's observation, that ammonia did not completely repress nitrite reductase.

When extracts of mycelia, previously exposed to a nitrogen-deficient medium, were assayed in the presence of ammonia, it was found that the specific activity of nitrite reductase was comparable to that observed in extracts of mycelia exposed to ammonia. When extracts of ammonia-grown mycelia were desalted on Sephadex G-10 the specific activity of nitrite reductase increased considerably and the activity became sensitive to ammonia *in vitro*. Nitrite reductase activity in extracts of mycelia grown on nitrate were only slightly sensitive to

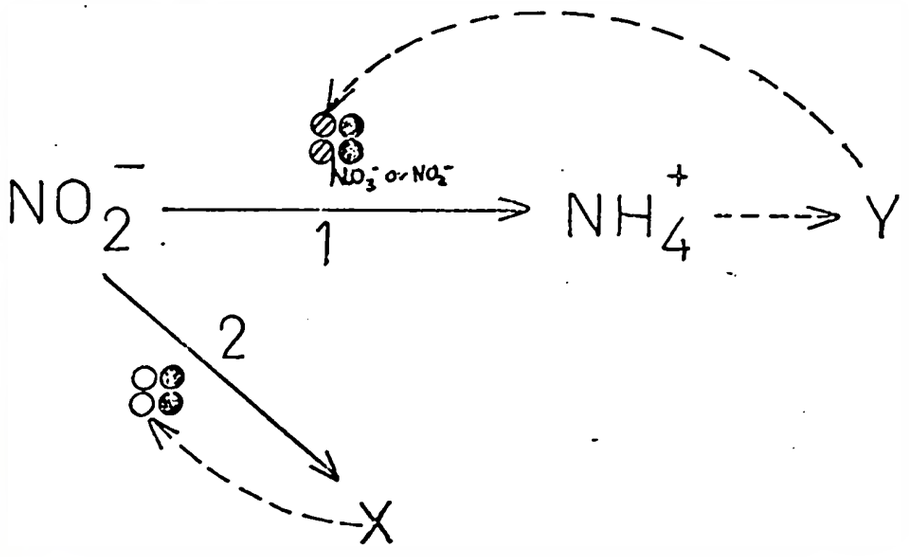
ammonia. From the foregoing, it would appear that the enzyme(s) present in extracts of mycelia exposed to ammonia and to a nitrogen-deficient medium may be similar or identical. The observed differences in specific activity of crude extracts would arise as a result of differences in intracellular concentrations of an inhibitor, which could be ammonia.

It is difficult to explain the catalysis of the production of low levels of ammonia from nitrite by extracts of ammonia-grown mycelia and by extracts of mycelia exposed to a nitrogen-deficient medium. This result could be explained by invoking the existence, in such extracts, of either two enzymes or two forms of aggregation of an enzyme responsible for nitrite reduction. In such extracts that enzyme or aggregate which does reduce nitrite to a product other than ammonia would be in greater abundance than that enzyme or aggregate which reduces nitrite to ammonia.

The model illustrated is suggested to explain the following:

1. Nitrite is not reduced stoichiometrically to ammonia.
2. At least three proteins are necessary for *in vivo* nitrite reduction.
3. Nitrate affects the initial rate of appearance of nitrite reductase activity.

4. Ammonia inhibits nitrite reductase activity from extracts of mycelia exposed to a nitrogen-deficient medium and from desalted extracts of mycelia grown on ammonia. Ammonia inhibits the activity present in extracts of nitrate-grown mycelia only slightly.
5. Sedimentation velocity gradient experiments resulted in the separation of at least two peaks of nitrite reductase activity.
6. An enzymatically inactive protein present in fractions eluted from a Sephadex G-100 column appears to enhance the nitrite reductase activity of S5000 crude extracts of mycelia exposed to any of the three mediums used for growth (i.e., ammonia, nitrate or nitrogen-deficient).



Model Used to Explain the Presence of Two Nitrite Reductase Activities in *Neurospora crassa*

It is suggested that the reduction of nitrite can be catalyzed by two different enzymes: enzyme 1 and enzyme 2. Ammonia is the product of one reaction and a postulated compound, X, is the product of the other. Both of these activities are necessary for growth on nitrate and on nitrite, and thus, the products X and ammonia are important to the cell.

