NITRITE REDUCTASE IN *NEUROSPORA CRASSA*:

A GENETIC AND BIOCHEMICAL STUDY

NITRITE REDUCTASE IN *NEUROSPORA CRASSA*: A GENETIC AND BIOCHEMICAL STUDY

by

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SCOPE AND CONTENTS:

Nitrite is an intermediate in the pathway of nitrate assimilation. Several questions about reduction beyond the level of nitrite remain to be answered.

The purpose of this investigation was to induce mutants deficient in nitrite reduction and to characterize these mutants phenotypically and genetically in an attempt to answer the following questions:

(a) How many enzymes are required *in vivo* for nitrite reduction?

(b) How many genes control nitrite reduction?

(c) Is nitrite reductase localized within a particle?

The results of this investigation showed that nitrite reductase is controlled by at least three genes and three cistrons on two linkage groups. None of the 'nitrite-mutants' were allelic with nitrate reductase mutants. There appeared to be more than one type of nitrite reductase activity in extracts of repressed wild type mycelia. Only one of these nitrite reductase species seemed to be necessary for the reduction of nitrite *in vivo*.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

INTRODUCTION

Green plants and many microorganisms are capable of synthesizing their nitrogenous compounds from inorganic nitrogen. Other forms of life must fulfil their nitrogen requirements from exogenous supplies of organic nitrogen. Organisms which can assimilate inorganic nitrogen thus provide the nitrogen in utilizable form to chemoorganotrophs.

There are two types of nitrate reduction: (a) nitrate respiration or dissimilatory nitrate reduction, in which nitrate replaces oxygen as the terminal electron acceptor in the respiratory electron transport chain, and (b) assimilatory nitrate reduction, in which nitrate is reduced to ammonia or amino level for the biosynthesis of cell constituents. It is with the second type of nitrate reduction that this study is concerned.

A proposed pathway of nitrate reduction to ammonia is shown in Fig. 1.

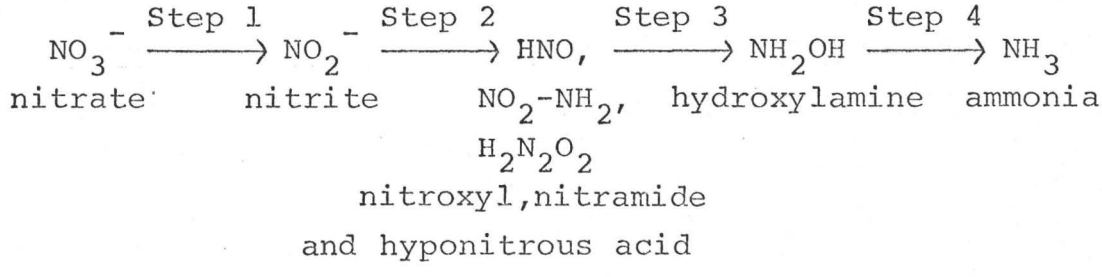


Fig. 1. A possible pathway for the reduction of nitrate (39).

Each step involves a two-electron transfer. The first step in this pathway, the reduction of nitrate to nitrite by nitrate reductase has been well studied (38,39, 49,60,61). The enzyme which catalyses the second step would logically be called nitrite reductase. However, the pathway and the intermediates beyond the level of nitrite remain uncertain. Nitrite reductase will here be used to refer to the enzyme which catalyses the reduction of nitrite to ammonia.

Assay of Nitrite Reductase: Electron donors and cofactors.

Nitrite reductase activity was first demonstrated by Yamagata in 1939 in cell free preparations of Pseudomonas aeruginosa. The enzyme has subsequently been detected in a variety of other organisms: Bacillus pumilis (64), Azotobacter agile (62), Neurospora crassa (9,35,40,41) and Glycine max (9,40).

A metalloflavoprotein, capable of reducing nitrite to ammonia in the presence of NADH or NADPH was reported in partially purified extracts of Glycine max and Neurospora crassa (40). Significant amounts of ammonia were produced even when nitrite was absent from the assay mixture. It is unlikely that this activity could have been due to endogenous nitrate and nitrite because of the purification procedure used.

Vadyanthan and Street (65) found that extracts of tomato roots catalysed the disappearance of nitrite in the

presence of NADH, FMN and Mn, but only 2% of the nitrite lost was recovered as ammonia.

Roussos and Nason (53) measured the nitrite-dependent oxidation of NADH in extracts from Glycine max but were unable to show either nitrite loss or ammonia production.

Medina and Nicholas (35) assayed nitrite reductase as the nitrite-dependent production of ammonia with NADH as electron donor and flavin as cofactor. They claimed that nitrite reductase in Neurospora was a flavoprotein which was dependent on Fe^{++} and Cu^{++} for maximum activity. In a later report, Nicholas et al. (44) showed that nitrite reductase activity in extracts of Neurospora crassa mycelia deficient in Cu, Fe, Mg or Zn was significantly depressed. No indication was given whether these metal effects were specific.

Spencer, Takahashi and Nason (62) reported a nitrite reductase from Azotobacter agile which used NADPH as electron donor and FAD as cofactor. Good stoichiometry was demonstrated between nitrite loss and ammonia production but no controls were included to detect how much ammonia production occurred in the absence of added nitrite.

Hageman et al. (11) and Cresswell (7) showed that extracts of nitrite reductase from Cucurbita pepo could reduce nitrite to ammonia using either reduced benzyl viologen or NADH and catalytic amounts of the dye as electron

donors. No reduction occurred with NADH or NADPH in the absence of the dye. The flavin requirement and response to Mn^{++} observed by other workers was not shown. Hageman et al. suggested that benzyl viologen was substituting for an unidentified natural cofactor.

Sanderson and Cocking (55), using the benzyl viologen assay of Hageman, showed with tomato leaf extracts that ammonia was the product of nitrite reduction. These workers observed that when NADH was present in the reaction mixture it was necessary to make a correction for the ammonia resulting from NADH oxidation. On the basis of this observation and their own more detailed results, Sanderson and Cocking (55) have pointed out the error in earlier assays which estimated pyridine nucleotide-dependent nitrite reductase activity by ammonia production. Ammonia is generally measured by the Conway microdiffusion technique (72). Reaction mixtures treated with strong alkali are placed in the outer well of a Conway microdiffusion dish. The unit is then sealed. Ammonia, made volatile by the alkali treatment, diffuses into acid in the inner well of the Conway unit and is estimated colorimetrically or by titration. NADH, NADPH, $NADP^+$ and NAD^+ are all partially degraded by alkali to release ammonia. $NAD(P)^+$ is significantly more labile than NAD(P)H. Thus the ammonia production reported by Nason et al. (40) probably resulted from $NAD(P)^+$ degradation rather than from nitrite reduction.

The validity of the reports of other workers (35,38,44,43, 67) who estimated nitrite reductase in this way is now open to question for the above reason.

FAD and manganous ions stimulate pyridine nucleotide oxidase. This effect could explain the observed stimulation of ammonia production by these substances.

Sanderson and Cocking (55) tested FAD, FMN, $MnCl_2$, vitamin K_3, reduced glutathione and sodium ascorbate in a nitrite reductase assay system with NAD(P)H as electron donor. No nitrite loss occurred in any of these tests. Sanderson and Cocking (55) and Cresswell (7) concluded that reduced pyridine nucleotides did not function as direct electron donors in nitrite reduction in higher plants.

Joy and Hageman (21) assayed nitrite reductase in extracts of Spinacea oleracea and Zea mays using dithionite and benzyl viologen as electron donors. Nitrite was reduced stoichiometrically to ammonia. No nitrite reductase activity was observed when reduced FAD was used as electron donor.

A new assay for nitrite reductase in Neurospora crassa was recently developed by Cook (4,5). This assay was based on that of Joy (21). Specific activities of nitrite reductase were greater than those reported by previous workers.

There are a number of reports on the reduction of nitrite by illuminated chloroplasts (2,12,14,17,18,21,23,28,

45,46,51,55,66). The reduction of nitrite in photochemical systems requires the presence of ferredoxin and illuminated grana as electron donors, and nitrite reductase.

The postulated electron pathways involved in nitrite reduction in vitro can be summarized as follows:

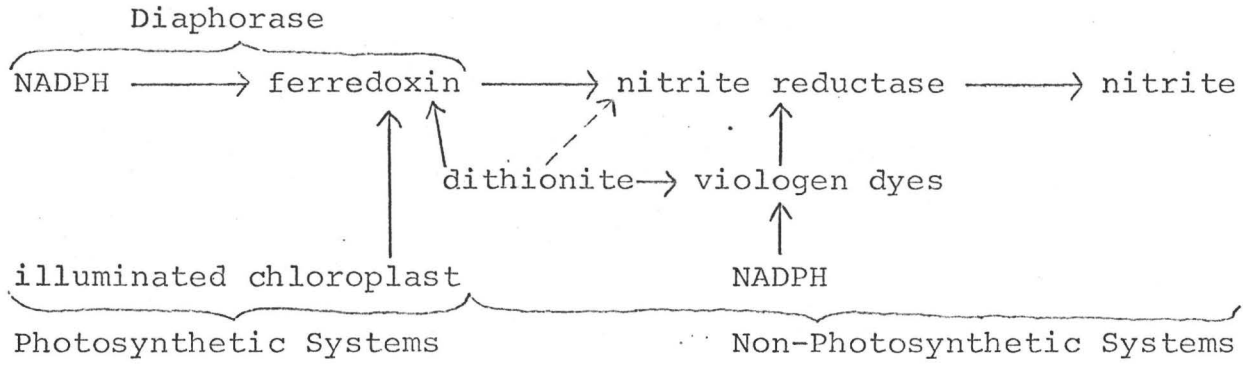


Fig. 2: Schematic representation of electron transport systems in nitrite reductase (13,21, modified by C.D.)

Intermediates.

The intermediates between nitrite and ammonia are unknown. Those which have been postulated are unstable or toxic or both.

The early experiments of Nicholas et al. (35,44) suggested a two electron step leading from nitrite to hyponitrite. This product was not demonstrated. Vadyanthan and Street (65) found that extracts of tomato roots catalyzed the disappearance of hyponitrite and hydroxylamine in the presence of NADH, nicotinamide, FMN and Mn. However, only 2% of the hyponitrite lost and 30 to 35% of the hydroxylamine

lost was recovered as ammonia.

Fewson and Nicholas (10) purified a nitric oxide reductase from Pseudomonas aeruginosa and claimed that nitric oxide was an intermediate in the pathway of nitrate assimilation. Nitric oxide is very unstable and would be an unlikely free intermediate.

Role of Hydroxylamine; Multiplicity of Hydroxylamine Reductase Enzymes

Hydroxylamine has been detected in nutrient media and has been reduced to ammonia in vivo by a number of organisms (58,39). These observations implicated hydroxylamine as a possible intermediate in the reduction of nitrite to ammonia. Enzymes capable of catalysing the hydroxylamine-dependent oxidation of reduced pyridine nucleotides have been studied in preparations of Salmonella typhimurium (56), Escherichia coli (31,26,22), Azotobacter agile (62), Neurospora crassa (70) and Glycine max (53).

Cresswell found that a nitrite reductase from Cucurbita pepo was able to reduce nitrite to ammonia at a much faster rate than it could reduce hydroxylamine under identical conditions. Studies with extracts of Escherichia coli (26) and Spinacea oleracea (2) also showed that the rate of reduction of nitrite always exceded the reduction of hydroxylamine. The combined data of kinetics, Km values for both substrates, and inhibition effects of nitrite on hydroxylamine reductase were regarded as evidence that

hydroxylamine was not a free intermediate in the reduction of nitrite to ammonia. This argument would not necessarily be valid if hydroxylamine reductase and nitrite reductase were separate enzymes. Additional evidence that free hydroxylamine was not an obligate intermediate in the reduction of nitrite was obtained by Lazzarini (26). His studies with nitrite reductase in extracts of E. coli showed that when N^{15} labelled nitrite was reduced in the presence of unlabelled hydroxylamine, the ammonia present at the end of the experiment was heavily labelled, while residual hydroxylamine was labelled only to a small extent.

Kemp (22) and Mager (31) have presented evidence suggesting that in Escherichia coli, the reduction of hydroxylamine to ammonia and the reduction of sulphite to hydrogen sulphide are functions of the same enzyme systems. Hydroxylamine reductase and sulphite reductase were both repressed by cysteine, an end product of sulphite metabolism. Both activities were absent in extracts of sulphite non-utilizing mutants (22). Similar results were obtained in Salmonella typhimurium by Siegel (56). The sulphite non-utilizing mutants described in these studies were able to grow on nitrate. These results suggested that sulphite reduction was the physiological function of the enzyme, whereas the reduction of hydroxylamine was an incidental capacity of the same enzyme system.

Zucker (69) isolated a flavoprotein from Neurospora crassa which catalyzed the hydroxylamine-dependent oxidation of NADH. Since this enzyme was also demonstrated in extracts of mycelia of nitrate- and nitrite-non-utilizing mutants, it is difficult to explain how this enzyme could play a major role in nitrite utilization.

Three species of hydroxylamine reductase were separated when extracts of Neurospora crassa (27,57) were centrifuged through sucrose density gradients. One of the hydroxylamine reductases (species A) was identical with sulphite reductase, and specifically required NADPH as electron donor. The second hydroxylamine reductase (species B) catalyzed the hydroxylamine-dependent oxidation of either NADH or NADPH. This enzyme was adaptive to the presence of nitrate in the medium and its activity was stimulated by FAD. The third hydroxylamine reductase (species C) could also accept electrons from either NADPH or NADH. It was present in low levels in all Neurospora extracts examined. Species B and C were present in extracts of a sulphite-non-utilizing mutant which lacked both sulphite reductase and species A of hydroxylamine reductase activity. It would seem therefore, that species B and C were not associated with sulphite reductase, whereas species A was identical with it.

Hewitt (15,16) showed two major hydroxylamine reductase activities in extracts of Cucurbita pepo and

Spinacea oleracea which were separable by Sephadex gel filtration (G100 and G200). These hydroxylamine reductases had different molecular weights and different electron donor specificities. One of these, HR-1, was associated with nitrite reductase and used Fd, FMN and reduced benzyl viologen as electron donors. The other major species of hydroxylamine reductase, HR-2, accepted electrons from reduced benzyl viologen but not from physiological donors. Hewitt et al. (16) have suggested that the multiple hydroxylamine reductases may be involved in some sort of metabolic control.

Multiplicity of Nitrite Reductase Enzymes

There is some evidence that some organisms may have more than one nitrite reductase. Czygan (8) 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.

Organic Pathway

The studies described above all assume an inorganic pathway for the reduction of nitrite to ammonia, but the possible existence of other pathways cannot be excluded.

The presence of nitrite and hydroxylamine reductases in extracts of nitrite-non-utilizing mutants of Neurospora crassa (58,70) can be explained by the existence of an

alternative pathway. Silver and McElroy found that extracts of Neurospora 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 differences were only observed 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.

The isolation of Neurospora mutants which required pyridoxine for growth on nitrate but not for growth on ammonia led Silver and McElroy (58) to postulate an organic pathway leading from hydroxylamine through pyridoxal oxime phosphate and pyridoxamine phosphate to amino nitrogen. A pyridoxine requiring mutant of Neurospora crassa employed by Nicholas (44) also required pyridoxine when grown on ammonia. Nicholas (44) reported that the nitrite reductase in crude extracts of the 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 (4) was unable to show a response of nitrite reductase activity to pyridoxine in his assay.

Organization and Localization of Enzymes Involved in Nitrite Reduction

Sims (59) proposed that individual enzymes for nitrate reduction in Candida utilis are organized in an oligomeric complex called a nitrosome. This nitrosome is believed to be capable of carrying out the reduction of nitrate or nitrite to ammonia without the formation of free intermediates (59). This idea was supported by the following evidence: (a) The extremely low level of detectable intermediates (e.g. hydroxylamine) suggested that the reduction of nitrite was catalyzed by a single enzyme complex (or aggregate) and that intermediates were not released. (b) The Km of hydroxylamine reductase for its substrate was very high. If fast rates of reduction of nitrite to ammonia were to be maintained in a system of separate enzymes therefore high concentrations of hydroxylamine would be expected. (c) The nitrate reducing enzyme system could be fractionated on agarose columns into species which were capable of reducing nitrate and nitrite to ammonia. The molecular weights of these components ranged from 65,000 to 500,000. Another species of molecular weight 17,000 was only able to reduce nitrate

and may represent a subunit dissociated from the complex. (d) Compounds which inhibited ammonia production from nitrate effected nitrite and hydroxylamine reductases equally.

Ritenour (52) showed that nitrite reductase in leaf tissue was localized within chloroplasts. (Nitrate reductase was shown to be a soluble enzyme.) This report has been confirmed by several other workers (2,51,28,46). It is also in agreement with a report by Schraeder et al. (71) that chloroamphenicol inhibited the synthesis of nitrite reductase but not nitrate reductase in Zea mays seedlings. (Chloramphenicol binds to mitochondrial and chloroplast ribosomes but has no effect on cytoplasmic ribosomes.) Miflin's work (36,70) indicates that nitrite reductase in barley preparations is associated with mitochondria. Since nitrate and nitrite reductases in higher plants do not have the same intracellular localization, nitrosomes probably do not function here.

A nitrosome may not function in Neurospora since here nitrate and nitrite reductases are different enzymes. It is not known however whether the enzymes are aggregated. A single enzyme could be responsible for the reduction of nitrite to ammonia. The localization of nitrite reductase in Neurospora has not been studied.

Regulation of Nitrite Reductase

Investigations into the regulation of nitrite

reductase have not been attempted until recently because of the lack of a suitable assay system.

There is evidence that nitrite reductase is repressed by ammonia. Pateman (67) observed that the specific activity of nitrite reductase in extracts of Aspergillus nidulans grown on ammonia was lower than it was in extracts of the mold grown on nitrate. Sims (59) found that ammonia completely repressed nitrite reductase in Candida utilis extracts but this effect was believed to be due to an increase in the concentration of amino acids and nucleotides made available by ammonia assimilation. Changes in the level of nitrite reductase were shown to be directly correlated with changes in the size of the total amino acids pool. Cook (4,5) demonstrated that the nitrite reductase of Neurospora crassa was partially repressed when the organism was grown in medium containing casamino acid digest as nitrogen source.

Pateman (47) claimed that nitrite reductase was induced by nitrate and nitrite because levels of nitrite reductase activity were higher in extracts of Aspergillus grown on urea plus nitrate or plus nitrite than in extracts of the mold grown on urea alone. Nitrite reductase levels in preparations of mycelia exposed to nitrogen deficient media were not included in these studies. A derepressible nitrite reductase, which requires the absence of repressor molecules rather than the presence of an inducer molecule

for its formation, would also be consistent with these results. The nitrite reductase assay used by these workers measured the nitrite-dependent oxidation without the nitrite itself being reduced (53).

Ingle (19) compared nitrite reductase levels in extracts of Raphanus sativus cotyledons grown for five days on various nitrogen sources, with nitrite reductase levels in preparations of cotyledons grown for the same time in nitrogen deficient media. Sims (59) observed that cell free homogenates of nitrogen-starved Candida utilis had only minute amounts of nitrite reductase. It was concluded (19,59) that nitrite reductase was induced by nitrate and nitrite. It seems doubtful that nitrogen-starved cells would have sufficient nitrogen reserves for the synthesis of new enzymes.

Cook (4,5) reported evidence suggesting that nitrite reductase in Neurospora crassa is a derepressible rather than an inducible enzyme. Maximum levels of nitrite reductase were obtained when Neurospora was exposed to nitrogen deficient medium.

Sims (59) reported a wide range of compounds that inhibited nitrite reductase in vitro in extracts of Candida utilis: ammonia, most amino acids and nucleotides, pyridoxine phosphate, and carbamyl phosphate. However, at the concentrations at which these substances occurred in the cell, only partial inhibition of the nitrosomal activity was

demonstrated.

Nitrate reductaseless (Nia) mutants of Aspergillus nidulans (6,48) synthesized nitrite and hydroxylamine reductases constitutively. Mutants at a regulatory gene (nr^c) which were constitutive with respect to nitrate reductase synthesis were also isolated. The constitutive (nr^c) mutants and the wild type strain were grown on urea with and without nitrate and the levels of nitrite reductase activity in their extracts compared. The specific activity of nitrite reductase in extracts of nr^c mutants grown on urea without nitrate was 50% of that of preparations of wild type grown on urea plus nitrate. The specific activity of mutants grown on urea plus nitrate was two to three times greater than that of cell free homogenates of wild type grown on nitrate. They proposed that a substance produced by the regulatory gene is converted into a repressor by ammonia or by nitrate reductase when the enzyme is not complexed with nitrate. This repressor prevents expression of the structural genes for nitrate reductase and nitrite reductase. When nitrate reductase is complexed with nitrate, it interferes with the conversion of the substance to a repressor.

Cook (4,5) has evidence that there may be a constitutive as well as a repressible nitrite reductase in Neurospora crassa. The low levels of nitrite reductase activity observed in extracts of repressed mycelia (that is, mycelia grown on casamino acids digest as nitrogen source)

can be interpreted in several ways.

(a) This activity represents a constitutive enzyme in Neurospora crassa,

(b) This activity could be due to another enzyme that can reduce nitrite in vitro but which is unrelated to in vivo nitrite reduction,

(c) Nitrite reductase cannot be completely repressed by the components of the casamino acids digest. Attempts to find a specific co-repressor molecule were unsuccessful.

Further studies in this and other systems are required before a conclusion about the mechanism for the regulation of nitrite reductase can be made.

Genetic Studies

The demonstration that an enzyme is capable of catalyzing a reaction does not necessarily mean that the enzyme performs the same function in vivo. A logical method of determining whether an enzyme activity is representative of an in vivo function is to study mutants defective in that function.

Silver and McElroy examined extracts of nitrite-non-utilizing mutants (58,73) for nitrite reductase activity. All except one of the mutants tested had nitrite reductase activity. Two mutants were excluded from the survey because of the high concentration of nitrite present in their extracts. The level of nitrate reductase in extracts of

these two mutants was higher than in extracts of the wild type strain and may explain the nitrite accumulation.

These investigators combined nitrite-non-utilizing mutants of Neurospora in heterokaryons. They concluded that five genes controlled nitrite reduction because five of the 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. (49) isolated mutants at two gene loci (nii A and nii B) which could not grow on nitrite and lacked nitrite reductase activity. Extracts of mutants at the nii A locus had normal nitrate reductase activity but lacked both nitrite and hydroxylamine reductase activities. Extracts of mutants in the nii B locus had low levels of all three activities. It is interesting to note that of forty five nitrite-non-utilizing mutants, none were singly affected in an independent structural gene for nitrite or hydroxylamine reductase. Pateman (47) regarded this as evidence that a single enzyme reduced nitrite to ammonia. The nitrite reductase assay used here measured the nitrite dependent oxidation of NADH. Nitrite-stimulated oxidation of NADH is known to occur without the concomitant reduction of nitrite (53).

Summary

It has been shown in a number of organisms and with a variety of assays that nitrite is reduced to ammonia possibly by a single enzyme. Nitrite reductase in Neurospora crassa appears to be a derepressible enzyme (4,5).

The following questions remain to be answered:

(a) Does the nitrite reductase assay which uses dithionite and benzyl viologen as electron donors measure an in vivo activity?

(b) How many enzymes are required for the reduction of nitrite in vivo?

(c) How many genes control the reduction of nitrite?

(d) What is the natural cofactor(s) in non-photosynthetic systems of nitrite reduction?

(e) What are the intermediates between nitrite and ammonia?

Purpose of This Project

The object of this project was to examine mutants defective in nitrite utilization in an attempt to answer the following questions:

(a) How many enzymes are required for the reduction of nitrite to ammonia in vivo?

(b) How many genes control nitrite reduction?

(c) Is nitrite reductase localized within an organelle?

Abbreviations

EDTA, disodium ethylene diamine, tetraacetate.

Nitrosoguanidine, or NG, N, methyl N' nitro-N'-nitrosoguanidine.

Benzyl viologen or BV, N, N' dibenzyl-4-dipyridylium dichloride.

TMPD, N, N, N'N tetramethyl-p-phenylene diamine.

NAD^+, NADH, Nicotinamide adenine dinucleotide oxidized and reduced forms respectively.

$NADP^+$, NADPH, Nicotinamide adenine dinucleotide phosphate oxidized and reduced forms respectively.

FMN, flavin mononucleotide.

FAD, flavin adenine dinucleotide.

METHODS AND MATERIALS

Fungal Strains

A pantothenic acid-requiring strain of *Neurospora crassa*, pan-2-B-36A was used as the "wild type". Strain 467 ad pan-2-a was used for backcrossing newly isolated mutants. Nitrate reductase-less mutants, referred to as nit mutants, were obtained from Dr. G. J. Sorger's stock collection (60). All strains were in St. Lawrence background.

Media

The basic medium was that described by Sorger and Giles (60). The nitrogen source was either 4 g/l NH_4Cl (*Ammonia Medium*), 5 g/l vitamin-free casamino acids (Difco) (*Casamino acids Medium*), 5 g/l KNO_3 (*Nitrate medium*), or 0.5 g/l $NaNO_2$ (*Nitrite medium*). The nitrite solution used in the *Nitrite medium* was filter sterilized before it was added to neutralized nitrogen free basic medium. *Ammonium-Nitrate Medium* consisted of basic medium plus 1 g/l NH_4Cl and 10 g/l KNO_3. *Ammonia Plating Medium* was composed of *Ammonia Medium* with 0.5 g/l dextrose, 0.5 g/l fructose and 10 g/l sorbose as carbon source. Solidification was achieved by the addition of 1 1/2% (w/v) agar (Difco). Plating media for complementation studies contained 2% (w/v) sucrose as carbon source. Slants were made from solidified

Casamino acids Medium. Supplements were added where necessary in the following concentrations: calcium pantothenate, 20 µg/ml; adenine, 100 µg/ml and histidine, 100 µg/ml.

Mutagenesis and Selection of Nitrite-Non-Utilizing Mutants

Suspensions containing 10^8 conidia/ml of Nitrate Medium were incubated without shaking, in the dark, at room temperature, for two hours, in the presence of 20 µg/ml final concentration of nitrosoguanidine. This treatment resulted in 10% conidial survival (Fig. 3). The conidial suspension was then centrifuged at 3000 g for 30 minutes and the conidia resuspended in pantothenate-supplemented Nitrate Medium. The culture was subsequently incubated on a rotary shaker and subjected to filtration enrichment (68). Wild type spores can germinate in Nitrate medium while nitrate-non-utilizing spores cannot. When 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 nitrate had been removed by repeated filtration, the remaining conidia in the final filtrate were plated on pantothenate-supplemented Ammonia Medium. Colonies from these plates were isolated into tubes of liquid pantothenate-supplemented Ammonia Medium and after conidiation were tested for the ability to grow on liquid pantothenate supplemented Nitrate Medium. Cultures were incubated in tubes at 27°C or 37°C for three days. Wild type, pan-2-B36A, and mutant

nit-1 were included for comparison. Strains which grew on ammonia but grew poorly on nitrate were kept as presumptive mutants for further analysis.

Physiological Tests:

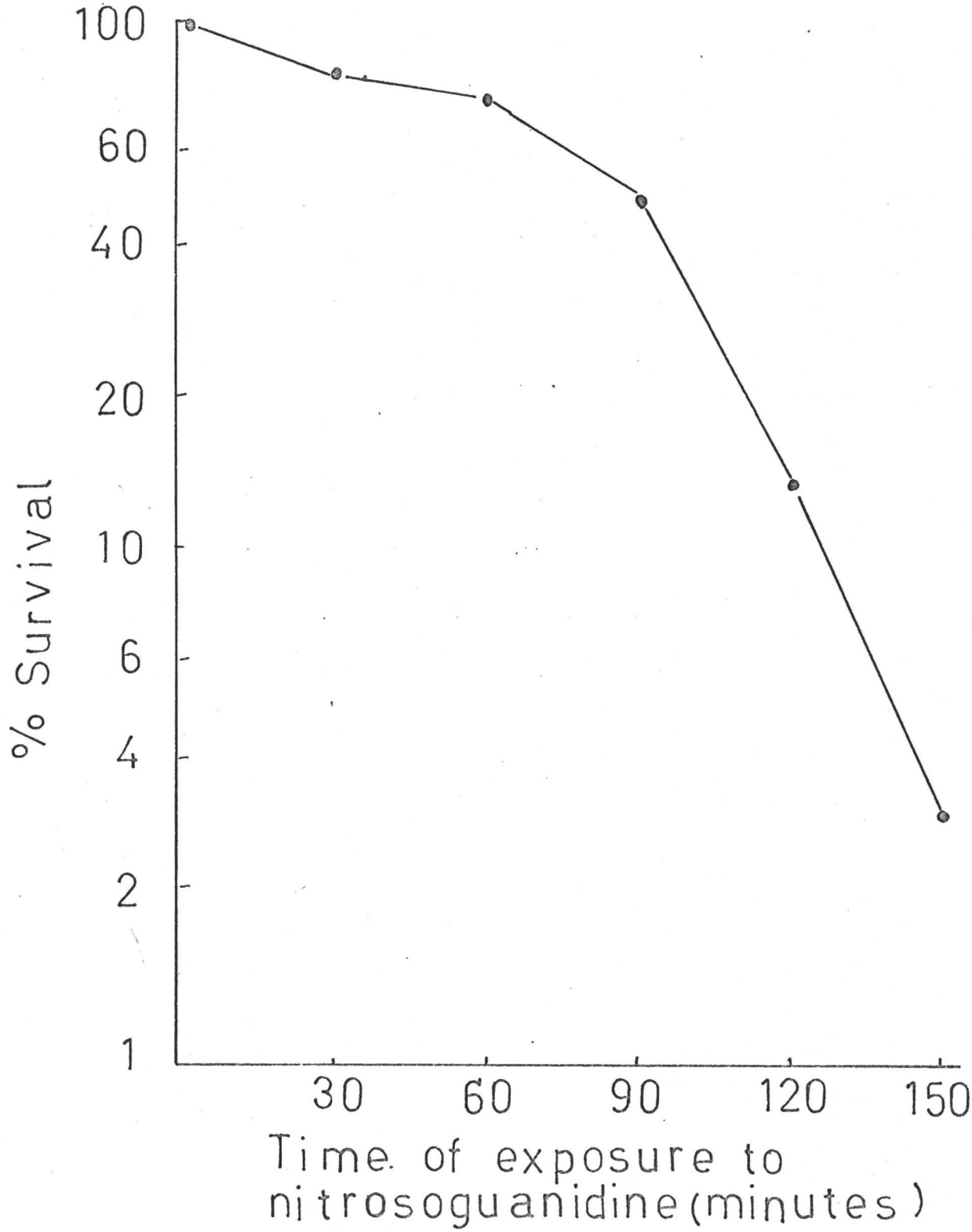
A. Estimation of Nitrite Accumulation

Aliquots of conidial suspensions were pipetted into 250 ml flasks containing 50 ml of Ammonium-Nitrate Medium. The flasks were incubated on a rotary shaker at 27°C or 37°C. At appropriate times 0.1 ml samples were removed from the incubation flasks and pipetted into 7.9 ml of distilled water. Nitrite was determined by a modification of the diazo-coupling method of Sanderson (55). One ml each of 1% (w/v) sulphanilamide in 3.2 M HCl and of 0.02% (w/v) aqueous N-1 napthylethylene diamine dihydrochloride were added to the samples in distilled water. The resulting purple colour was measured after 20 minutes in a Klett-Summerson colorimeter (#54 filter) and the reading related to a standard curve (Fig. 4). In the linear range, one Klett unit is equivalent to 0.4 mμ moles of nitrite.

Large numbers of strains were surveyed for their ability to accumulate nitrite by incubating conidia in small tubes of Ammonium-Nitrate Medium for three days at 27°C. At the end of the incubation period 0.5 ml each of 1% sulphanilamide in 3.2 M HCl and of 0.02% aqueous N-1 napthylethylenediamine dihydrochloride were added to each culture tube and the presence or absence of purple

Fig. 3. Effect of Nitrosoguanidine on the Survival of Conidia.

A suspension of Pan 2-B-36A containing 10^8 conidia per ml was treated with 20 µg/ml (final concentration) of Nitrosoguanidine. At zero time and at intervals thereafter, the treated suspension, diluted appropriately, was pipetted into molten (42°C) Ammonia plating medium and plated. After incubation for two days at 27°C the colonies were counted.


100
60
40
20
10
6
4
2
1
% Survival
30
60
90
120
150
Time of exposure to nitrosoguanidine (minutes)

colour noted.

B. Growth Studies

(a) Semiquantitative.

Selected mutants were surveyed for their ability to grow on various nitrogen sources. Aliquots of conidial suspensions were introduced into 250 ml flasks containing 50 ml of the appropriate medium and incubated on a rotary shaker at 27°C or 37°C for 70 hours. Mycelia from each culture were collected by filtration through a Buchner funnel blotted between paper towels, dried in an oven for 12 hours at 60°C, and weighed on a microbalance.

(b) Quantitative.

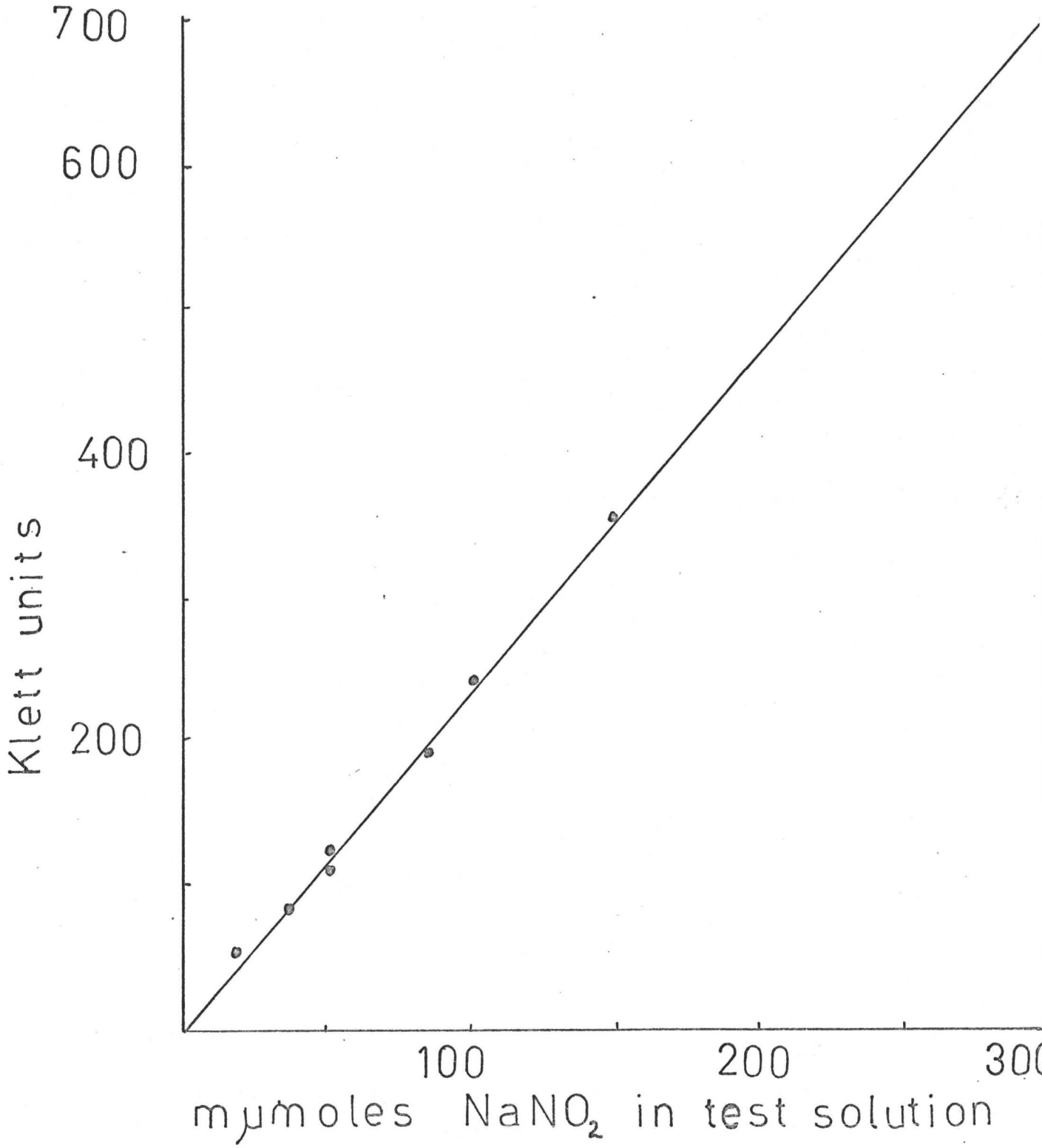
Flasks of liquid media were inoculated and incubated as described above. At appropriate time intervals over a period of 70 hours, cultures were removed and the mycelial dry weights measured as described above.

Repression and Derepression

The procedure of Cook (4,5) was used. Mycelia were grown into stationary phase (approximately 30 hours) in liquid Casamino Acids Medium on a rotary shaker at 27°C or 37°C. Mycelial pads were harvested by filtration through a Buchner funnel, washed with distilled water and cut into sectors. Sectors of wet weight 0.2 g to 0.4 g were transferred either to liquid Nitrate Medium (derepressed cultures) or to liquid Casamino acids Medium (repressed cultures) and incubated at 27°C or 37°C for 15 to 18 hours

Fig. 4. Reference Curve for Nitrite Concentration.

Samples (0.1 ml) of solutions containing different concentrations of $NaNO_2$ were pipetted into 7.9 ml of distilled water. Nitrite was measured as described in the text.


700
600
400
200
Klett units
100
200
300
mμmoles NaNO2 in test solution

on a rotary shaker.

Genetic Methods

A. Crosses

Crosses were made on corn meal agar (Difco) and allowed to mature 4 to 7 weeks before harvesting. Strips of filter paper and 0.05% (w/v) acetate were added to the crossing medium to improve fertility and germination (20). Ripe, black spores were scraped off the sides of the culture tubes, suspended in buffer and counted in a haemocytometer, and introduced into small tubes of 0.1% agar. The spore suspensions in agar were heat shocked for 45 minutes at 60°C and then plated on appropriately supplemented Ammonia Medium. Colonies from these plates were transferred to small tubes of supplemented Casamino acids Medium and incubated at 27°C until conidiation. Conida from these cultures were then examined for their ability to grow on Nitrate Medium and to accumulate nitrite on Ammonium-Nitrate Medium. The total number of recombinants in a cross was calculated as twice the number of wild type spores observed among the progeny of that cross.

B. Complementation

In Neurospora, a heterokaryon is a mycelium containing more than one genetically distinct type of nucleus in a common cytoplasm. Incompatability factors exist which prevent heterokaryon formation. Therefore, forcing markers were used to ensure that heterokaryons were formed.

The nitrite-non-utilizing mutants all required pantothenate. Each of the new mutants was crossed to 467 ad-pan-a, and nitrite-non-utilizing mutants which also required adenine for growth were isolated.

Conidial suspensions of approximately equal optical density were prepared from each pantothenate-requiring nitrite-non-utilizing mutant and from each adenine-requiring, nitrite-non-utilizing mutant. Aliquots of these suspensions were mixed in all pairwise combinations and plated on unsupplemented Ammonia Medium with 2% sucrose as carbon source. Heterokaryons between the two strains were forced on this unsupplemented medium because one type of nucleus supplied the function that the other nucleus lacked. Small sections were cut from the growing points of each heterokaryon and transferred to tubes of liquid unsupplemented Ammonia Medium. Conidia from the preformed heterokaryons were tested for growth on unsupplemented liquid Nitrate Medium and for accumulation of nitrite on unsupplemented liquid Ammonium-Nitrate Medium. Complementation was recorded as positive if growth occurred on nitrate and if there was no nitrite accumulation, and as negative if no growth occurred on nitrate and there was nitrite accumulation.

Determination of Proportions of Nuclear Types in Heterokaryons

Nuclei segregate randomly into conidia. A heterokaryon can thus give rise to homokaryotic vegetative spores. Nuclear ratios were determined by the method of

Prout (49) which is based on the above principle. Conidia from heterokaryons were plated on various types of media:

(a) X medium which supported the growth of heterokaryotic macroconidia and was selective for the growth of macroconidia homokaryotic for one type of nucleus (X).

(b) Y medium which supported the growth of heterokaryotic macroconidia and was selective for the growth of macroconidia homokaryotic for the other type of nucleus (Y).

(c) Unsupplemented media which supported the growth of heterokaryons only.

The plates were incubated at 27°C until colonies appeared. The number of colonies arising from heterokaryotic conidia equals the number of colonies able to grow on unsupplemented media. The number of colonies arising from homokaryotic conidia with type X (or Y) nuclei equals the number of colonies able to grow on type X (Y) medium minus the number of colonies able to grow on unsupplemented medium.

Biochemical Procedures:

Extraction Procedure

Mycelial pads were harvested by filtration through a Buchner funnel, washed with distilled water and pressed manually between paper towels to remove as much moisture as possible. The blotted pad was wrapped in aluminium foil and submerged in liquid nitrogen. The frozen pad was then ground in an ice cold mortar using silica (240 mesh,

Fisher Scientific Co., Fairlawn, New Jersey) as abrasive. When a smooth paste had been formed, three volumes of cold potassium phosphate buffer (0.1 M, pH 7.0) were added. The resulting slurry was centrifuged at 5000 g in a refrigerated centrifuge for 20 minutes. The supernatant designated Crude extract was kept on ice and used within five hours.

Isolation of Mitochondria

The technique of Suyama (63) was used. It was hoped that disruption of mitochondria would be prevented by not using silica as abrasive and by omitting the liquid nitrogen step in the extraction procedure. The pad was ground in an ice cold mortar in three volumes of extraction buffer (potassium phosphate, 0.1 M, pH 7.0) containing 0.3 M sucrose and 0.001 M EDTA. The resulting slurry was centrifuged at 1085 g to remove nuclei and cell debris. The supernatant was then centrifuged at 5000 g for ten minutes. The 'mitochondrial' pellet thus obtained was resuspended in extraction buffer containing 0.3 M sucrose and 0.001 M EDTA, kept on ice, and used within five hours.

Sucrose Density Gradient Analysis

Preparation of Homogenate

The method of Luck (30) was used. The mycelial pad collected as described previously was ground in a mortar with five volumes of potassium phosphate buffer (0.1 M, pH 7.0) containing 0.44 M sucrose and 0.001 M EDTA. The resulting slurry was centrifuged at 500 g to remove nuclei

and cell debris. The mitochondria were in the supernatant.

Preparation of Continuous Sucrose Density Gradients

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 3 M sucrose was pipetted into the bottom of each tube to provide a cushion for pelleting cellular debris and nuclei. 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 0.001 M EDTA. Gradients were routinely made twelve to fifteen hours before centrifugation and stored at 4°C.

Centrifugation

One ml of the homogenate, rendered free of nuclei and cell debris as described above, was layered on top of the preformed gradient and centrifuged at 39,000 rpm for five hours, at 4°C, in a SW50 rotor in a Spinco Model L Ultracentrifuge. Equilibrium is reached within three hours (74). After centrifugation, the bottom of each tube was perforated and forty fractions of approximately 0.1 ml each (10 drops) were collected. All operations were at 4°C.

Nitrite Reductase Assay

The method of Cook (4,5) was used. This assay measures the reductant-dependent disappearance of nitrite.

The assay mixture contained in a volume of 3 ml: potassium phosphate buffer, pH 7.5, 120 µmoles; benzyl viologen, 0.5 µmoles; $NaNO_2$, 3.3 µmoles; sodium dithionite, 7.5 µmoles; and between 0.2 and 0.4 ml of extract. The dithionite, which is rapidly oxidized, was added last with minimum turbulence. The reaction mixture was incubated in open test tubes for ten minutes at 30°C and the reaction terminated by vigorous shaking on a vortex mixer (Scientific Products, Evanston, Illinois) until all the benzyl viologen was oxidized (became colourless). Samples (0.1 ml) of the reaction mixture were pipetted into 7.9 ml of distilled water and the nitrite content estimated as previously described. Reagent blank and zero reaction time controls were included. Nitrite accumulation from the reduction of endogenous nitrate was measured in a similar reaction mixture to which no nitrite had been added. Calculations of nitrite reductase activity were then corrected for nitrite accumulation. One unit of nitrite reductase activity is defined as the reduction of 1 mµ mole of nitrite per minute at 30°C. The specific activity of crude extracts of derepressed cells is constant between 0 and 1.3 mg of crude extract protein in the assay mixture (Fig. 5). All measurements were made in this linear range. The error in the measurement of nitrite reductase activity was ±6.0 units of activity (or 5 Klett units).

Estimation of Mitochondrial Content of Fractions from Sucrose Density Gradient Centrifugation

Fractions were collected from equilibrium sucrose density gradients as described previously. The mitochondrial content of each fraction was estimated by (a) Janus Green B staining and (b) Polarographic measurement of mitochondrial respiration.