Mutant Z would be affected in the ○ protein and mutant O would be affected in ◎ protein. A common protein, ● which is affected in t-13 would be necessary for both activities.

It is suggested that ammonia is not the inhibitor of nitrite reductase activity *in vivo*, but that the compound X, which would be similar in structure to ammonia, and may be an amine, is the true inhibitor of enzyme 2. This reasoning might explain why a high concentration of ammonia is necessary to completely inhibit nitrite reductase activity, *in vitro*. It is possible that enzyme 1 is under a feedback type of control. It is postulated that a compound, Y, a product of ammonia assimilation, would inhibit this activity. This inhibition would only take place if enzyme 1 were in the associated state, in the presence of nitrate or nitrite.

The presence of nitrate or nitrite would be necessary for full enzyme 1 activity. In the absence of nitrite or nitrate the proteins would be dissociated.

Both enzymes would be derepressible and would be present in extracts of mycelia exposed to a nitrate, ammonia or a nitrogen-deficient medium, assuming that ammonia is not the repressor. Differences in specific activities from these three sources would be due to:

1. the presence or absence of inhibitors, and
2. the presence or absence of nitrate or nitrite.

Each enzyme may be a dimer with each monomer composed of two proteins. This would be consistent with the dilution effect noted for each activity (Figure 4).

Sedimentation velocity gradient experiments suggested the presence of two nitrite reductases in S16000 extracts of nitrate-grown mycelia. However, there was an indication of a third peak. It is possible that at least one of the dimers is partially dissociated into monomers, during the centrifugation, and that the monomers can reduce nitrite.

Column chromatography of S5000 extracts on Sephadex G-100 may result in the complete dissociation of either or both enzyme 1 and 2. It is possible that the S5000 extraction procedure results in at least the partial dissociation of enzyme 1. Thus, the enhancement obtained when PV is added to SE, could be due to a re-association of proteins, which were inactive in the dissociated state.

This model places nitrite reductase in perspective

with the nitrate assimilatory pathway. Nitrate reductase, the first enzyme in the pathway is not feedback inhibited by ammonia and the only control which appears to be in effect is repression by ammonia. A control mechanism may be desirable, then, to channel the nitrite which is being produced by nitrate reductase into a compound other than ammonia. This would effectively decrease the amino acid pool. Under normal nitrate assimilatory conditions, ammonia would be the main product formed, but an amount of X would also be made. If the pool of Y, assuming ammonia is at least indirectly incorporated into Y, becomes too large, enzyme 1 could be inhibited so that nitrite reduction would now proceed via the enzyme 2 pathway. Similarily, if the pool of X (or a metabolite of X) became too large, inhibition of enzyme 2 by X (or a metabolite of X) could result in a switch to the catalysis of nitrite reduction to ammonia. In either case, nitrite, which is toxic, would not accumulate.

A number of questions arise from this model:

1. What are X and Y?
2. Is nitrite reductase derepressible, and if so, what is the natural repressor?
3. How does X fit into other metabolic pathways?

The techniques used in this study to separate nitrite reductase activities were not satisfactory. Further

work, with separation and purification techniques, must clarify the presence of two enzyme activities.

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APPENDIX

Isolation and Testing of Mutant 25

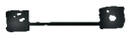
Mutant 25 was the only strain selected for testing. Mutant 25 grew on a casamino acids and ammonia medium like the wild type strain. Although the mutant did grow to some extent on nitrate and nitrite, the rate of growth was much slower than that of the wild type, indicating that 25 has a defect in the utilization of nitrite. Figures 25 and 26 give the growth patterns of wild type and mutant 25 on different nitrogen sources.

Fig. 25.

Growth curves of *pan-2B-36A* on different nitrogen sources at 27°C.

Casamino acids 

Ammonium tartrate 

Potassium nitrate 

Sodium nitrite 

Filter-sterilized sodium nitrite (0.5 g/l) was added to neutralized medium.