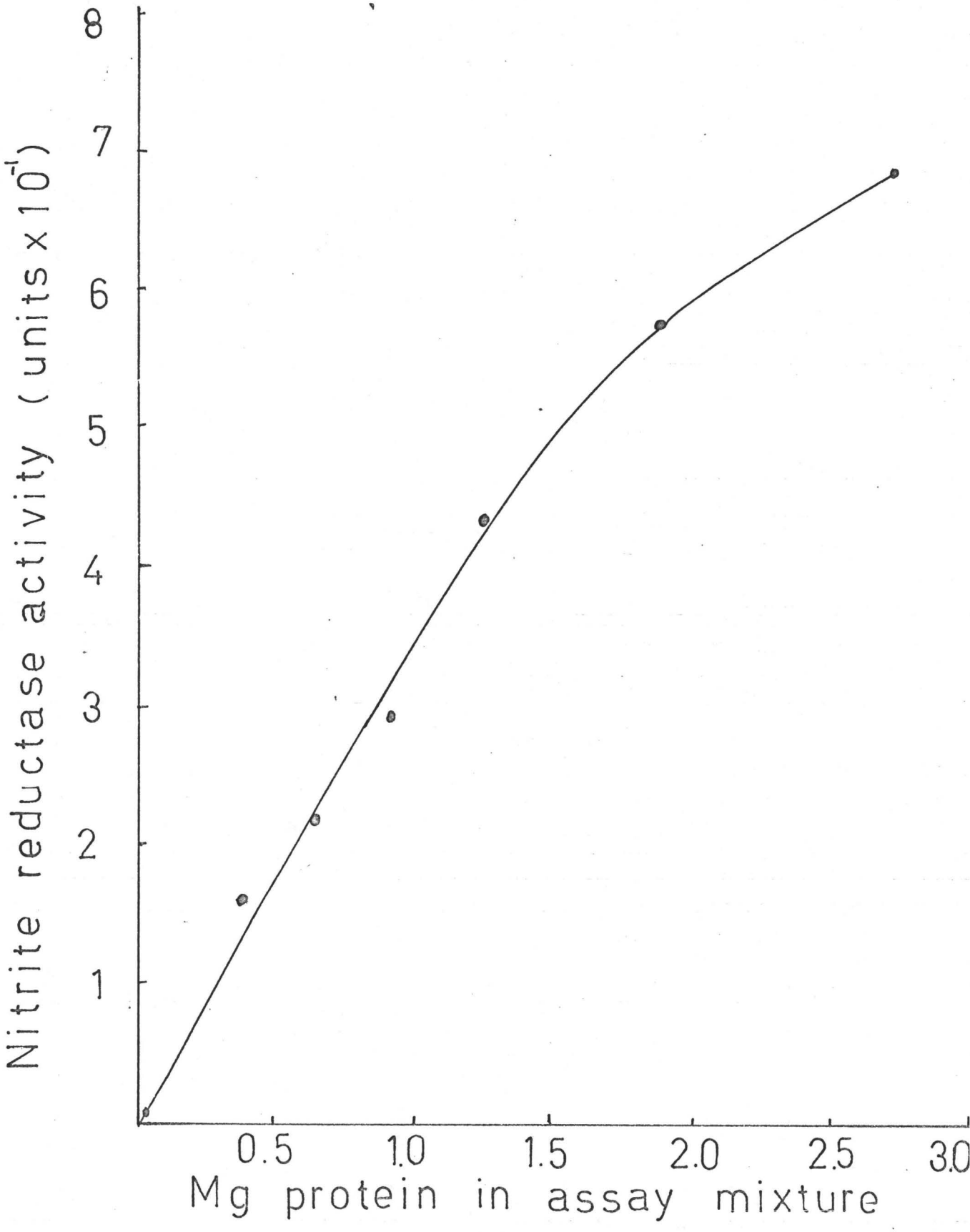
(a) Janus Green B Method

Mitochondria are stained greenish blue by dilute solutions of Janus Green B (diethylsafranineazodimethylaniline). Colouration is due to an oxidation-reduction process. The blue dye is successively reduced to leuco-Janus Green B (diethylsafranine) and finally to a colourless compound (leucosafranine) (25). Mitochondria reduce Janus Green B at a slower rate than the rest of the cell and are thus selectively stained. The dye is maintained in its oxidized (coloured) form in mitochondria but is rapidly reduced to the colourless leucosafranine compound by cytoplasmic enzymes.

The staining procedure was as follows. A drop of Janus Green B solution (0.001% in 0.85% NaCl solution) was applied to a clean prewarmed slide. A drop of the fraction to be analyzed was put on the stained slide and the whole preparation covered with a coverslip. Slides were examined under oil with a light microscope (100 X 8 X ocular) and the stained mitochondria counted in several fields.

Fig. 5. Effect of Concentration of Extract Protein in the Reaction Mixture on Nitrite Reductase Activity.

Extracts of wild type strain, Pan-2-B-36A mycelia were prepared and assayed for nitrite reductase activity as described in the text.


Nitrite reductase activity (units x 10⁻¹)
1
2
3
4
5
6
7
8
0.5
1.0
1.5
2.0
2.5
3.0
Mg protein in assay mixture

Polarographic Measurement of Oxygen Uptake

The polarographic oxygen electrode technique for measuring oxygen utilization is described in 'Methods in Enzymology' (3). The basis of this method is that TMPD reduced by ascorbate can donate electrons to cytochrome c in the mitochondrial electron transport chain. Electrons are then transferred to oxygen whose reduction is measured as oxygen disappearance.

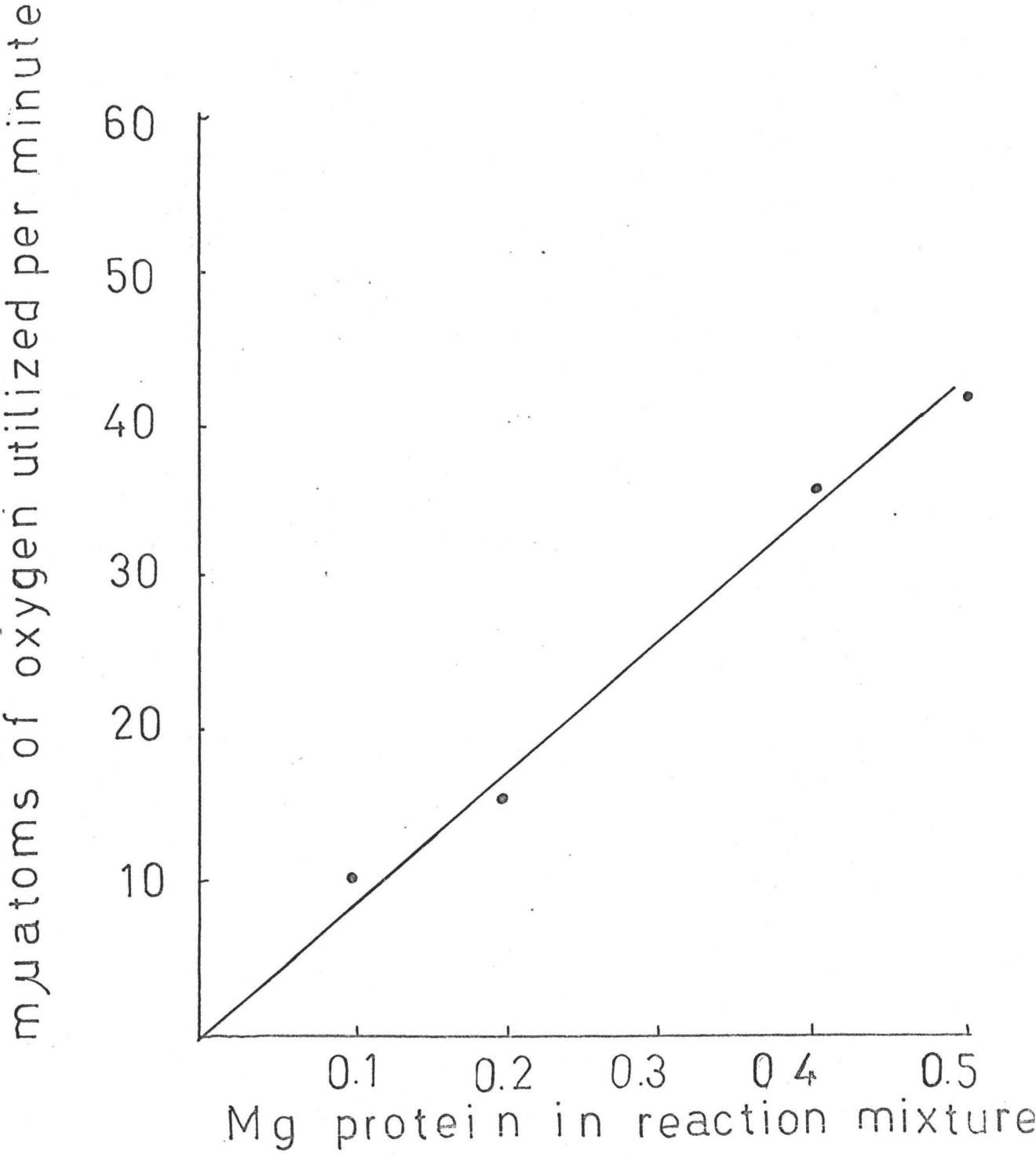
The Clark electrode apparatus was equilibrated with air-saturated buffer of constant temperature and the reactants added through a small opening at the top of the reaction vessel. The assay system contained in a volume of 2.6 ml potassium phosphate buffer, pH 7.3, 50 μmoles; sucrose, 1.25 m moles; EDTA, 2.5 μmoles; ascorbate, 10 mμ moles; TMPD, 2.5 mμ moles and 0.1 ml of extract suitably diluted in buffer where necessary. Oxygen uptake was measured over a period of ten minutes. Microatoms of oxygen utilized were calculated from the oxygen electrode data as described in Methods in Enzymology (3). A reference curve of oxygen utilization with increasing extract concentration in the reaction mixture is shown in Fig. 6. All measurements were made in the range of constant specific activity.

Protein Estimation

Protein concentrations were determined with either the Biuret test (24) or by measuring optical density at 280 mμ. Bovine serum albumen was used as standard.

Fig. 6. Effect of Concentration of Extract Protein in Reaction Mixture on Rate of Oxygen Utilization.

Extracts of wild type mycelia derepressed at 27°C were prepared and assayed polarographically for oxygen utilization as described in the text.


mμatoms of oxygen utilized per minute
60
50
40
30
20
10
0.1
0.2
0.3
0.4
0.5
Mg protein in reaction mixture

Sources of Materials

Sulphanilamide and N-1 napthylethylenediamine dihydrochloride were obtained from Eastman Organic Chemicals, Rochester, New York; benzyl viologen was from Mann Research Laboratories Inc., New York, New York; L-ascorbic acid and TMPD were purchased from British Drug Houses, Ltd., Poole, England; nitrosoguanidine was bought from Aldrich Chemical Ind., Milwaukee, Wisconsin; bovine serum albumen was obtained from Sigma Chemical Co., St. Louis, Missouri. Inorganic compounds, except where indicated were from Fisher Scientific Co., Fairlawn, New Jersey.

RESULTS

Section I. Induction and Selection of Mutants

The purpose of this part of the project was to induce mutants defective in the reduction of nitrite and to characterize them phenotypically. Mutagenesis was carried out in pantothenate requiring strain, Pan 2-B-36A, as described in METHODS. This strain is wild type with respect to nitrite reduction. The results of three filtration experiments are shown in Table 1. The second filtration experiment was done in the hope of finding temperature sensitive mutants, that were defective in nitrite reduction at 37°C but not at 27°C.

Characterization of Mutants

Mutants deficient in nitrite reductase would be expected to have the following properties:

(a) growth like wild type on Ammonia Medium or Casamino Acids Medium,

(b) no growth on Nitrate or Nitrite Medium,

(c) Accumulation of nitrite when grown on Ammonium-Nitrate Medium,

(d) defective or missing nitrite reductase activity in crude extracts.

TABLE 1

Nitrate-Non-Utilizing Mutants Collected by Filtration Enrichment

Mutant hunt	I	II	III
Strain Used	Pan 2-B-36A	Pan 2-B-36A	Pan 2-B-36A
Initial conidial concentration (per ml)	1×10^8	1×10^7	2×10^7
Mutagen	Nitroso-guanidine	Nitroso-guanidine	Nitroso-guanidine
Total filtration time (hours)	68	56	56
Total number of colonies tested	1,700	1,142	301
Nitrate non-utilizers found	26	49	33

The details of the procedure for mutagenesis are described in METHODS.

Wild type, Pan 2-B-36A, on the other hand, should grow on both Nitrate and Nitrite Medium, should not accumulate nitrite on Ammonium-Nitrate Medium and should have nitrite reductase activity in crude extracts. Mutants blocked at nitrate (nit mutants) should be unable to grow on nitrate but should be like the wild type strain with respect to growth on nitrite, accumulation of nitrite, and the presence of nitrite reductase in crude extracts.

All of the presumptive mutants isolated in the three filtration concentration experiments were surveyed for the ability to grow on Nitrate and on Nitrite Medium and for the accumulation of nitrite on Ammonium-Nitrate Medium (see Appendix). Those mutants which showed the most marked differences from the wild type strain were selected for further study. The phenotypic characteristics of selected mutants are shown in Table 2. Wild type and nit-1 behave as expected. All of the mutants accumulated nitrite and are of two main types: those which can grow on nitrate and nitrite (typified by T_{23}) and those which are unable to grow on nitrate and nitrite (typified by mutant 'O').

The growth of the selected mutants, of wild type and of nit-1 on various nitrogen sources was measured quantitatively as described in METHODS and is shown in Figs. 7 to 18. The patterns of nitrite accumulation in the mutants and in the wild type strain are shown in Figs. 19 to 22.

TABLE 2

Summary of Preliminary Growth and Accumulation Tests of Selected Mutants

Strain	Accumulation of Nitrite mμmoles per ml of media Per mg dry weight of culture at 70 hrs.		Growth of mycelium on Ammonium, Nitrate or Nitrite as sole nitrogen source % of dry weight on casamino acids medium								Category
	27°C	37°C	Amino acids	NH_3	NO_3^-	NO_2^-	Amino acids	NH_3	NO_3^-	NO_2^-	
			27°C				37°C				
T_{13}	0.89	9.40	100	40	11	21	100	37	1	4	Temperature sensitive non grower and temperature sensitive accumulation
0	4.10	-	100	34	4	2	-	-	-	-	Non grower
Z	1.80	-	100	34	2	2	-	-	-	-	Poor grower*
T_{23}	0.11	0.90	100	40	74	44	100	34	27	39	Temperature sensitive accumulator
nit-1	0.00	0.00	100	40	1	12	100	40	0	12	Control
Wild type (Pan 2 B-36A)	0.04	0.15	100	37	76	48	100	34	55	59	Control

* See Quantiative Data

Growth experiments were semiquantitative (see METHODS). Nitrite accumulation was determined in 70 hour cultures as described in METHODS.

Mutant '0' cannot grow on nitrate or nitrite (Figs. 11 and 12) and accumulates very high levels of nitrite (Fig. 21). Mutant Z also accumulates nitrite (Fig. 19) but grows a little on nitrate and nitrite (Figs. 17 and 18). Mutant T_{13} appears to be temperature sensitive with respect to nitrite accumulation (Fig. 22) and with respect to growth on Nitrate and Nitrite Medium (Figs. 13 and 14). Mutant T_{23} can grow on nitrate and nitrite at both 27°C (Fig. 15) and at 37°C (Fig. 16) but appears to be temperature sensitive with respect to nitrite accumulation (Fig. 20).

Fig. 7. Growth of wild type, Pan 2-B-36A on various nitrogen sources.

Aliquots of a suspension of wild type conidia were inoculated into 250 ml flasks containing 50 ml of liquid Casamino Acids Medium (Δ), Ammonia Medium (0), Nitrate Medium (□), or Nitrite Medium (.) (see METHODS). The cultures were incubated on a rotary shaker at 27°C. At the indicated times, mycelia were harvested by filtration through a Buchner funnel and their dry weights determined as described in METHODS.

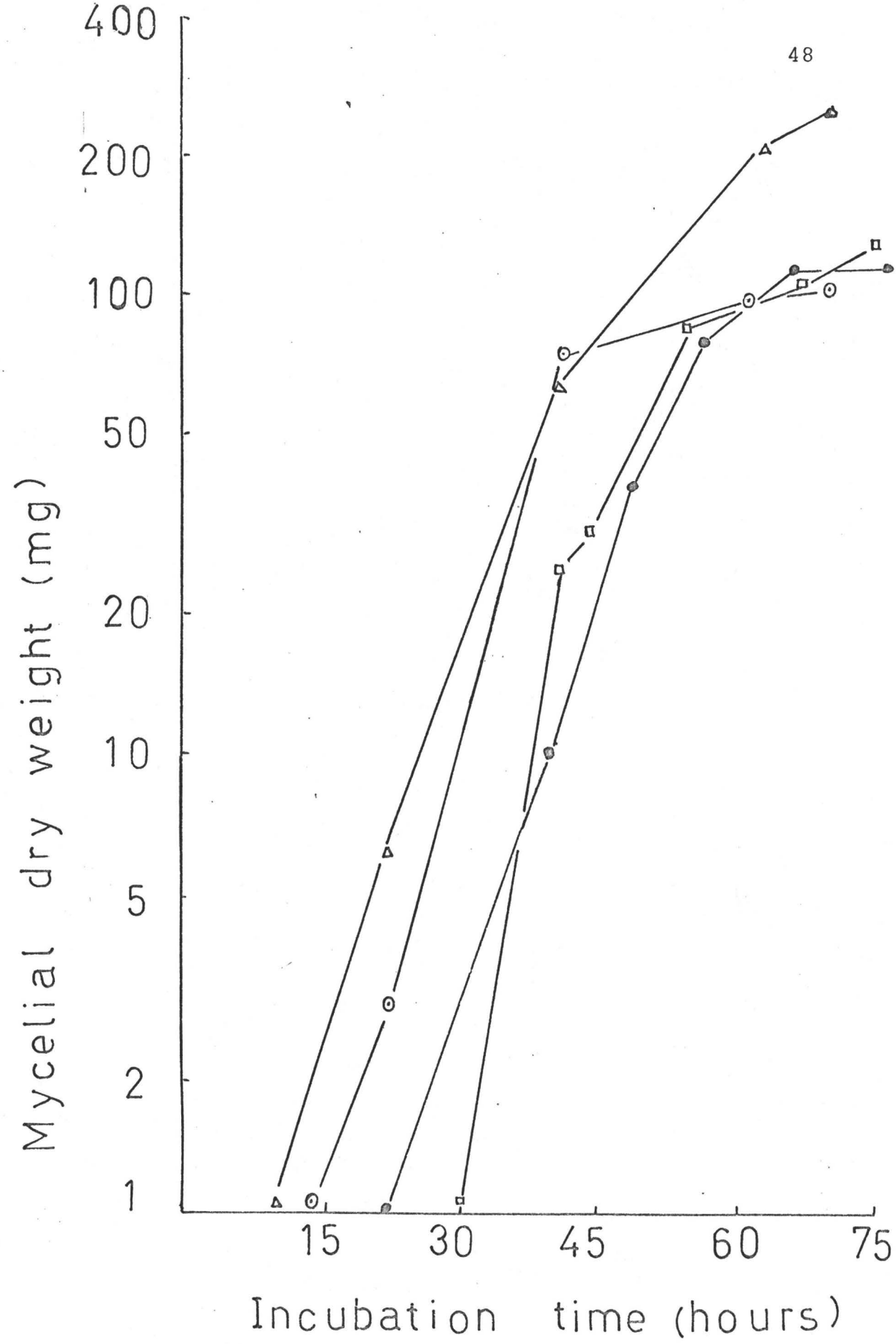

400
200
100
50
20
10
5
2
1
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 8. Growth of wild type, Pan 2-B-36A on various nitrogen sources at 37°.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (θ), Nitrate (□) and Nitrite (✓).

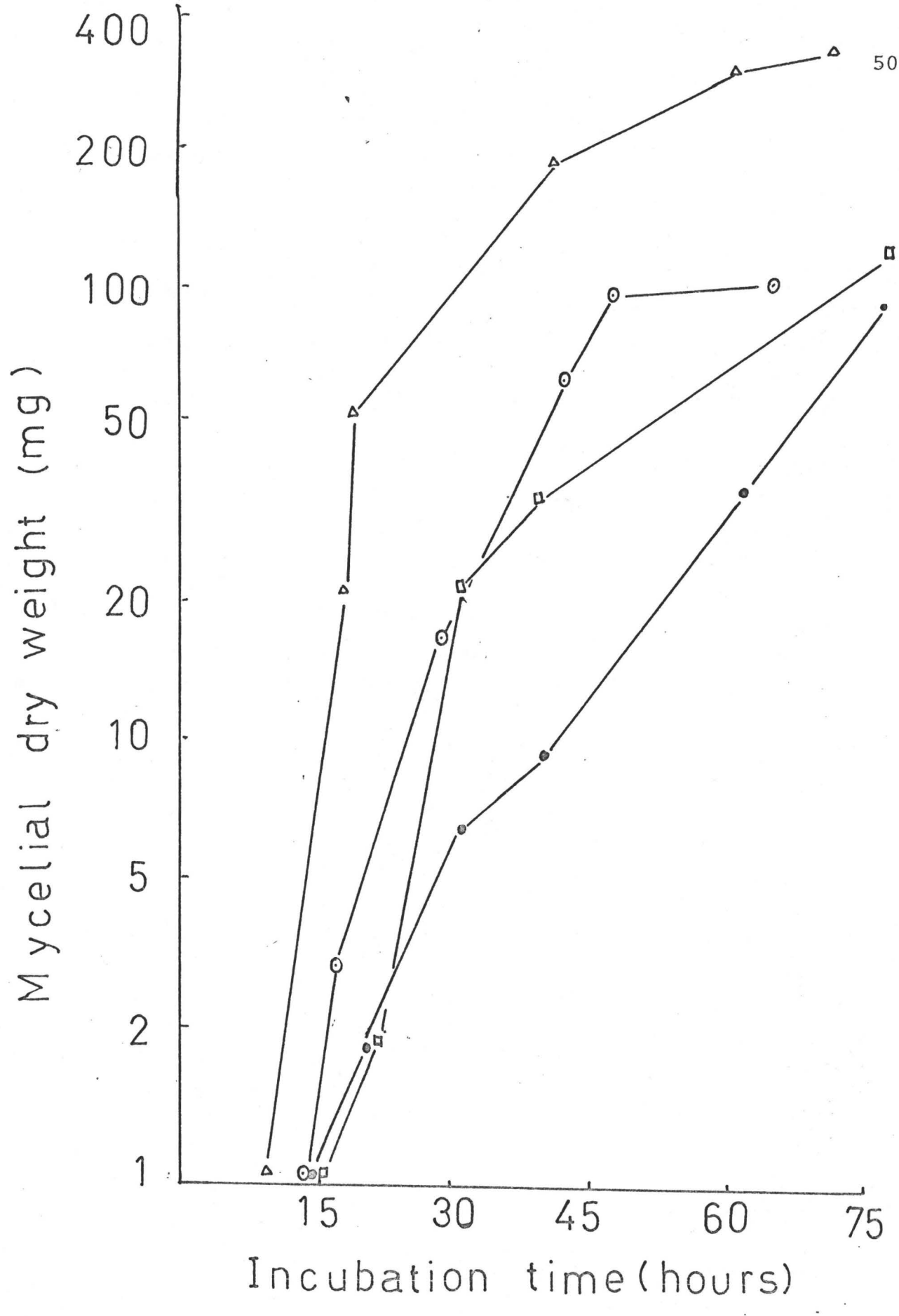

400
200
100
50
20
10
5
2
1
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)
50

Fig. 9. Growth of Mutant nit-1 on various nitrogen sources at 27°C.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (⊙), Nitrate (□) and Nitrite (•).

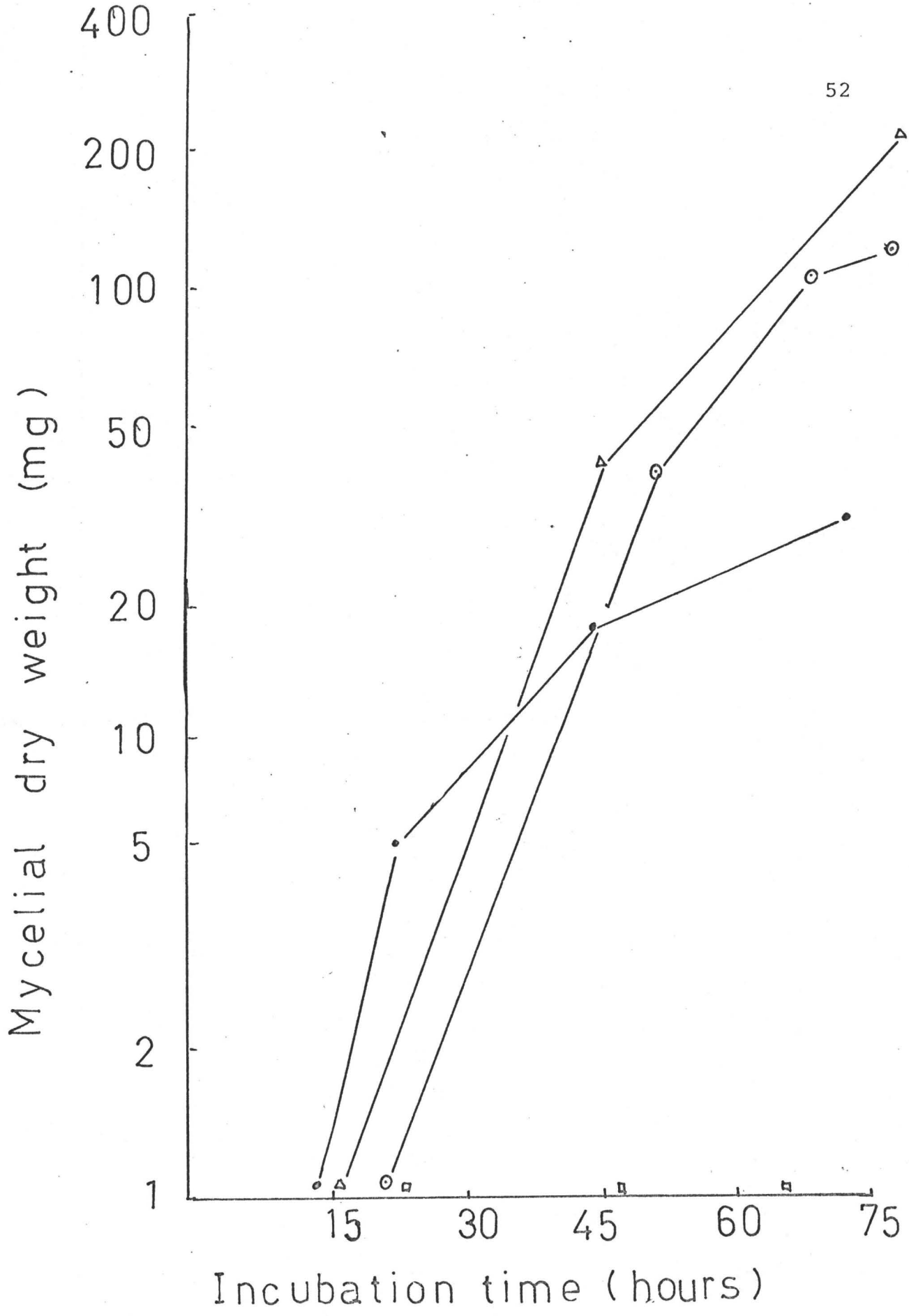

400
200
100
50
20
10
5
2
1
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 10. Growth of mutant nit-1 on various nitrogen sources at 37°C.

The procedure described in the legend of Fig. 7 was used. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (□) and Nitrite (•).

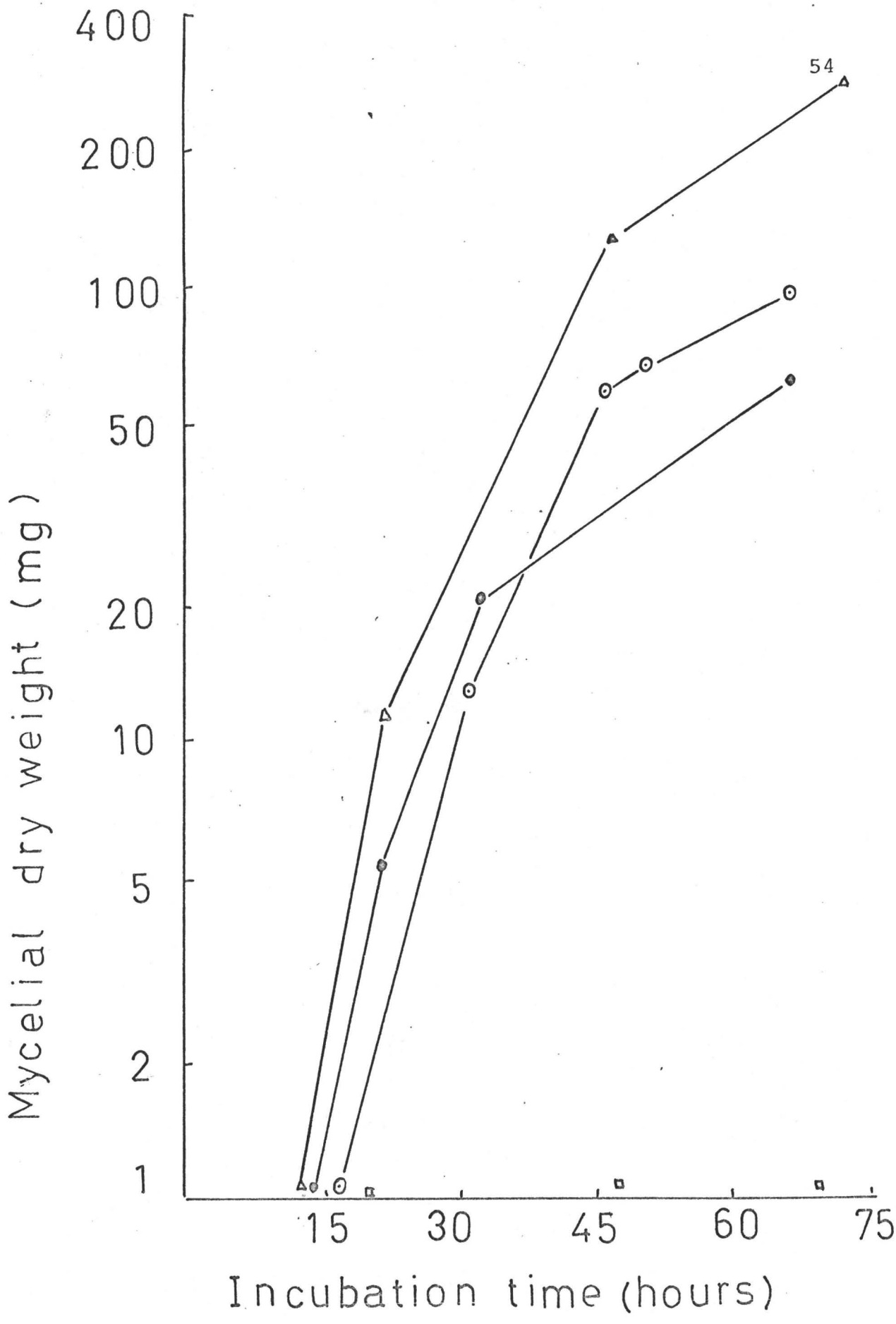

400
200
100
50
20
10
5
2
1
54
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 11. Growth of mutant 'O' on various nitrogen sources at 27°C.

The procedure described in the legend of Fig. 7 was used. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (■) and Nitrite (•).

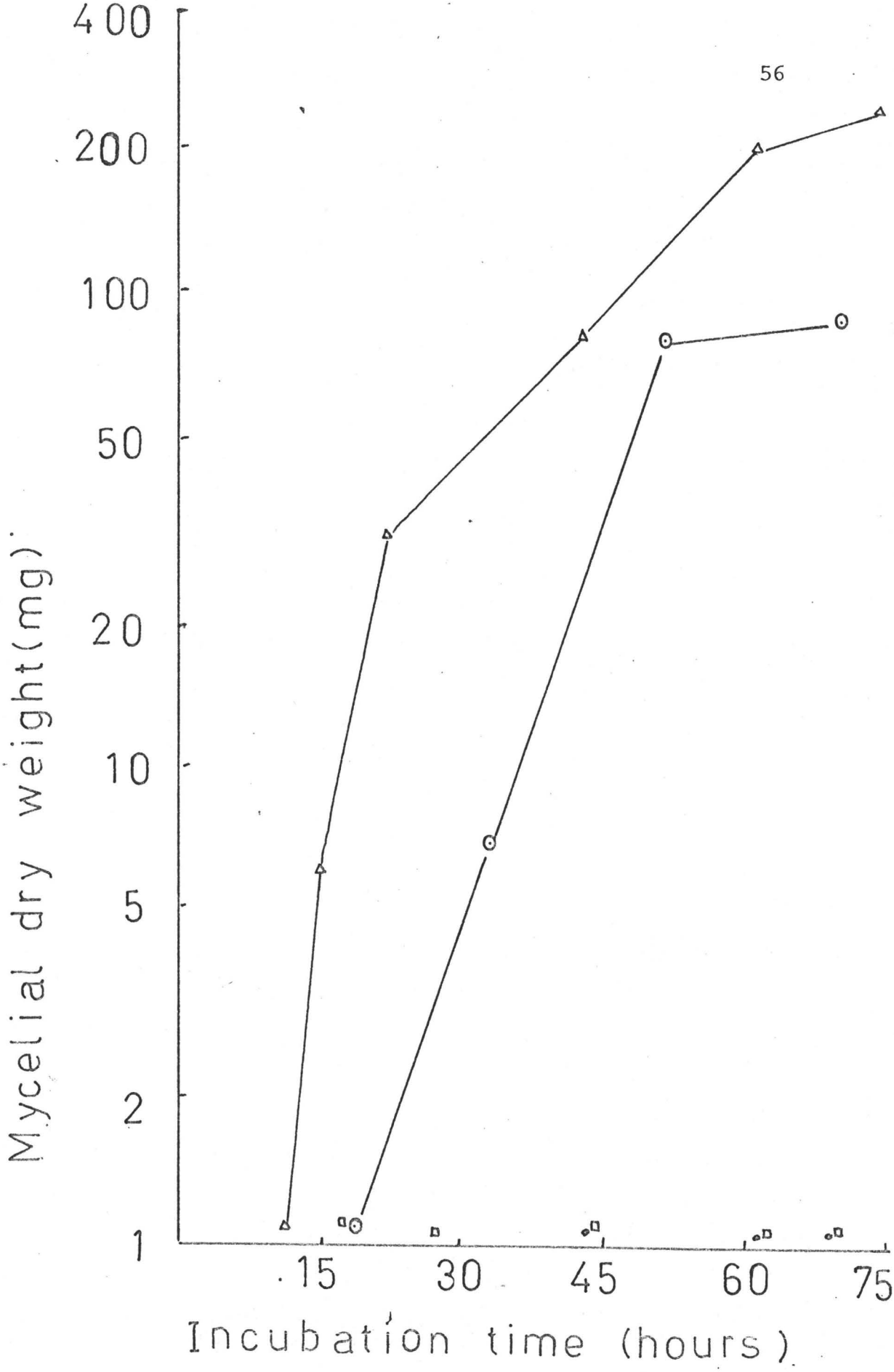

400
200
100
50
20
10
5
2
1
Mycelial dry weight(mg)
15
30
45
60
75
Incubation time (hours)

Fig. 12. Growth of Mutant 'O' on various nitrogen sources at 37°C.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (□) and Nitrite (•).

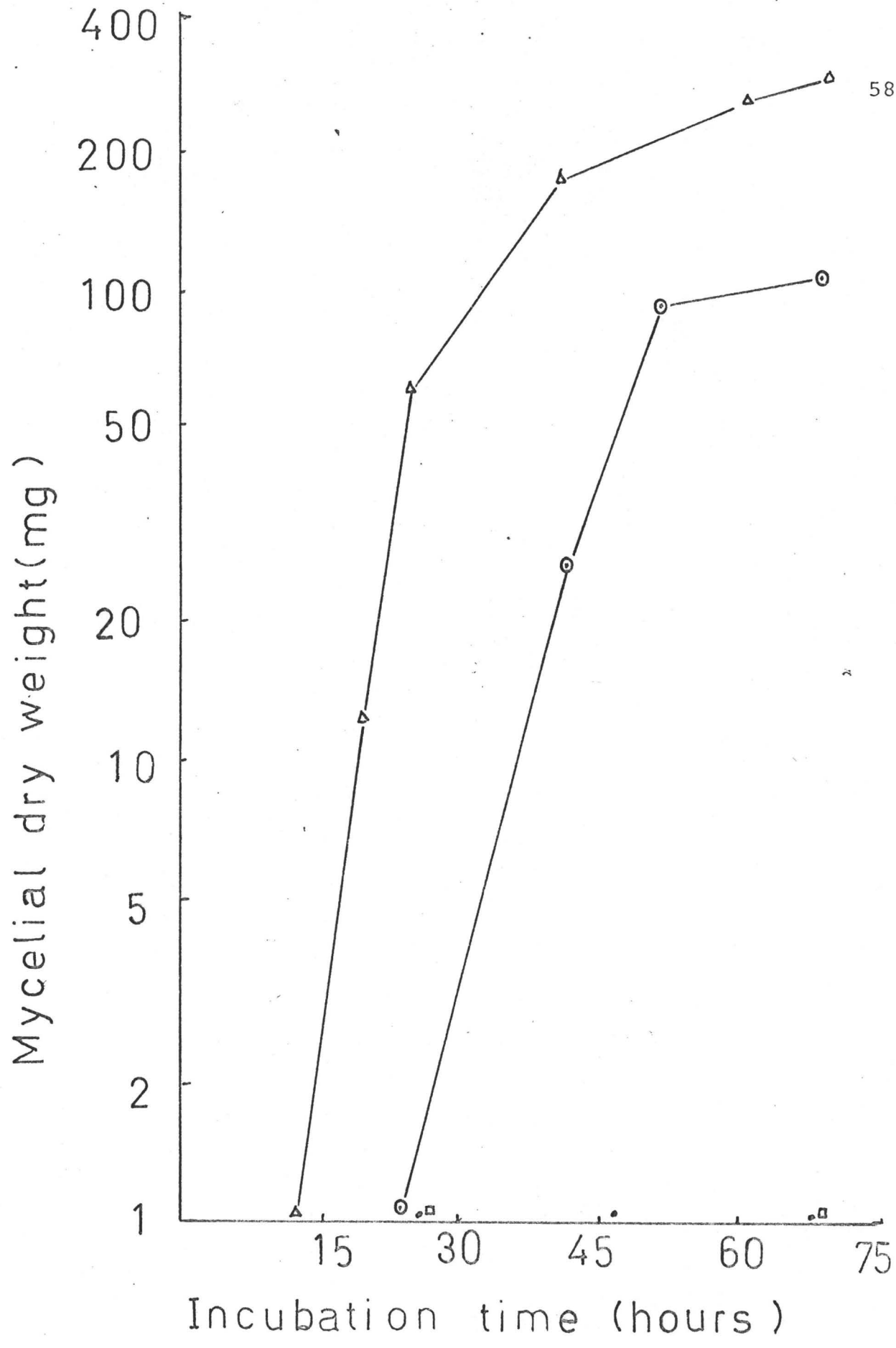

400
200
100
50
20
10
5
2
1
Mycelial dry weight(mg)
15
30
45
60
75
Incubation time (hours)

Fig. 13. Growth of Mutant T_{13} on various nitrogen sources at 27°C.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (□) and Nitrite (•).

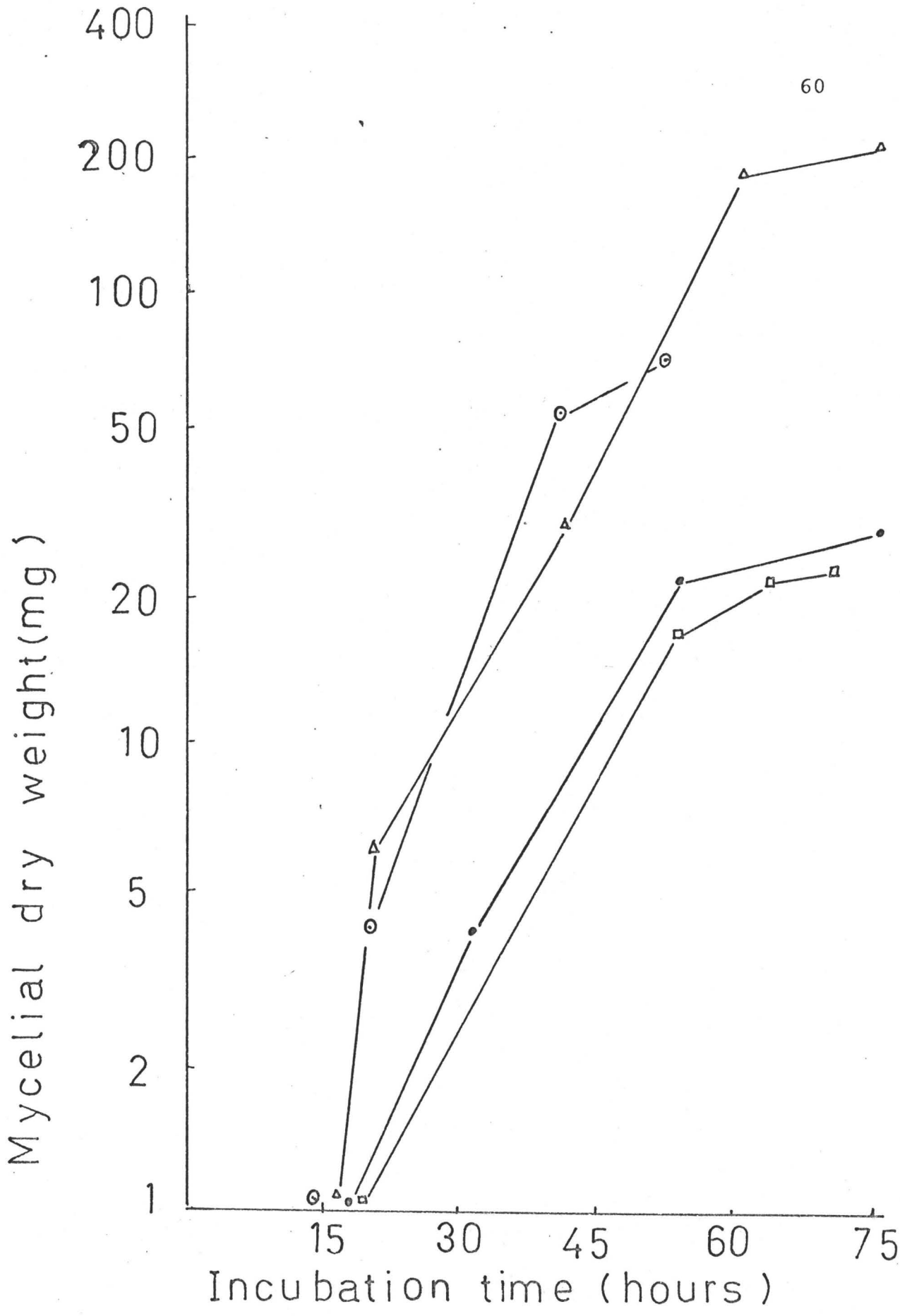

400
200
100
50
20
10
5
2
1
Mycelial dry weight(mg)
15
30
45
60
75
Incubation time (hours)

Fig. 14. Growth of Mutant T_{13} on various nitrogen sources at 37°C.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (□) and Nitrite (•).

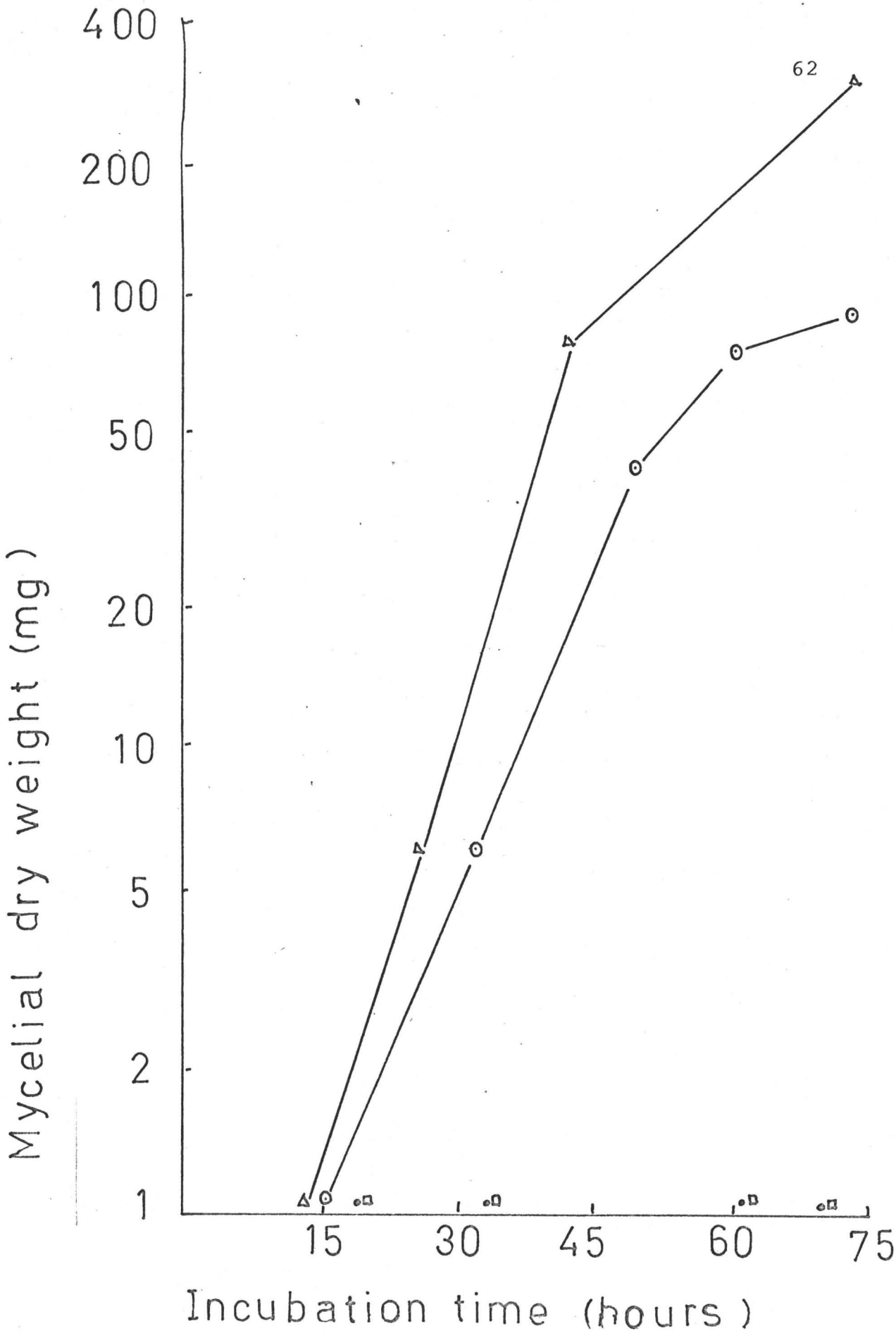

400
200
100
50
20
10
5
2
1
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 15. Growth of mutant T_{23} on various nitrogen sources at 27°C.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (■) and Nitrite (•).