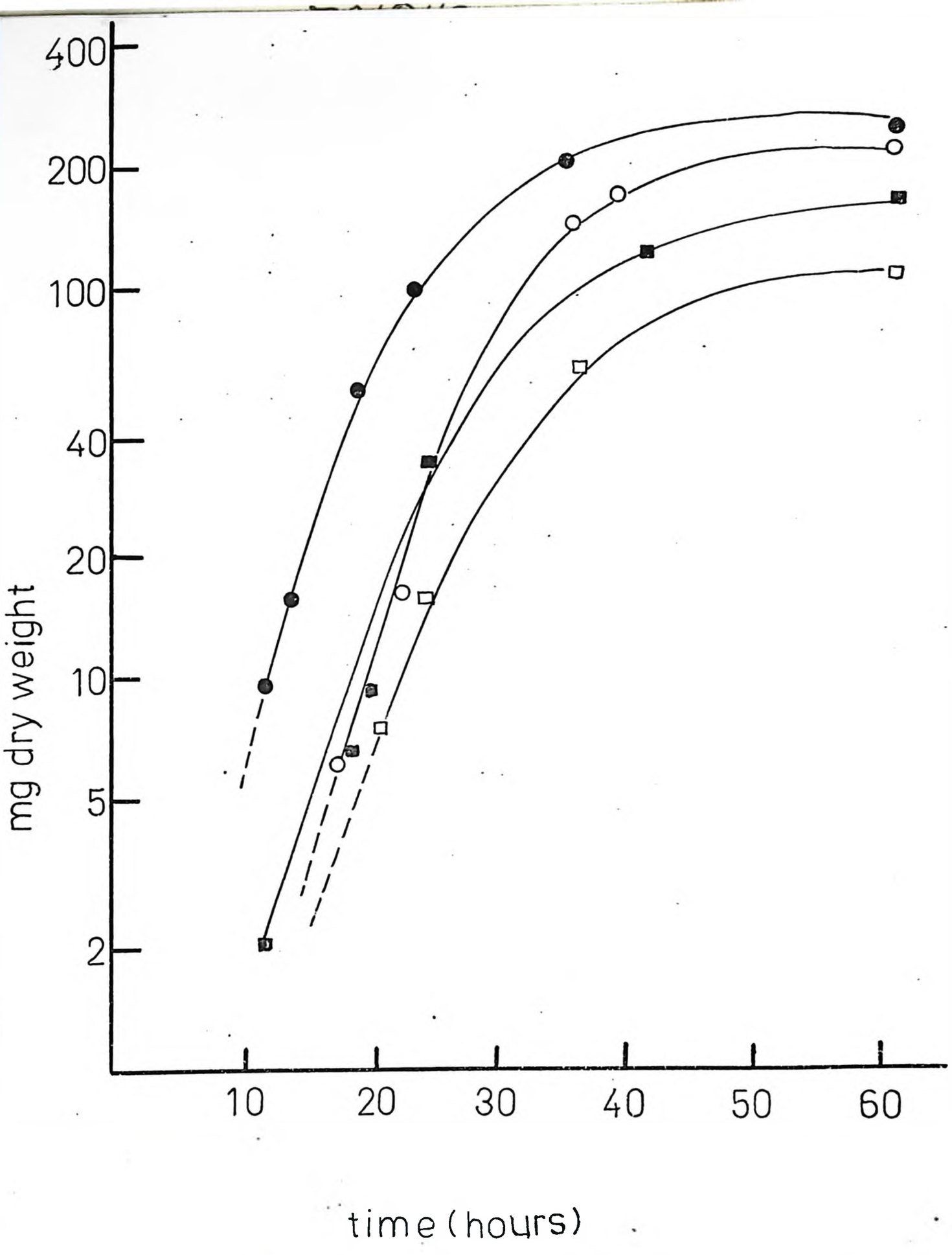
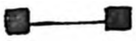

400
200
100
40
20
10
5
2
mg dry weight
10
20
30
40
50
60
time (hours)

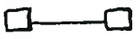
Fig. 26.

Growth curves of mutant 25 on different nitrogen sources at 27°C.