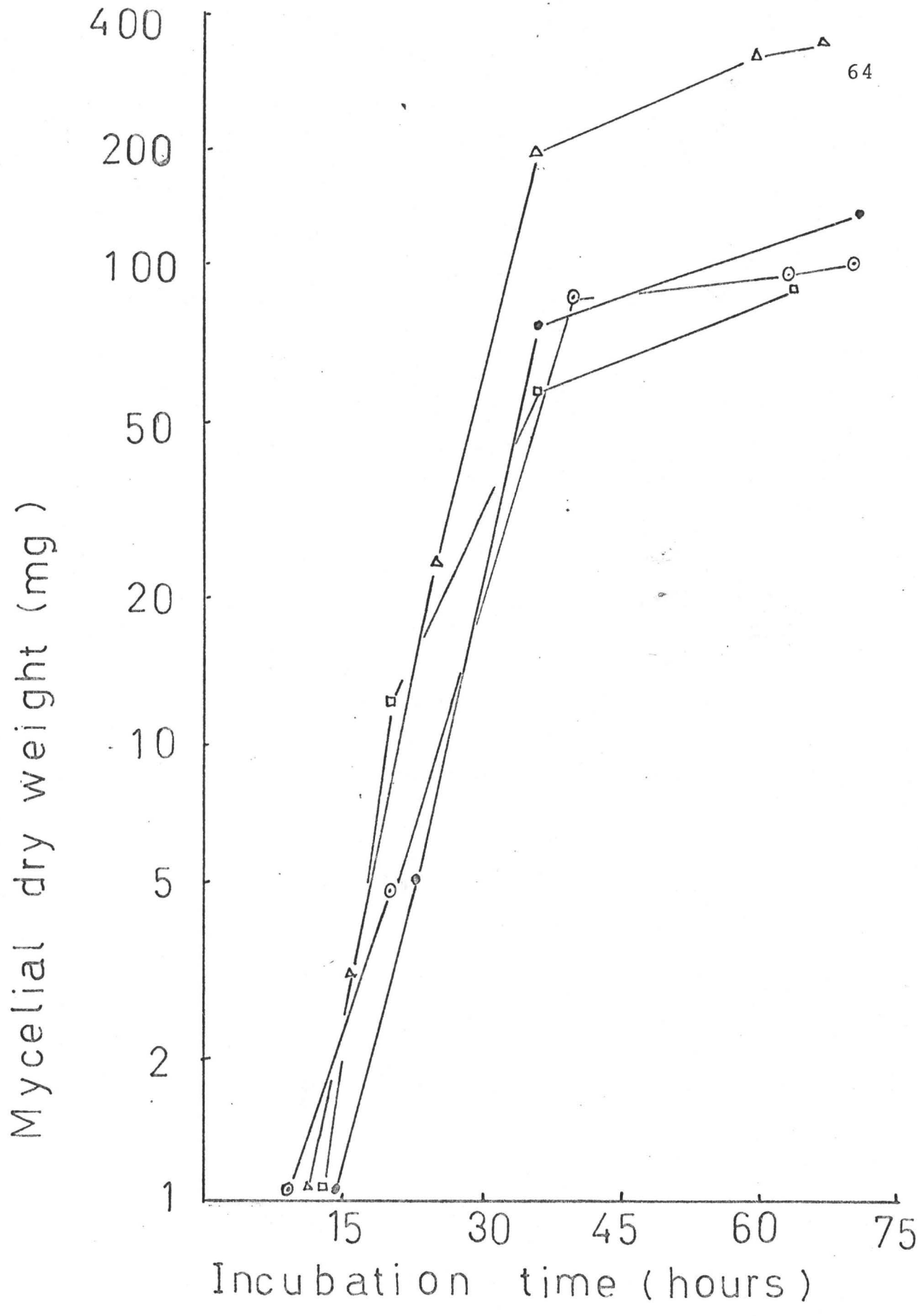

400
200
100
50
20
10
5
2
1
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 16. Growth of mutant T_{23} on various nitrogen sources at 37°C.

The procedure described in the legend of Fig. 7 was used. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (□) and Nitrite (●).

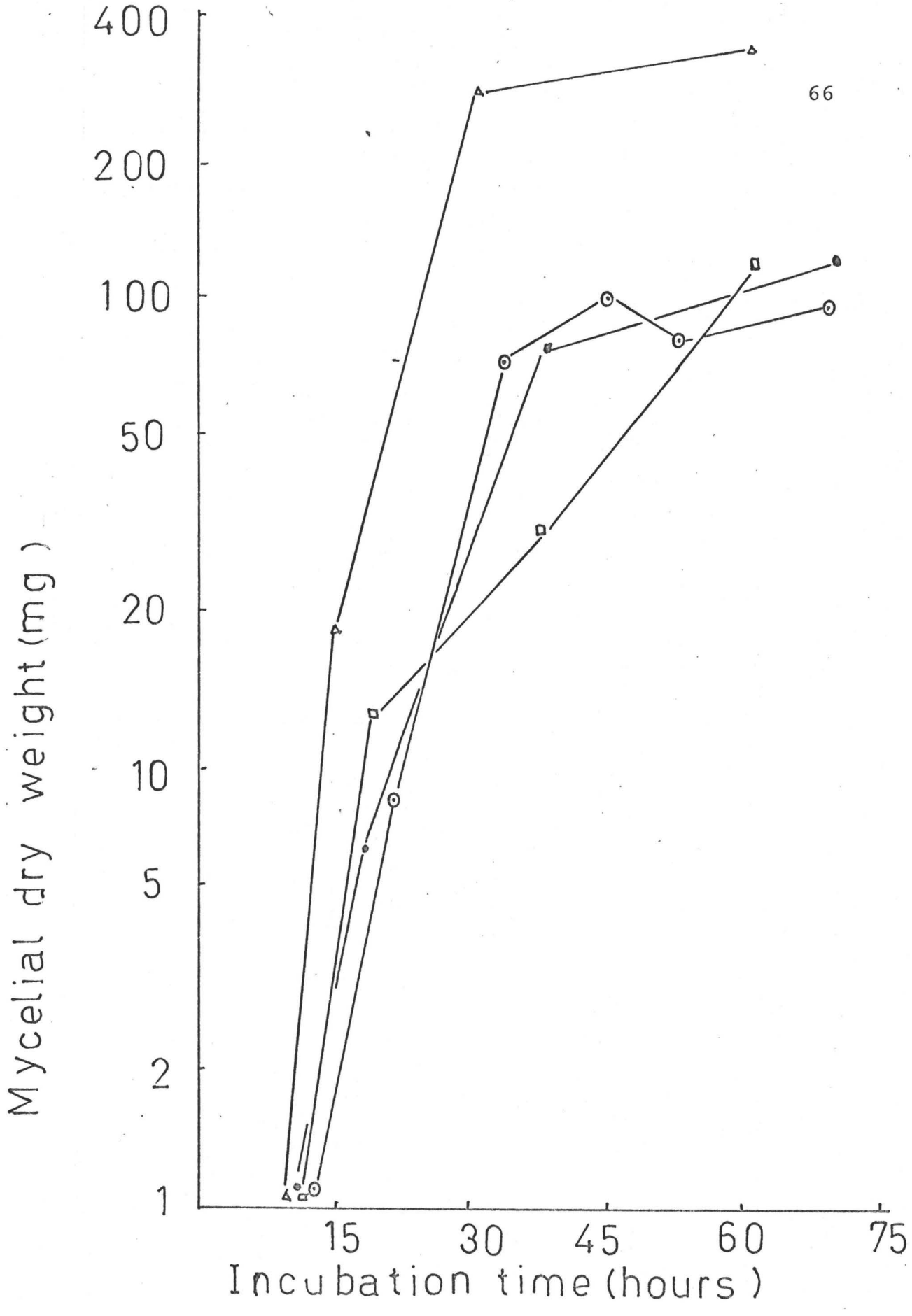

400
200
100
50
20
10
5
2
1
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 17. Growth of mutant Z on various nitrogen sources at 27°C.

The procedure described in the legend of Fig. 7 was used. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (▫) and Nitrite (•).

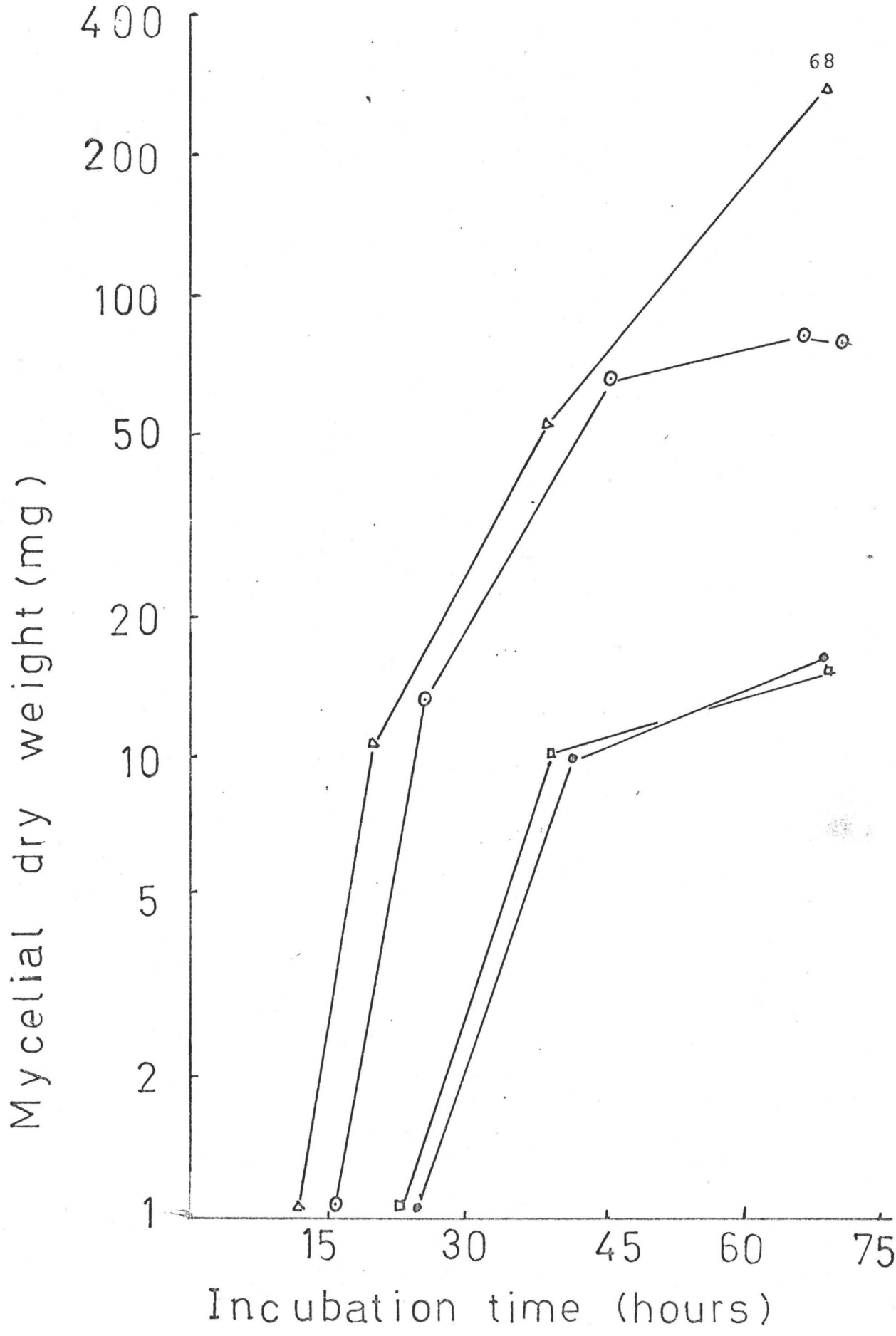

400
200
100
50
20
10
5
2
1
68
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 18. Growth of mutant Z on various nitrogen sources at 37°C.

The procedure described in the legend of Fig. 7 was followed. Growth on Casamino Acids (Δ), Ammonia (O), Nitrate (□), and Nitrite (•).

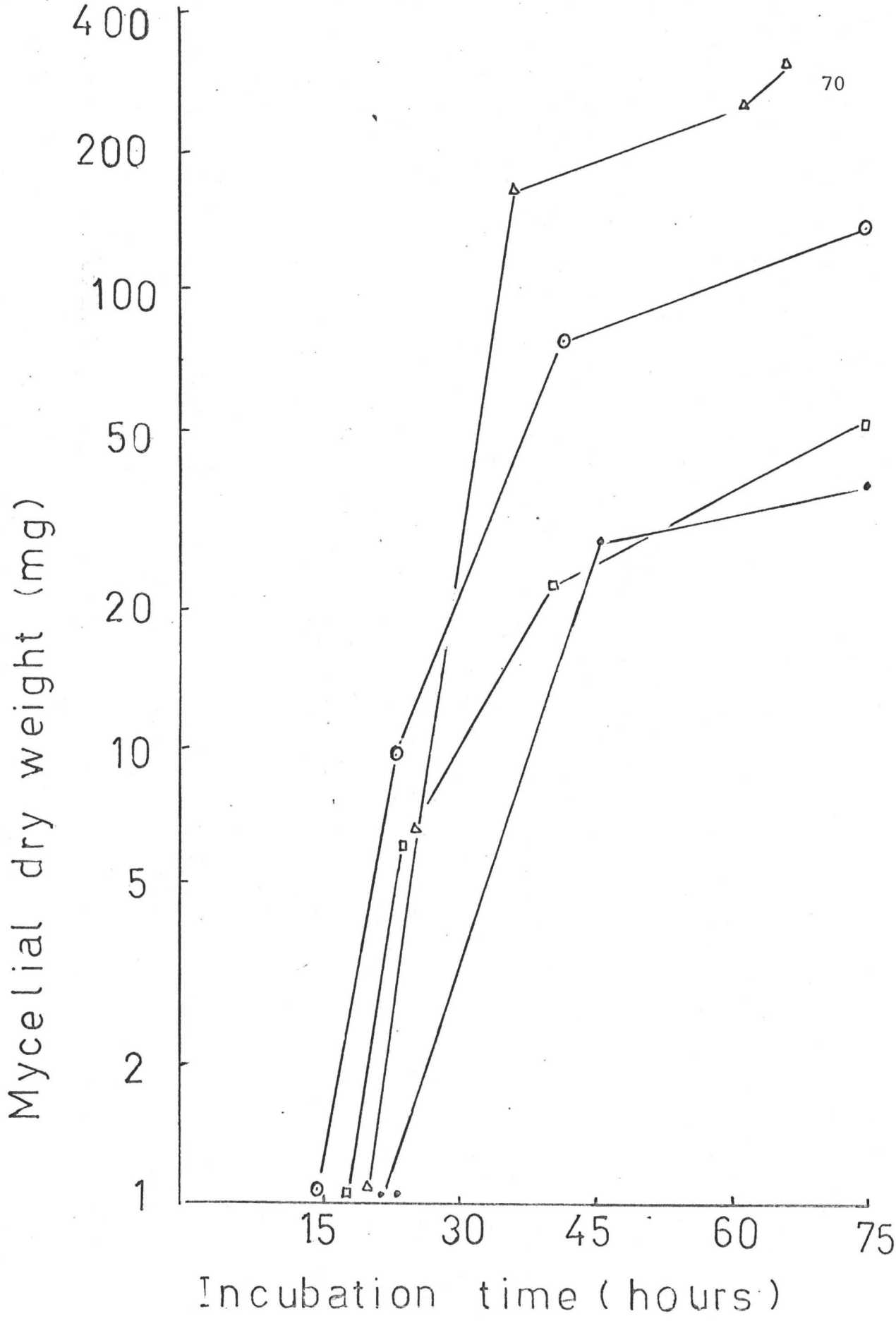

400
200
100
50
20
10
5
2
1
70
Mycelial dry weight (mg)
15
30
45
60
75
Incubation time (hours)

Fig. 19. Accumulation of nitrite by wild type, Pan 2 B-36A and by mutant Z.

Aliquots of conidial suspensions of each strain were inoculated in Ammonium Nitrate Medium (see METHODS) and incubated on a rotary shaker at 27°C and at 37°C. At intervals, mycelial pads were collected and their dry weights measured as described in METHODS. Samples (0.1 ml) of media from the same culture were tested for nitrite content (see METHODS). Nitrite accumulation in mutant Z at 37°C (●—●), in mutant Z at 27°C (□···□), in wild type at 37°C (▲—▲) and in wild type at 27°C (△—△).

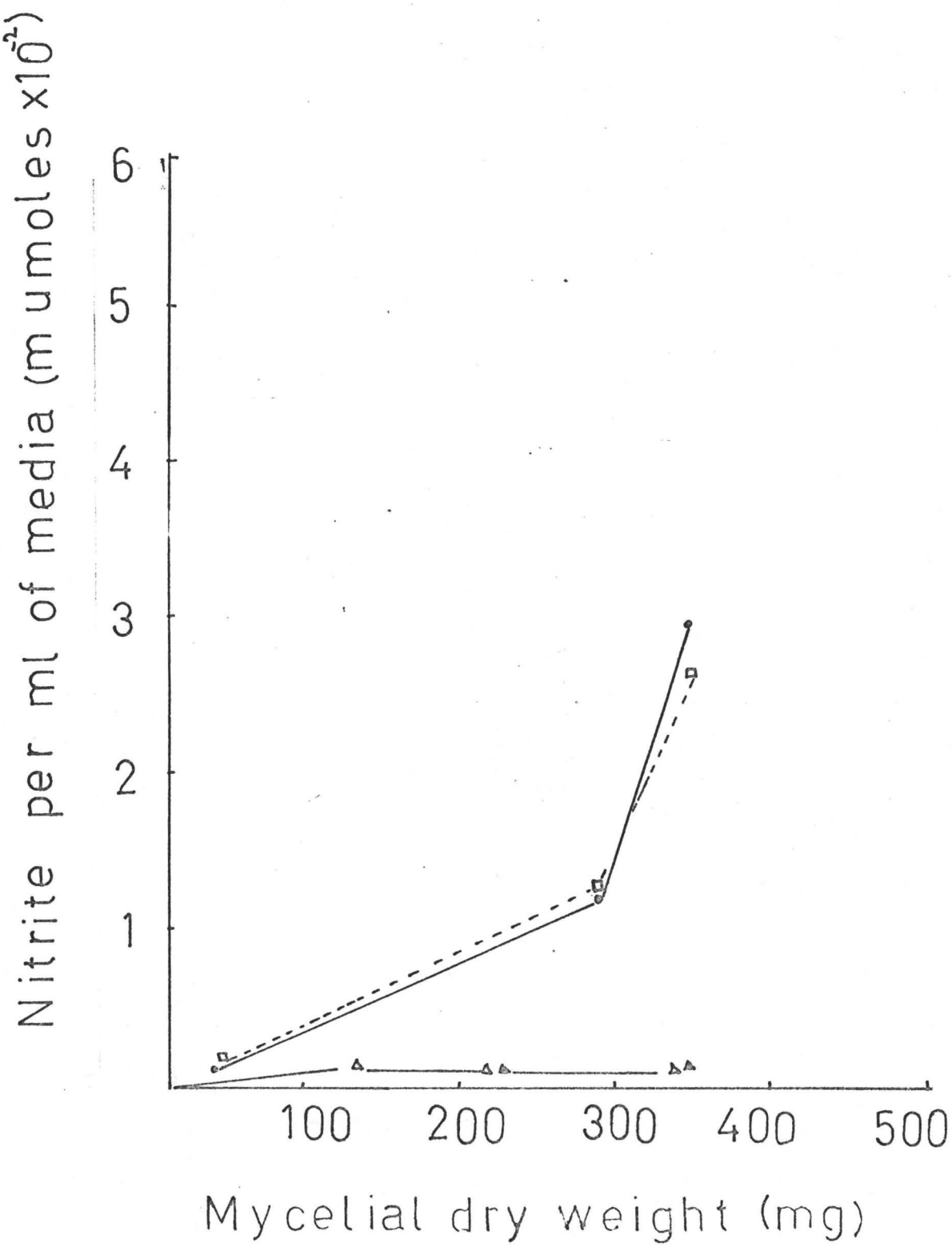

Nitrite per ml of media (m umoles x10⁻²)
6
5
4
3
2
1
100
200
300
400
500
Mycelial dry weight (mg)

Fig. 20. Accumulation of nitrite by mutant T_{23} at 27°C and at 37°C.

The procedures was as described in the legend of Fig. 19. Nitrite accumulation in T_{23} at 37°C (•—•) and at 27°C (□—□).

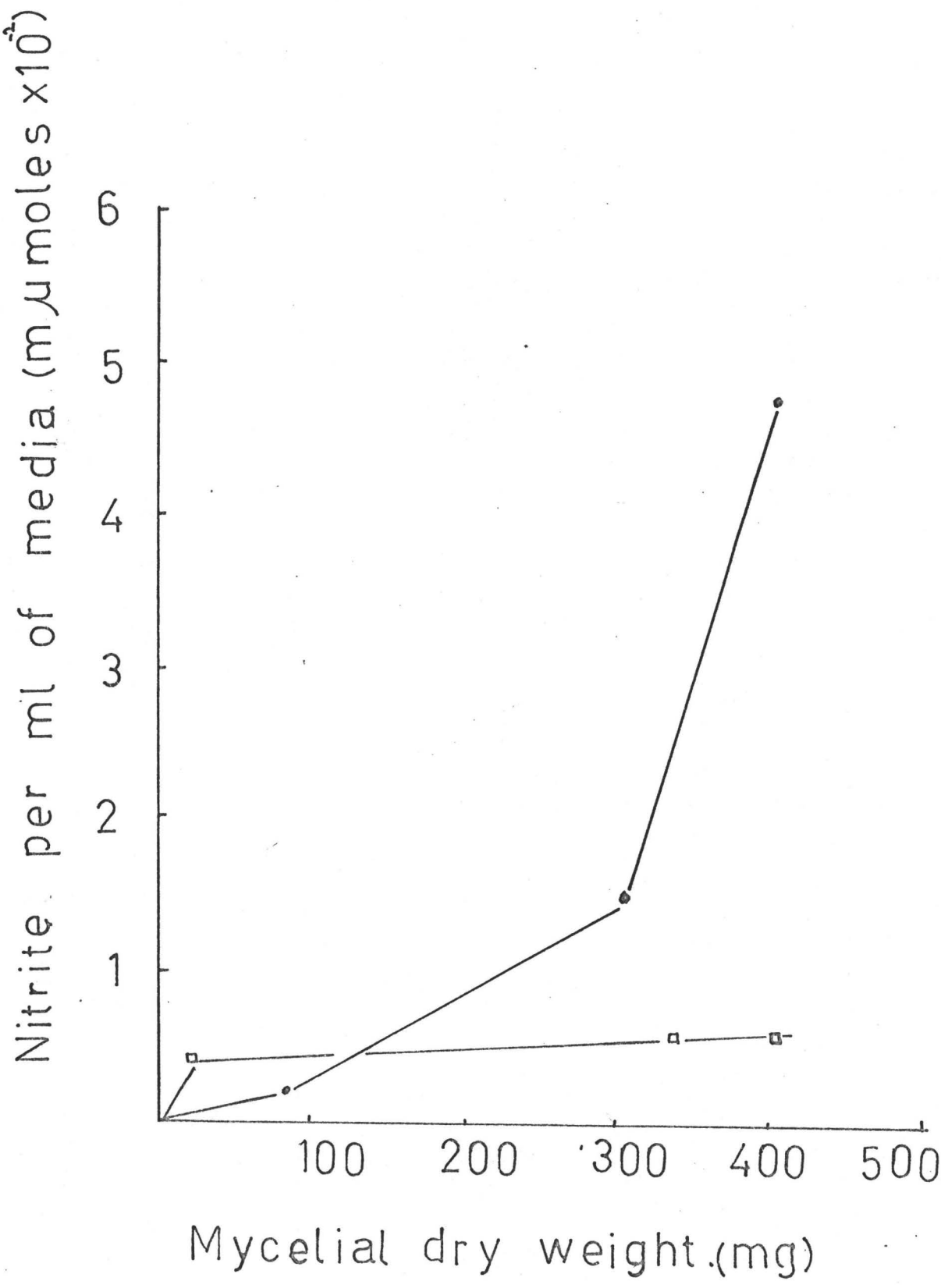

Nitrite per ml of media (mμmoles x10⁻²)
6
5
4
3
2
1
100
200
300
400
500
Mycelial dry weight (mg)

Fig. 21. Accumulation of nitrite by mutant 'O' at 27°C and at 37°C.

The procedure described in the legend of Fig. 19 was used. Nitrite accumulation in mutant 'O' at 27°C (□—□) and at 37°C (•—•).

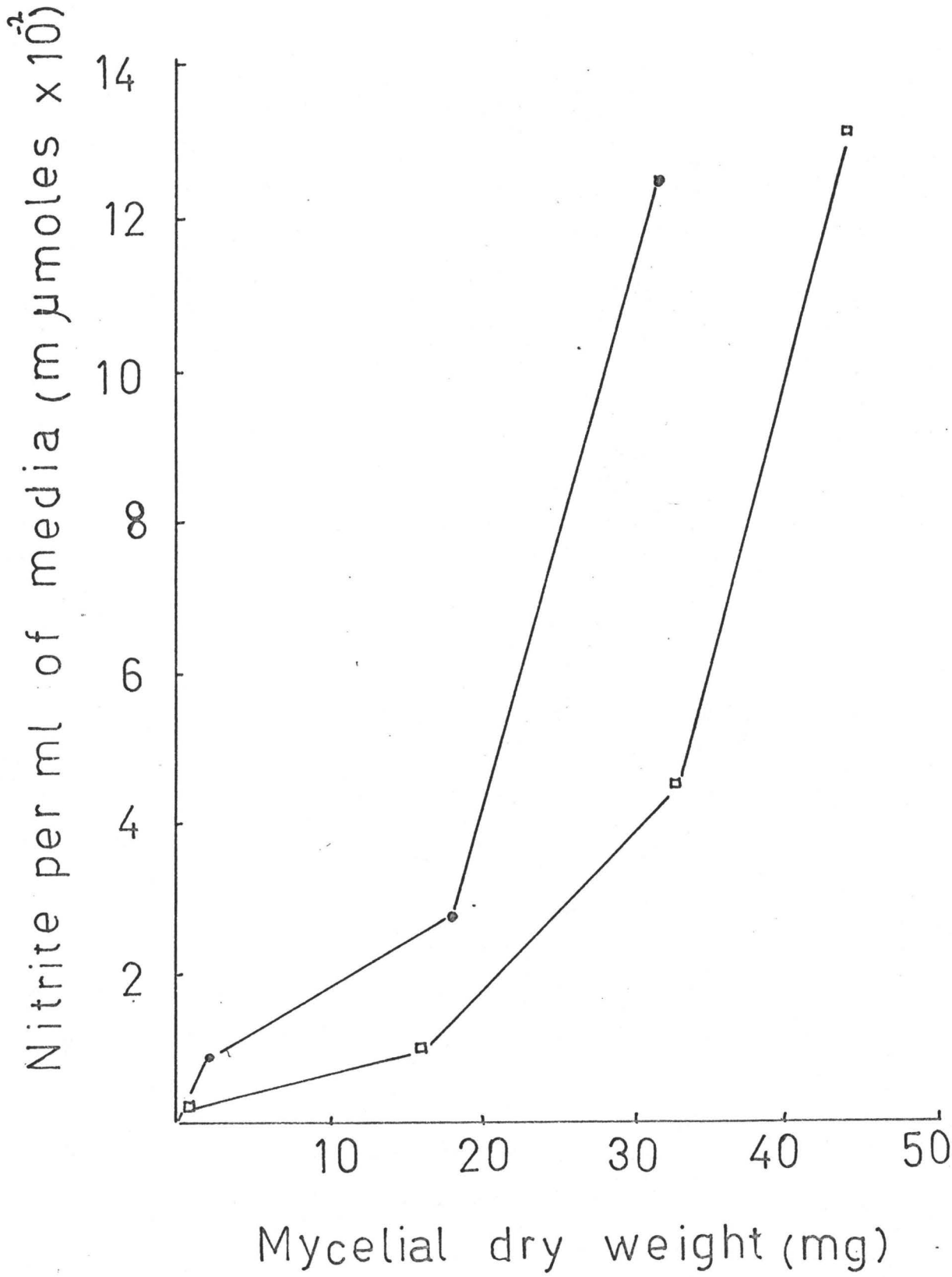
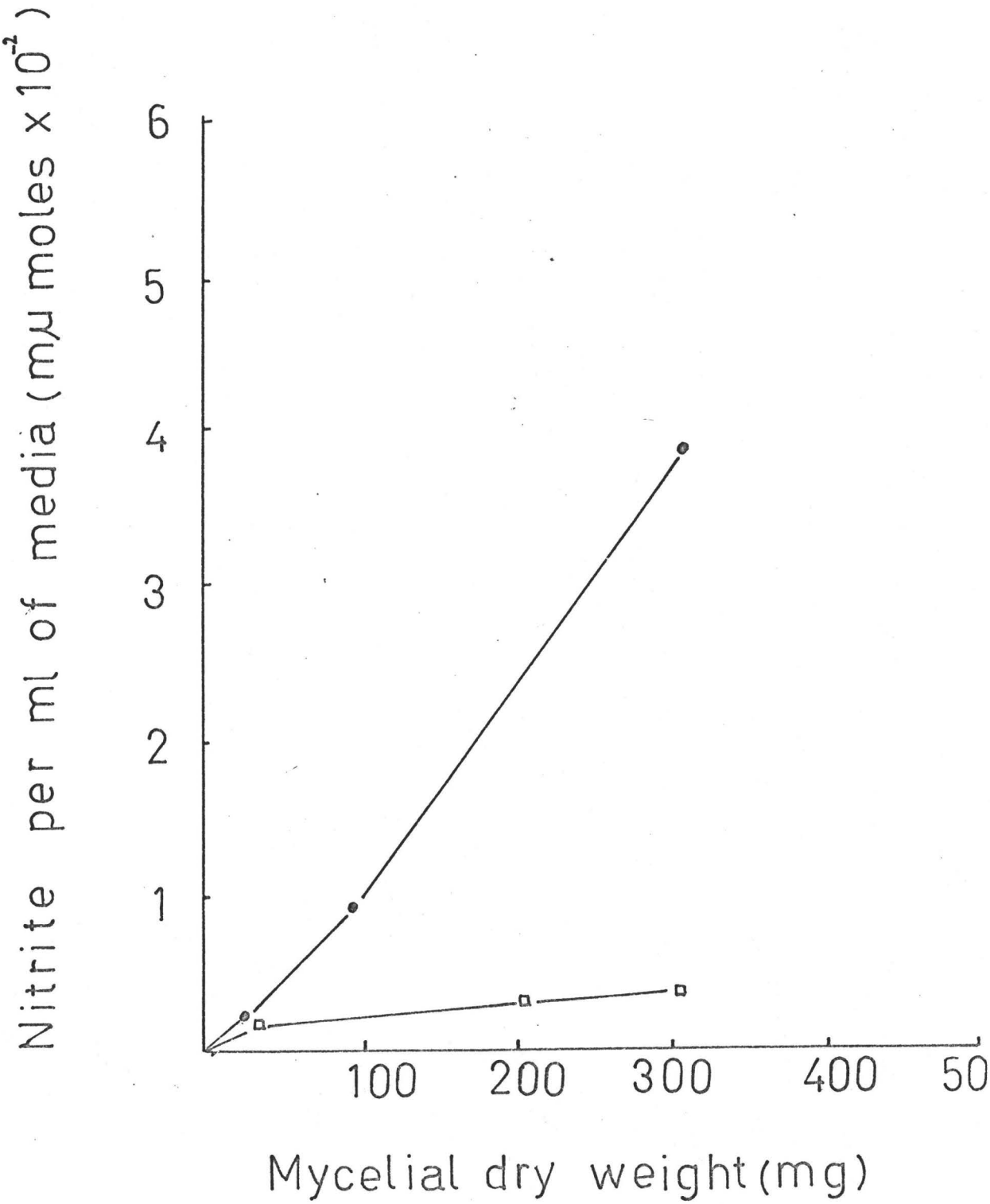

Nitrite per ml of media (m µmoles x 10^{-2})
14
12
10
8
6
4
2
10
20
30
40
50
Mycelial dry weight (mg)

Fig. 22. Accumulation of nitrite by mutant T_{13} at 27°C and at 37°C.

The procedure was as described in the legend of Fig. 19. Nitrite accumulation in mutant T_{13} at 37°C (•—•) and at 27°C (□—□).


Nitrite per ml of media (mμ moles x 10⁻²)
6
5
4
3
2
1
100
200
300
400
500
Mycelial dry weight (mg)

Section II. Genetic Relationships Among Newly Isolated Mutants.

The second part of this project was to determine how many genes control nitrite reduction.

When each of the new mutants was backcrossed to wild type (467 ad pan a) only the two parental phenotypes were observed among the progeny. This result suggested that each mutant was defective in a single gene function (Table 3). Backcrosses were made with either the mutant or the wild type strain as the protoperithecial parent. The similar results obtained with both types of crosses indicated that the mutations were not transmitted by maternal inheritance (Table 3).

Crosses were made between pairwise combinations of the selected newly isolated mutants (Table 4).

Mutant Z shows more than 50% recombination with mutants 'O', T_{13} and T_{23} and is, therefore, probably on a different linkage group. Mutant 'O' shows less than 17% recombination with mutants T_{13} and T_{23} and may, therefore, be on the same linkage group with them.

Crosses between mutants T_{13} and T_{23} were made but could not be analysed because of fertility and germination problems.

The high levels of recombination observed (Table 4) between the mutants suggests that at least three genes [(i) O, (ii) Z, and (iii) T_{13} and T_{23}] are represented.

TABLE 3

Backcrosses of Newly Isolated Mutants to "Wild Type"

Cross Parents A	Cross Parents B	Number of ascospores tested	% germination	% different Phenotypes Parental Types A	Parental Types B	Non Parental Types*
467	23	80	29	40	60	0
23	467	68	28	45.6	54.3	0
0	467	85	43	47	53	0
467	0	100	37	46	54	0
467	2	52	43	61.9	38.1	0
2	467	81	33	40.7	59.3	0
467	13	64	30	56	44	0
13	467	100	20	57	43	0

* With respect to nitrite utilization

Crosses were made as described in METHODS. Spore isolates from each cross were cultured and analysed for the ability to grow on nitrate and for the accumulation of nitrite on Ammonium-Nitrate Medium. The crosses were made in two ways with one or the other strain as the protoperithecial parent.
467 = adenine requiring mutant
23, 13, 0, Z = nitrite-non-utilizing mutants.

TABLE 4

Genetic Relationships Among Newly Isolated Mutants

#	Cross Parents A	B	Number of colonies or ascospores tested	% germination	% different phenotypes among progeny: Parental Types A	B	Recombinants wild type	% Recombination
(1)	0	Z	73+	8	*	*	27	52
(2)	0	13	85+	21	43.5	48.1	8.4	16.8
(3)	0	23	96+	51	42.6	50.0	7.4	14.8
(4)	0	23	58±	58	56.2	36.2	5.2	10.3
(5)	Z	23	109+	17.7	36.4	22.7	40.9	82
(6)	Z	13	88+	48	28.5	39.7	31.8	64

Crosses were made as described in METHODS. Vegetative cultures of spore isolates from each cross were analysed for their ability to grow on nitrate and to accumulate nitrite on Ammonium Nitrate Medium. The percent recombination is twice the observed percent of prototrophs among the progeny.

* 0 and Z phenotypes were not distinguishable; + colonies; ± ascospores.

It is not possible from the available data to determine whether mutants T_{13} and T_{23} are allelic.

The conclusions regarding linkage are made with the reservation that there may have been differential germination of certain phenotypes. If differential germination is not involved, the number of spores of Parental type A (Table 4) which would include double mutant recombinants, should be equal to the number of spores of Parental type B (Table 4), plus the number of wild type spores among the progeny of the cross. This relationship was roughly observed in crosses (2), (3) and (4) but not in crosses (5) and (6) (Table 4). Cross #1 could not be analysed in this way because mutants 'O' and Z are indistinguishable from each other and from 'O', Z double mutants.

Attempts were made to localize the new mutants by crosses to:

(a) nit mutants (60),

(b) multiply marked strains: al-2-cr a (linkage group I), pyr-1 - pdx-1A (linkage group IV), Pdx-1-col a (linkage group IV) and ylo-pan-tryp A (linkage group VI) obtained from the Fungal Genetics Stock Center (via R. Subden), and

(c) reciprocal translocation markers or alcoy linkage testers obtained from the Fungal Genetics Stock Center.

Poor germination and phenotypic ratios which suggested the involvement of differential germination made the results

of these crosses impossible to interpret. These crosses have, therefore, not been included in this report.

Complementation

In order to determine how many functional groups are involved in nitrite reduction, complementation tests were performed (see METHODS). Table 5 shows the results of tests on conidia from forced heterokaryons. Heterokaryons between the new mutants and a strain wild type with respect to nitrite utilization (Pan-2-B-23A) grew on nitrate and did not accumulate nitrite on Ammonium Nitrate Medium. The mutant defects were thus not dominant over their wild type alleles. Heterokaryons between the new mutants and nit mutants were all able to grow on nitrate and did not accumulate nitrite, suggesting non-allelism between the nitrite-non-utilizing mutants and nit mutants. Mutant 'O' complemented mutants T_{13}, T_{23} and Z, mutant Z complemented all of the new mutants except T_{23}; mutant T_{13} complemented all of the new mutants and mutant T_{23} complemented all of the new mutants except mutant Z.

These results suggest that at least three complementation groups control nitrite reduction. The failure to observe complementation between mutant strains T_{23} and Z may have been due either to dominance of T_{23} over Z (the heterokaryon displayed a T_{23} phenotype) or to a preponderance of T_{23} type nuclei in the T_{23} plus Z heterokaryon. Nuclear

TABLE 5

Complementation Among Newly Isolated Mutants and Nit Mutants

	O ad A	Z ad A	T_{13} ad A	T_{23} ad A	nit-1 ad A	nit-2 ad A	nit-3 his A	nit-5 ad A	"wild type" Pan 2-B-36A
O pan A	-	+	0	0	+	+	+	+	+
Z pan A	+	-	0	0	+	+	+	+	+
T_{13} pan A	+	+	-	+	+	+	+	+	+
T_{23} pan A	+	-	+	-	+	+	+	+	+
nit-1 ad A	0	0	0	0	-	+	+	+	+
nit-2 ad A	0	0	0	0	+	-	+	+	+
nit-3 his A	0	0	0	0	+	+	-	+	+
nit-5 ad A	0	0	0	0	+	+	+	-	+
"wild type" Pan 2-B-36A	+	+	+	+	+	+	+	+	+

Complementation among newly isolated mutants and nit mutants. A heterokaryon between each adenine-requiring-nitrite-non-utilizing mutant and each pantothenate-requiring-nitrite-non-utilizing mutant was preformed on unsupplemented Ammonia Medium. Cultures from conidia of these preformed heterokaryons were then tested for nitrite accumulation in unsupplemented Ammonium Nitrate Medium and for growth on minimal Nitrate Medium. The results shown in this Table refer to tests on conidia from preformed heterokaryons.
+ = growth on nitrate and not nitrite accumulation; - = no growth on nitrate and accumulation of nitrite; 0 = test not done.

ratios were determined (49) by plating conidia from a sample of heterokaryons on various types of media (unsupplemented media, adenine-supplemented media, pantothenate-supplemented media and adenine-plus pantothenate-supplemented media). Nuclear ratios near unity were observed for most of the heterokaryons tested. However, there were almost three times as many T_{23} nuclei as Z nuclei in the heterokaryon between these two strains.

The genetic studies suggest, with the reservations already made, that at least three different complementation groups and at least three different genes are involved in nitrite reduction. The simplest overall interpretation of the results is that four genes representing four cistrons are represented among the newly isolated mutants.

TABLE 6

Nuclear Ratios in Heterokaryons Between New Mutants and Nit Mutants

Heterokaryon X	Heterokaryon Y	A Conidia with Type X Nuclei no/10^6 conidia ($X10^{-4}$)	B Conidia with Type Y Nuclei no/10^6 conidia ($X10^{-4}$)	Nuclear Ratios A/B
Z pan	nit-5 ad	32.2	38.8	0.83
0 pan	Z ad	21.8	17.3	1.3
0 pan	nit-5 ad	15.6	15.6	1.0
T_{13} pan	Z ad	14.6	14.1	1.03
T_{13} pan	nit-5 ad	13.3	16.7	0.9
T_{23} pan	Z ad	17.0	6.2	2.8

Aliquots of conidial suspensions from these heterokaryons were plated on selective media to determine the numbers of the different types of nuclei present in each heterokaryon (see METHODS). Approximately 1,000 colonies were examined in each test.

Section III. Biochemical Characterization of the Newly Isolated Mutants.

The third part of the project was to answer the questions:

(1) how many enzymes are required for the reduction of nitrite *in vivo*, and

(2) where are the enzyme(s) localized intracellularly?

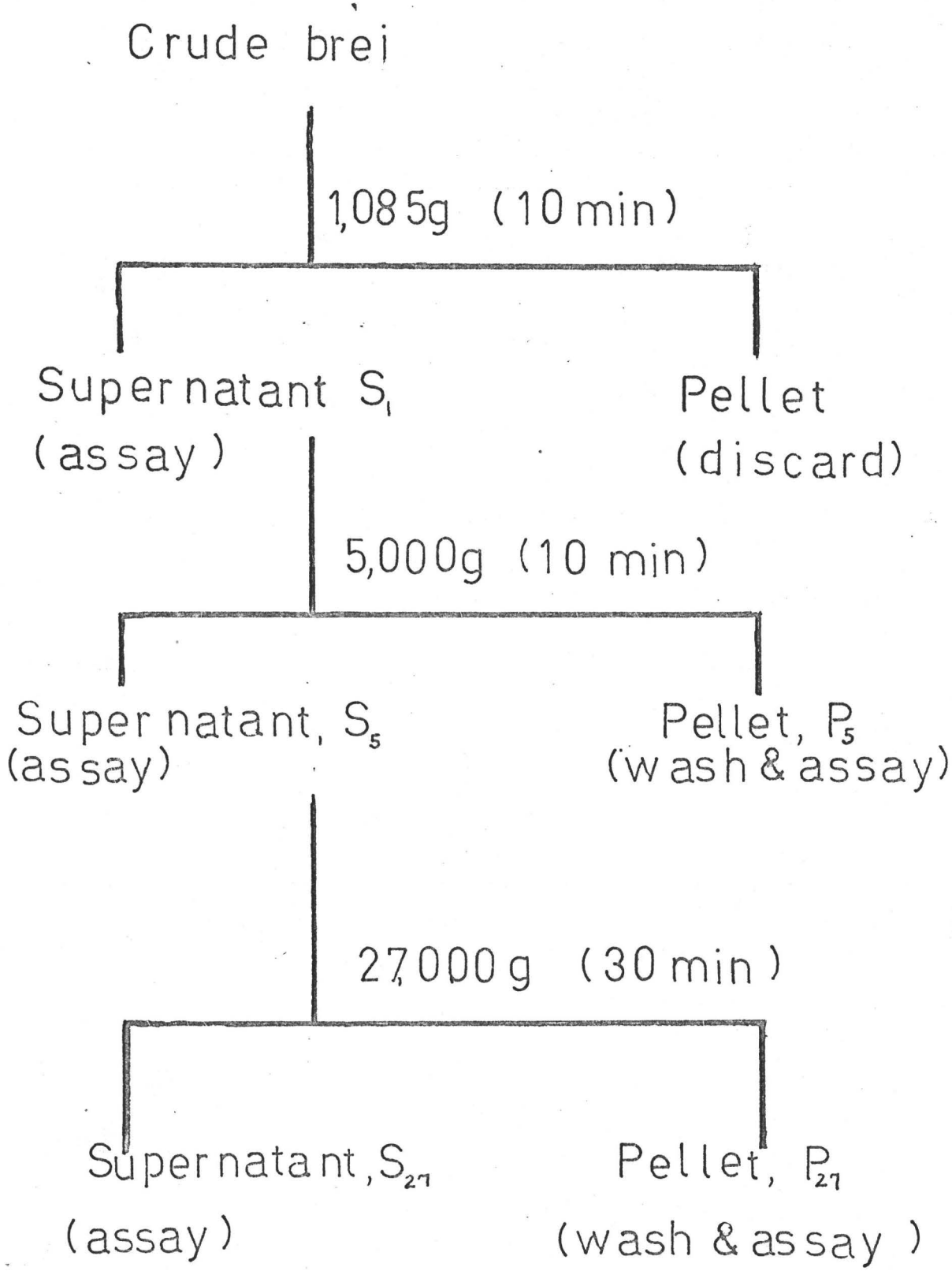
A preliminary, almost qualitative experiment was carried out to determine whether nitrite reductase was present in more than one subcellular organelle. Extracts of wild type mycelia, derepressed at 27°C and prepared so as to preserve mitochondria (63) were centrifuged differentially as outlined in Fig. 23. All of the fractions assayed had nitrite reductase activity.

Seventy seven percent of the activity associated with fraction P_5 was removed with one washing, showing that a major portion of activity was loosely bound to P_5. The supernatant from the washed P_5 (w_5-1) had only 22% of the activity of unwashed P_5, suggesting that binding of nitrite reductase to P_5 may by necessary for maximum activity.

A substantial amount of activity (59%) remained associated with the P_{27} pellet even after ten washings. This result indicated that the activity observed in the P_{27} fraction was not due to loose sticking of the enzyme to subcellular particles and that the enzyme in this fraction may be different from the nitrite reductase in fraction P_5

Fig. 23. Protocol for differential centrifugation of a crude extract from wild type mycelia.

Homogenates of mycelia derepressed at 27°C were prepared according to the method of Suyama (63) (as described in METHODS) and subjected to differential centrifugation. The P_5 pellet was washed by resuspension in phosphate buffer (0.1 M, pH 7.0) containing 0.3 M sucrose and 0.001 M EDTA, followed by centrifugation at 10,000 g for 10 minutes. The resulting pellet was then resuspended in phosphate buffer containing sucrose. The P_{27} pellet was washed ten times by repeated resuspension in sucrose-EDTA buffer followed by centrifugation at 27,000 g for 10 minutes.


Crude brei
1,085g (10 min)
Supernatant S_1
(assay)
Pellet
(discard)
5,000g (10 min)
Supernatant, S_5
(assay)
Pellet, P_5
(wash & assay)
27,000g (30 min)
Supernatant, S_{27}
(assay)
Pellet, P_{27}
(wash & assay)

and in fraction S_{27}.

The nitrite reductase activity in fractions P_5 and S_5 was 115% of the activity in fraction S_1. Differences of 5 Klett units or 6 units of nitrite reductase activity are regarded as significant. The increase observed here could thus be simply due to variation in measurement.

Approximately 60% of the nitrite reductase activity was lost when fraction S_5 was centrifuged and divided into fractions S_{27} and P_{27}. This decrease in activity, which is significant could have been due to inactivation of nitrite reductase or to the separation of two components both of which are necessary for maximum activity. Measurement of nitrite reductase activity in mixed fractions may have resolved this question but since the observations were very preliminary, this was not done.

The very tentative conclusions from this experiment are that:

(1) there is more than one type of nitrite reductase activity,

(2) nitrite reductase activity is associated with more than on subcellular organelle: nitrite reductase activity appears to be loosely associated with P_5 ("mitochondria") and is bound fairly tightly to P_{27} ("microsomes").