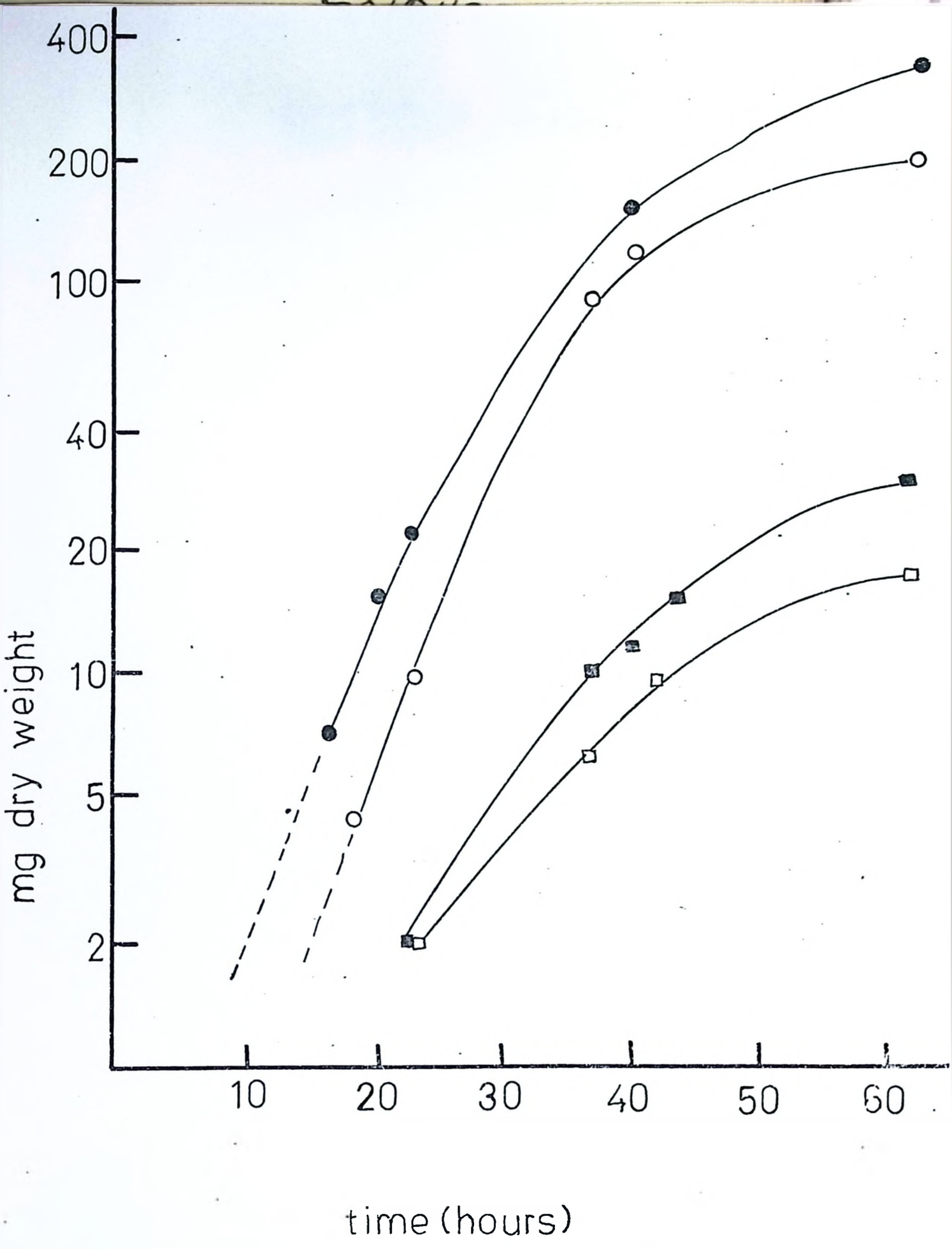
Casamino acids 

Ammonium tartrate 

Potassium nitrate 

Sodium nitrite 

Filter-sterilized sodium nitrite (0.5 g/l) was added to neutralized medium.


400
200
100
40
20
10
5
2
mg dry weight
10
20
30
40
50
60
time (hours)