To further explore the possibility that there was more than one nitrite reductase activity, perhaps in different subcellular particles, attempts were made to separate the enzymes or particle bound activities by

TABLE 7

Nitrite Reductase Activity in Fractions Obtained by Differential Centrifugation of Wild Type Crude Extract

Fraction	Total Volume of fraction ml	Mg Protein in assay mixture	Units of activity per ml of fraction	Units of activity in entire fraction	% of Total S_1 activity in entire fraction	Specific activity units/mg protein
S_1	37	1.92	32.4	1199	100	17
S_5	35	1.92	31.2	1092	90.8	16
P_5	6	0.63	40.8	244.8	24.2	64
P_5W_1	3	0.38	18.6	55.8	4.7	49
W_{5-1}	3	0.125	17.4	53.2	4.4	138
S_{27}	34	0.48	11.4	388	32.5	24
P_{27}	6	0.73	13.2	79.2	6.6	18
$P_{27}W_1$	4	0.30	18.0	72	6.0	60
$P_{27}W_3$	4	0.20	13.2	52.8	4.2	66
$P_{27}W_5$	4	0.20	14.4	57.6	4.8	72
$P_{27}W_7$	4	0.20	19.2	76.8	6.4	86
$P_{27}W_{10}$	3	0.13	15.6	46.8	3.9	120
W_{27-1}	3	0.23	10.8	32.4	2.7	47
W_{27-3}	3	0.23	4.2	12.6	1.1	18
W_{27-5}	3	0.13	3.6	10.8	0.9	27
W_{27-7}	3	0.13	2.4	7.2	0.6	18
W_{27-10}	3	0.11	0	0	0	0

Nitrite reductase activity was measured in fractions obtained by differential centrifugation of wild type crude extract (Fig. 23). Protein was measured by Biuret. Pellets washed once, three times, five times and then times are designated P_{w1}, P_{w3}, P_{w5}, P_{w7} and P_{w10} respectively. Supernatants from the first, their, fifth, seventh and tenth washes are designated W_1, W_3, W_5, W_7 and W_{10} respectively.

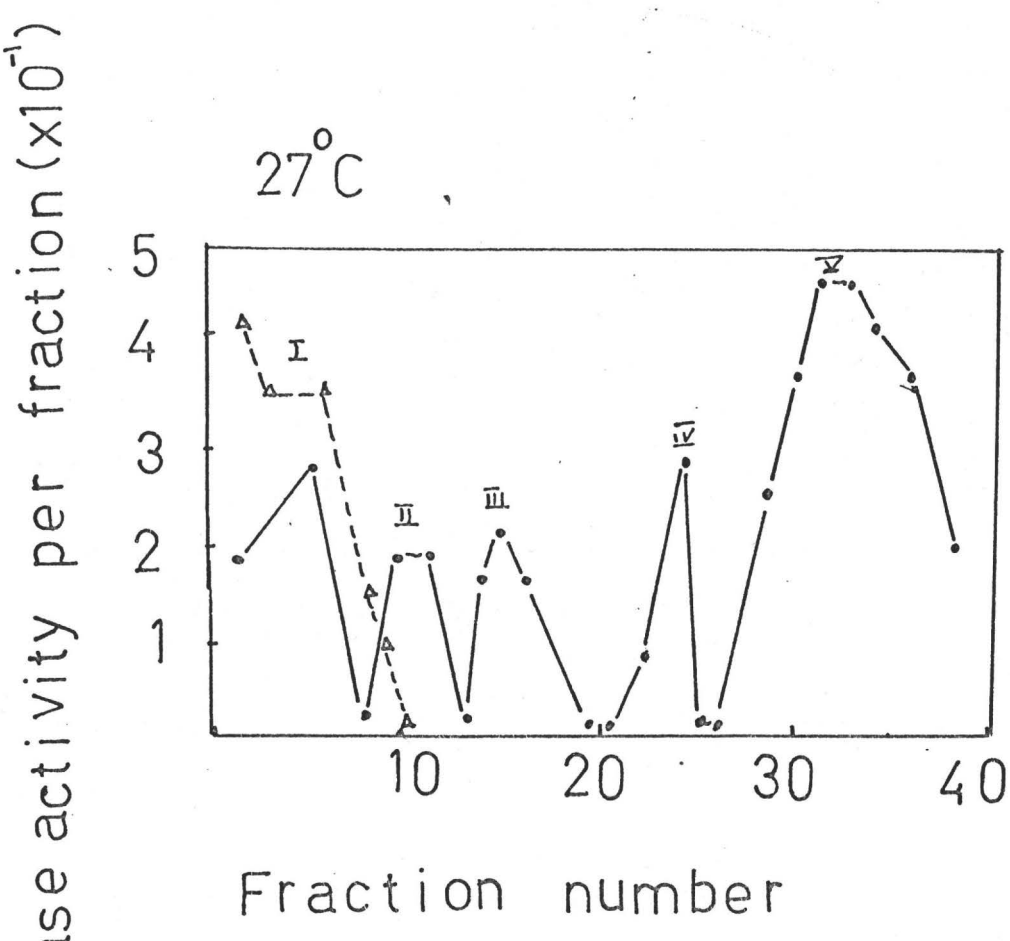
equilibrium sucrose density gradient centrifugation. Extracts of mycelia which had been derepressed or repressed at 27°C or 37°C were centrifuged through the gradients until equilibrium. Fractions were collected and nitrite reductase activity measured as described in METHODS. Species I nitrite reductase is defined as the peak of activity between fractions 3 and 11 of the gradients; species II as the peak of nitrite reductase activity between fractions 9 and 16 of the gradients; species III as the peak of nitrite reductase activity between fractions 15 and 22 of the gradients; species IV as the peak of nitrite reductase activity between fractions 20 and 30 of the gradients and species V as the peak of nitrite reductase activity between fraction 28 and 40 of the gradients.

Extracts of wild type mycelia derepressed at 27°C or 37°C had all five species of nitrite-reductase activity (Figs. 24 and 25). Preparations of wild type mycelia repressed at 27°C had only species I activity; extracts of mycelia repressed at 37°C had species I and species II nitrite reductase activity. These results suggest that the activities represented by species I at 27°C and species I and possibly species II at 37°C are not repressible under the conditions used in this study.

Extracts of nitrite-non-grower, nitrite accumulator, mutant '0' mycelia derepressed at 27°C lacked species IV and V of nitrite reductase activity (Fig. 26 (a) and (b)).

Fig. 24. Profiles of nitrite reductase activity in sucrose density gradients through which extracts of wild type mycelia derepressed and repressed at 27°C and at 37°C have been centrifuged until equilibrium.

Fractions one to three represent cell debris which has moved into the 3 M sucrose cushion at the bottom of the gradient. Units of nitrite reductase activity (X 10^{-1}) layered on the gradient and percent recovery from the gradient respectively: extracts of mycelia derepressed at 27°C, 37.2 units and 81%; extracts of mycelia repressed at 27°C, 20.4 units and 70%; extracts of mycelia derepressed at 37°C, 20.4 units and 79%; extract of wild type mycelia repressed at 37°C, 40.8 units and 81%. Extracts of derepressed mycelia (•—•); extracts of repressed mycelia (▲—▲).


27°C
Units of nitrite reductase activity per fraction (x10⁻¹)
5
4
3
2
1
I
II
III
IV
V
VI
10
20
30
40
Fraction number

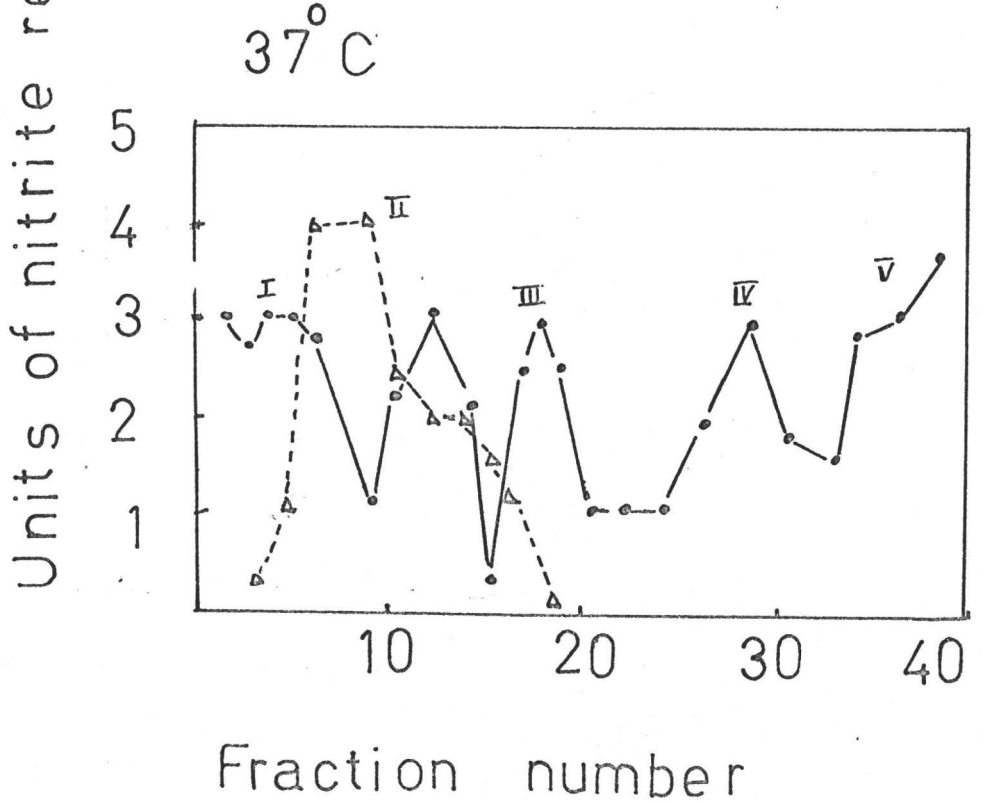
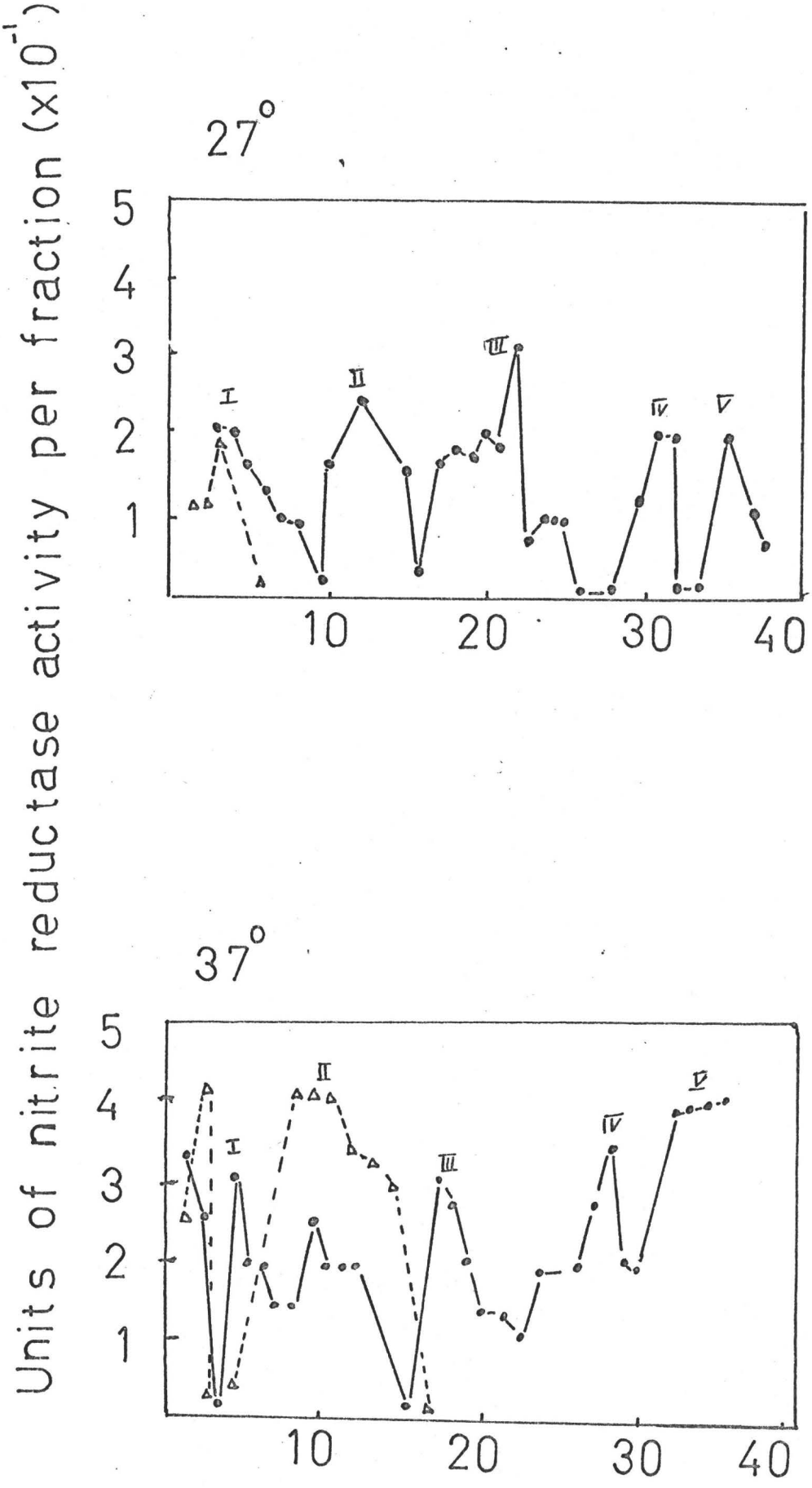

37°C
5
4
3
2
1
I
II
III
IV
V
10
20
30
40
Fraction number

Fig. 25. Profiles of nitrite reductase activity in sucrose density gradients through which extracts of wild type mycelia derepressed and repressed at 27°C and at 37°C have been centrifuged until equilibrium.

This figure shows a repetition of the experiment described in Fig. 24. Units of nitrite reductase activity (X 10^{-1}) layered on the gradient and percent recovery of from the gradient respectively: extracts of mycelia derepressed at 27°C, 40.8 units and 76%; extracts of mycelia repressed at 37°C, 20.4 units and 71%; extracts of mycelia derepressed at 37°C, 20.4 units and 81%; extracts of mycelia repressed at 37°C, 40.8 units and 69%. Extracts of derepressed mycelia (•—•); extracts of repressed (▲—▲) mycelia.


Units of nitrite reductase activity per fraction (x10⁻¹)
27°
37°
I
II
III
IV
V
1
2
3
4
5
10
20
30
40

Preparations of mutant 'O' mycelia repressed at 27°C had only species I activity. The absence of species IV and V activities in this nitrite non-utilizing mutant suggests that either or both of these activities were required for growth on nitrite.

Extracts of mutant T_{13} (temperature sensitive with respect to nitrite accumulation and the ability to grow on nitrite) mycelia derepressed at 27°C contained the five species of nitrite reductase activity (Figs. 27 and 28). Extracts of T_{13} mycelia repressed at 27°C and at 37°C had only species I nitrite reductase activity (Figs. 27 and 28).

Extracts of mutant T_{23} (which can grow on nitrite and is a temperature sensitive nitrite accumulator) mycelia repressed at 27°C and at 37°C had only species I activity (Figs. 29 (a) and (b). An extract of T_{23} mycelia derepressed at 27°C (Fig. 29) had a nitrite reductase activity profile which was quite different from those shown in Figs. 24 to 28. The whole profile appears to have shifted. This may be due to an error in the preparation of the gradient. Species I was present at both 27°C and 37°C; a second peak of activity was present between fractions 11 to 30 (Species II and III); a third peak of activity appeared between fractions 20 and 27 (part of Species III and part of Species IV); a fourth and fifth peak of activity were present between fractions 30 and 35 and fractions 35 and 40 respectively (Species V). Extracts of

Fig. 26. Profiles of nitrite reductase activity in sucrose density gradients through which extracts of mutant 'O' mycelia derepressed or repressed at 27°C have been centrifuged until equilibrium.

This figure shows two repetitions of the experiment. Units of nitrite reductase activity (X 10^{-1}) layered on the gradient and percent recovery from the gradient respectively: extracts of mycelia derepressed at 27°C, 34.8 units and 62% (Fig. (a)); extracts of mycelia repressed at 27°C, 19.2 units and 58% (Fig. (a)); extracts of mycelia derepressed at 27°C, 33.8 units and 52% respectively (Fig. (b)). Extracts of derepressed mycelia (•—•); extracts of repressed mycelia (▲—▲).

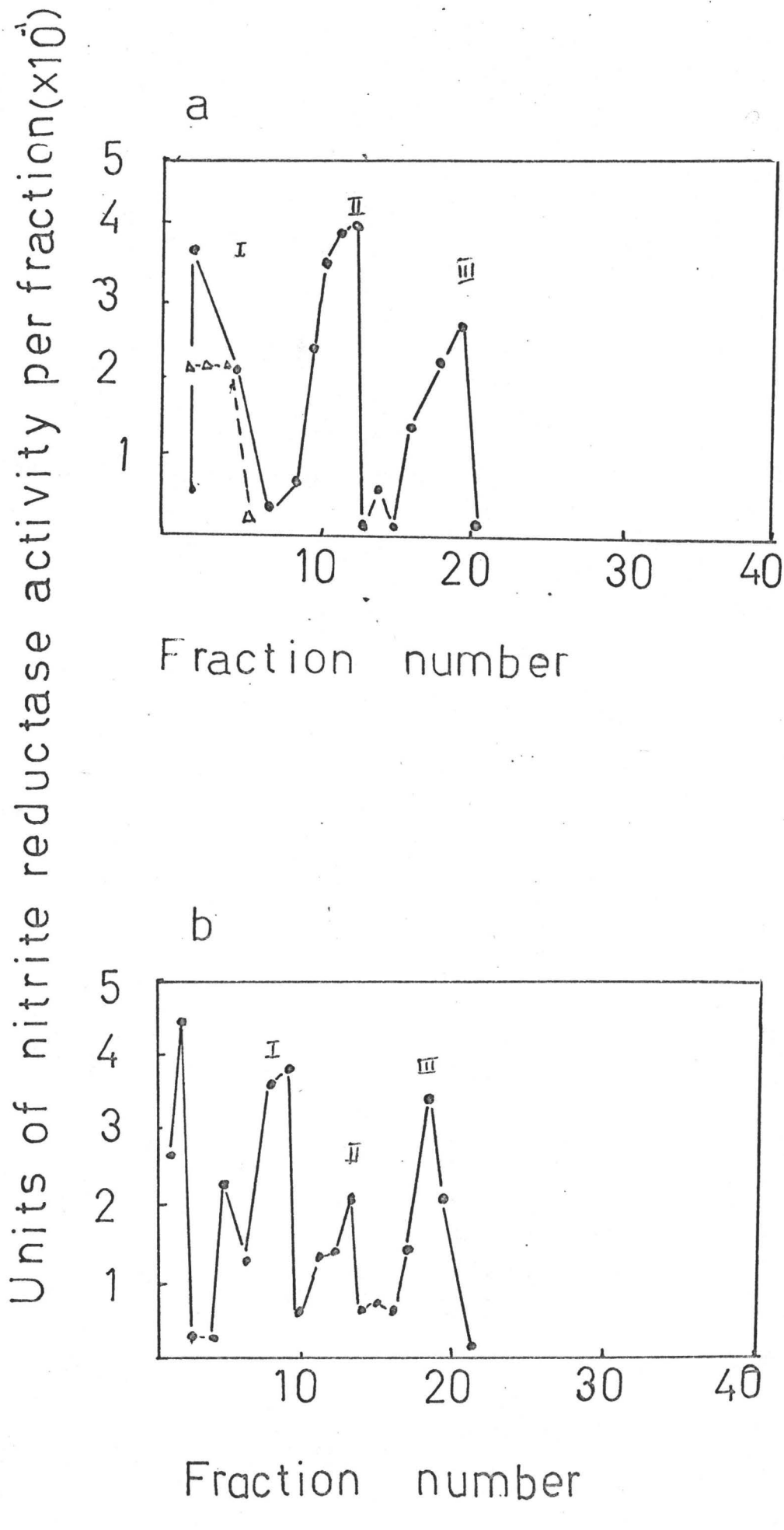


Fig. 27. Profiles of nitrite reductase activity in sucrose density gradients through which extracts of mutant T_{13} mycelia derepressed and repressed at 27°C and at 37°C have been centrifuged until equilibrium.

Units of nitrite reductase activity (X 10^{-1}) layered on the gradient and percent recovery from the gradient respectively: extracts of mycelia derepressed at 27°C, 48 units and 80%; extracts of mycelia derepressed at 37°C, 42 units and 78%; extracts of mycelia repressed at 27°C, 25.2 units and 56%; extracts of mycelia repressed at 37°C, 19.2 units and 96%. Extracts of T_{13} derepressed (•—•) and repressed (▵—▵) mycelia.

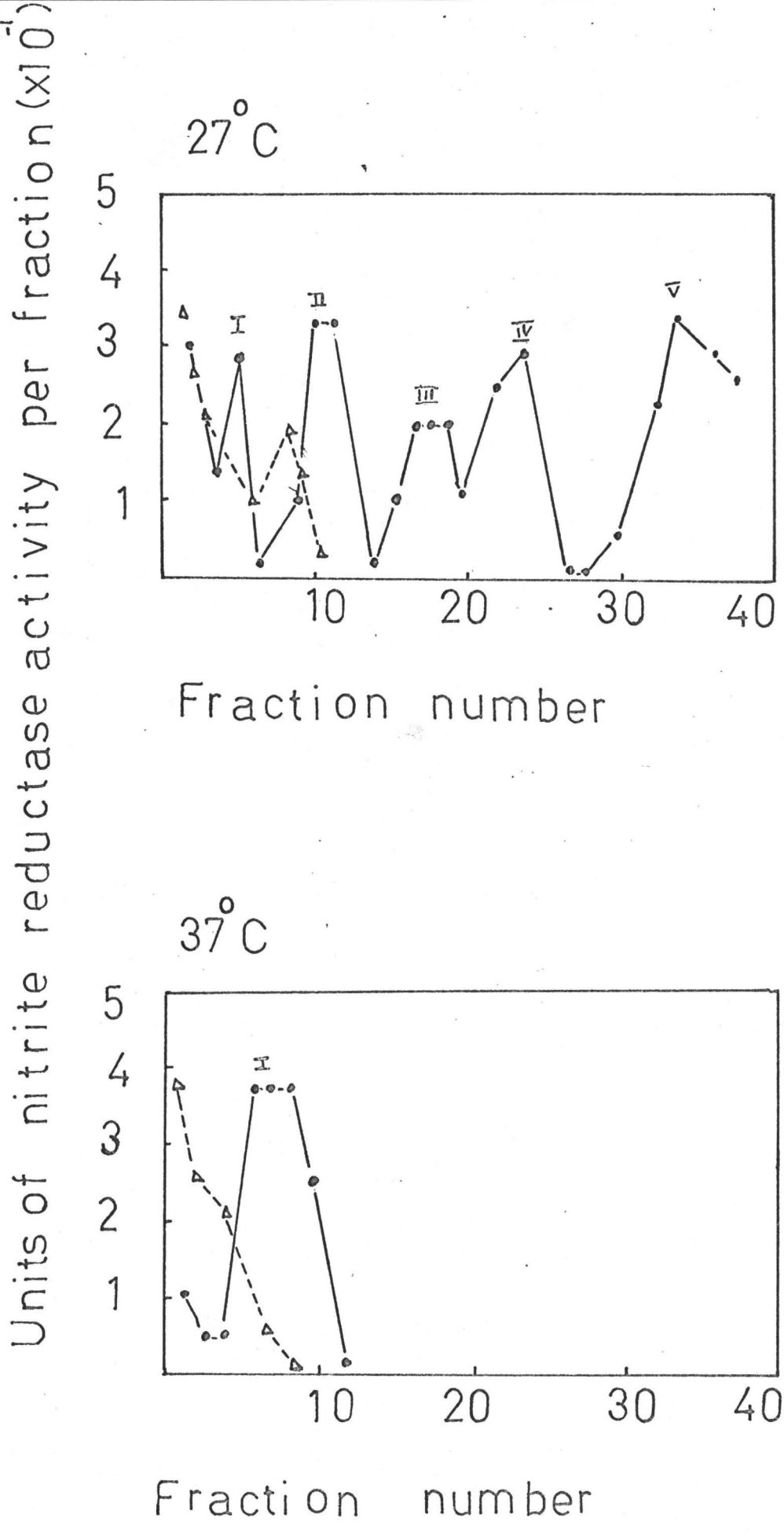
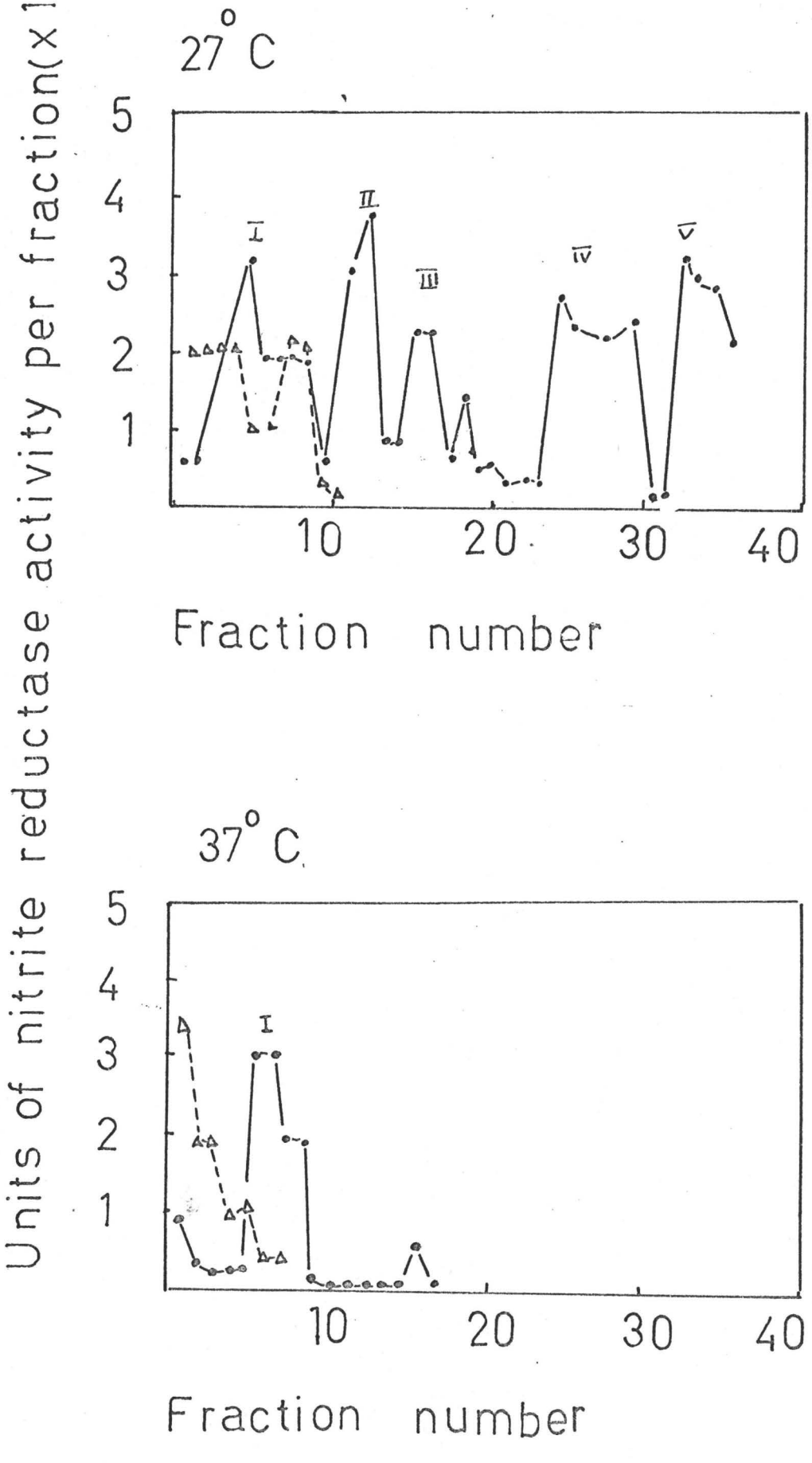

27°C
Units of nitrite reductase activity per fraction (x10⁻¹)
5
4
3
2
1
I
II
III
IV
V
10
20
30
40
Fraction number
37°C
5
4
3
2
1
I
10
20
30
40
Fraction number

Fig. 28. Profiles of nitrite reductase activity in sucrose density gradients through which extracts of mutant T_{13} derepressed and repressed at 27°C and at 37°C have been centrifuged until equilibrium.

This figure shows a repetition of the experiments described in Fig. 27. Units of nitrite reductase activity layered on the gradient and percent recovery from the gradient respectively: extracts of mycelia derepressed at 27°C, 48 units and 93%; extracts of mycelia derepressed at 37°C, 19.2 units and 63%; extracts of mycelia repressed at 27°C, 20.2 units and 56%; extracts of mycelia repressed at 37°C, 19.2 units and 92%. Extracts of T_{13} derepressed (•—•) and repressed (Δ—Δ) mycelia.


27° C
37° C
Units of nitrite reductase activity per fraction(x 10⁻¹)
Fraction number
Fraction number
I
II
III
IV
V
1
2
3
4
5
10
20
30
40

T_{23} mycelia derepressed at 37°C (Fig. 29 (b)) had nitrite reductase profiles like T_{23} mycelia derepressed at 27°C (Fig. 29 (a)) but lacked the fifth peak of activity.

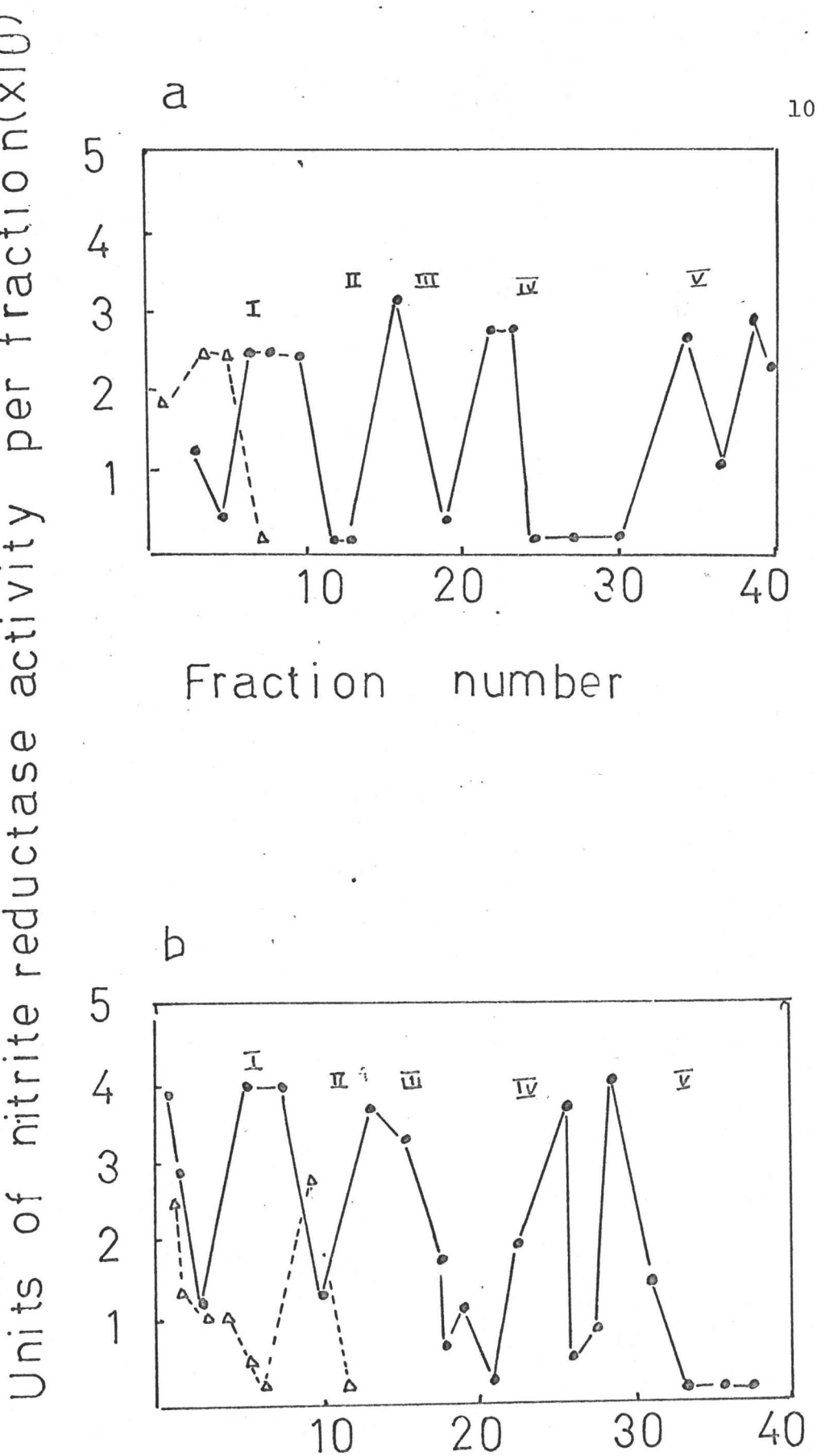
Large variations were observed in the positions in the gradient of the various species of nitrite reductase activity. These variations could have been due to small differences in growth conditions which have been found to drastically affect nitrite reductase activity and/or to variations in the preparation of gradient and the collection of fractions.

In view of these variations, it is difficult to conclude much from the sucrose density gradient data. Species I activity at 27°C and possibly species I and species II at 37°C may be constitutive. Species I activity is present in all of the mutants tested, it is probably not the activity necessary for the assimilation of nitrite in vivo. Little can be said about species III, IV and V except that they are repressible. The studies with mutants suggest that species IV of nitrite reductase activity which is present in extracts of strains able to grow on nitrite but absent in those unable to utilize nitrite (e.g. mutant 'O' at 27°C and mutant T_{13} at 37°C) may be the activity necessary for the reduction of nitrite in vivo.

Attempts were made to characterize some of the peaks of activity observed when extracts of wild type mycelia were

Fig. 29. Profiles of nitrite reductase activity in sucrose density gradients through which extracts of mutant T_{23} mycelia derepressed and repressed at (a) 27°C and (b) 37°C have been centrifuged until equilibrium.

Units of activity layered on the gradient and percent recovery from the gradient respectively: extracts of mycelia derepressed at 27°C, 40 units and 72%; extracts of mycelia repressed at 27°C, 20 units and 67%; extracts of mycelia derepressed at 37°C, 48 units and 83%; extracts of mycelia repressed at 37°C, 18 units and 74%. Extracts of derepressed (•—•) and repressed (Δ—Δ) mycelia.


a
b
I
II
III
IV
V
1
2
3
4
5
10
20
30
40
Fraction number
Units of nitrite reductase activity per fraction(x10⁻¹)

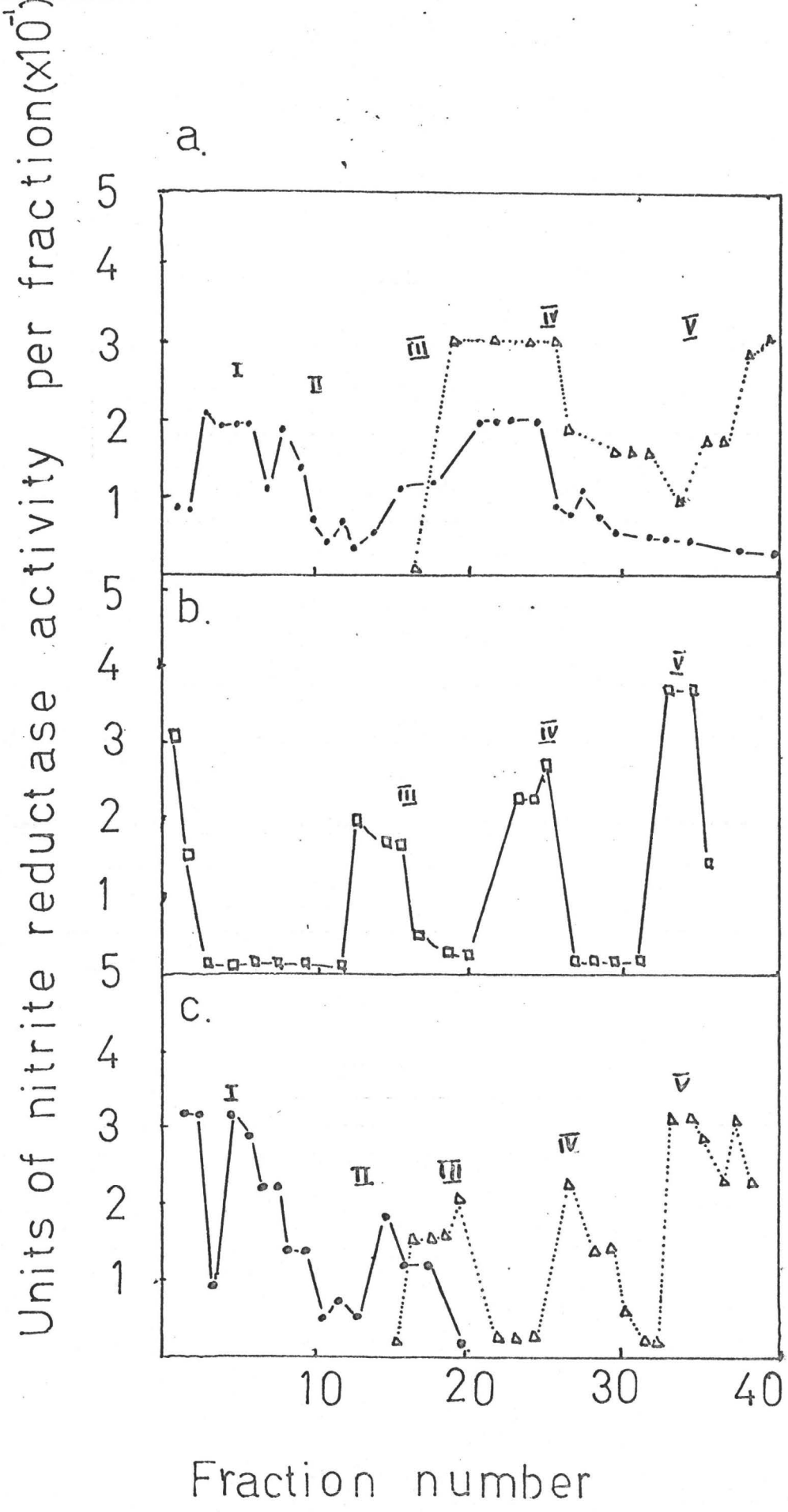
subjected to equilibrium sucrose density gradient centrifugation. Fractions P_5, P_{27} and S_5 (possibly representing "mitochondrial", "microsomal" and "soluble" fraction, respectively) were analysed as follows: Unwashed fraction P_5 (see Fig. 23) had Species I, II, III and part of IV (Fig. 30 (a)). Unwashed fraction P_{27} contained species III, IV and V (Fig. 30 (a)). Fraction S_5 had species IV and V and a third species which may have included both species II and III. A somewhat clearer picture was obtained when P_5 and P_{27} pellets were washed ten times before centrifugation through the gradient (Fig. 30 (c)). Fractions S_5 and P_{27} appear to be equivalent. Fraction P_5 differs from P_{27} in both its profile of nitrite reductase in equilibrium sucrose density gradients and in the ease with which nitrite reductase activity can be removed from it by washing.

The failure to clearly separate mitochondrial, microsomal and soluble fractions by differential centrifugation made this method unsuitable for characterizing the various peaks of nitrite reductase activity. The differences observed between fractions P_5 and P_{27}, however, are in agreement with the tentative conclusion that there is more than one type of nitrite reductase associated with different subcellular particles.

The preliminary experiment with differential centrifugation of extracts of wild type mycelia described above showed that there was nitrite reductase activity

Fig. 30. Profiles of nitrite reductase activity in sucrose denstiy gradients through which fractions P_5, P_{27} and S_5 (see Fig. 23) of treated extracts of wild type mycelia derepressed at 27°C have been centrifuged until equilibrium.

This figure represents two separate experiments: in one ((a) and (b)) fraction S_5 and unwashed fractions P_5 and P_{27} were analysed; in the other (c) P_{27} pellet was washed ten times before centrifugation through sucrose density gradients. Units of nitrite reductase activity layered on the gradient and percent recovery from the gradient respectively: extract of P_5 (Fig. a) 40 units and 80%; extract of P_{27} (Fig. a) 38.4 units and 80%; extract of P_5 (Fig. c) 28.5 units and 60%; extract of P_{27} (Fig. c) 45.6 units and 58%; and extract of S_5 (Fig. b) 29 units and 84%. Extracts of P_5 (•—•), P_{27} (Δ·····Δ) and S_5 (□—□).


a.
b.
c.
I
II
III
IV
V
Units of nitrite reductase activity per fraction(x10⁻¹)
1
2
3
4
5
10
20
30
40
Fraction number

associated with a fraction that presumably contained mitochondria. Attempts were thus made to relate the nitrite reductase peaks (obtained by equilibrium sucrose density gradient analysis of extracts) to the presence of mitochondria. It was also necessary to determine whether the mitochondria in all strains were normal. Fractions from sucrose density gradients through which extracts of wild type mycelia had been centrifuged until equilibrium were assayed for mitochondria by Janus Green B staining and by measuring mitochondrial respiration polarographically.

Janus Green B activity was demonstrated in a broad peak ranging from fractions 6 to 26 (Fig. 31). Fractions from equilibrium sucrose gradients through which extracts of wild type mycelia repressed and derepressed at 27°C and at 37°C (Figs. 32 and 33), of mutant 'O' mycelia derepressed at 27°C (Fig. 34 (b)); of mutant T_{13} mycelia derepressed at 37°C (Fig. 35) and of mutant T_{23} mycelia derepressed at 37°C were assayed for mitochondrial respiration polarographically. Mitochondrial respiratory activity in each experiment was found in a band extending from fraction one to about fraction twenty six of the gradients. The data from the Janus Green B experiment and the polarographic experiments agree.

It may be concluded from these experiments that: (i) the mutant strains are similar to the wild type strain with respect to mitochondria, and,

Fig. 31. Profile of Janus Green B mitochondrial activity in a sucrose density gradient through which an extract of wild type mycelia derepressed at 27°C was centrifuged until equilibrium.

Aliquots of each fraction were stained with Janus Green B as described in METHODS. The stained preparations were examined under oil with a light microscope. Mitochondria were counted in three microscopic fields for each aliquot and an average number of stained particles per field calculated. One ml of homogenate from which nuclei and cell debris had been removed (see METHODS) was layered on the gradient.

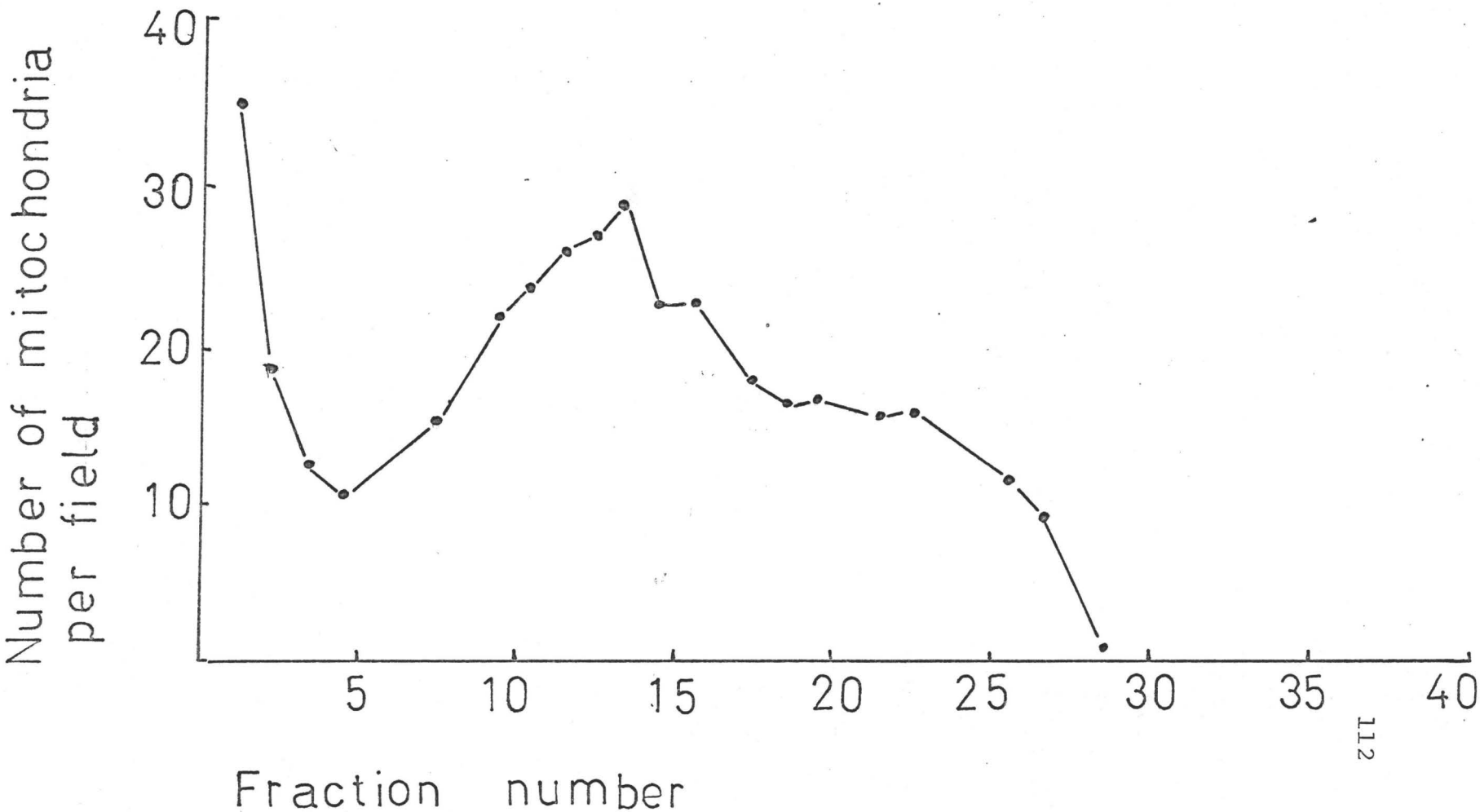

Number of mitochondria per field
40
30
20
10
5
10
15
20
25
30
35
40
Fraction number

Fig. 32. Profile of mitochondrial respiratory activity in sucrose density gradients through which extracts of wild type mycelia derepressed at 27°C and at 37°C have been centrifuged until equilibrium.

Respiration was measured in a Clark oxygen electrode as described in METHODS. One ml of the homogenate prepared as described in METHODS was layered on the gradient.

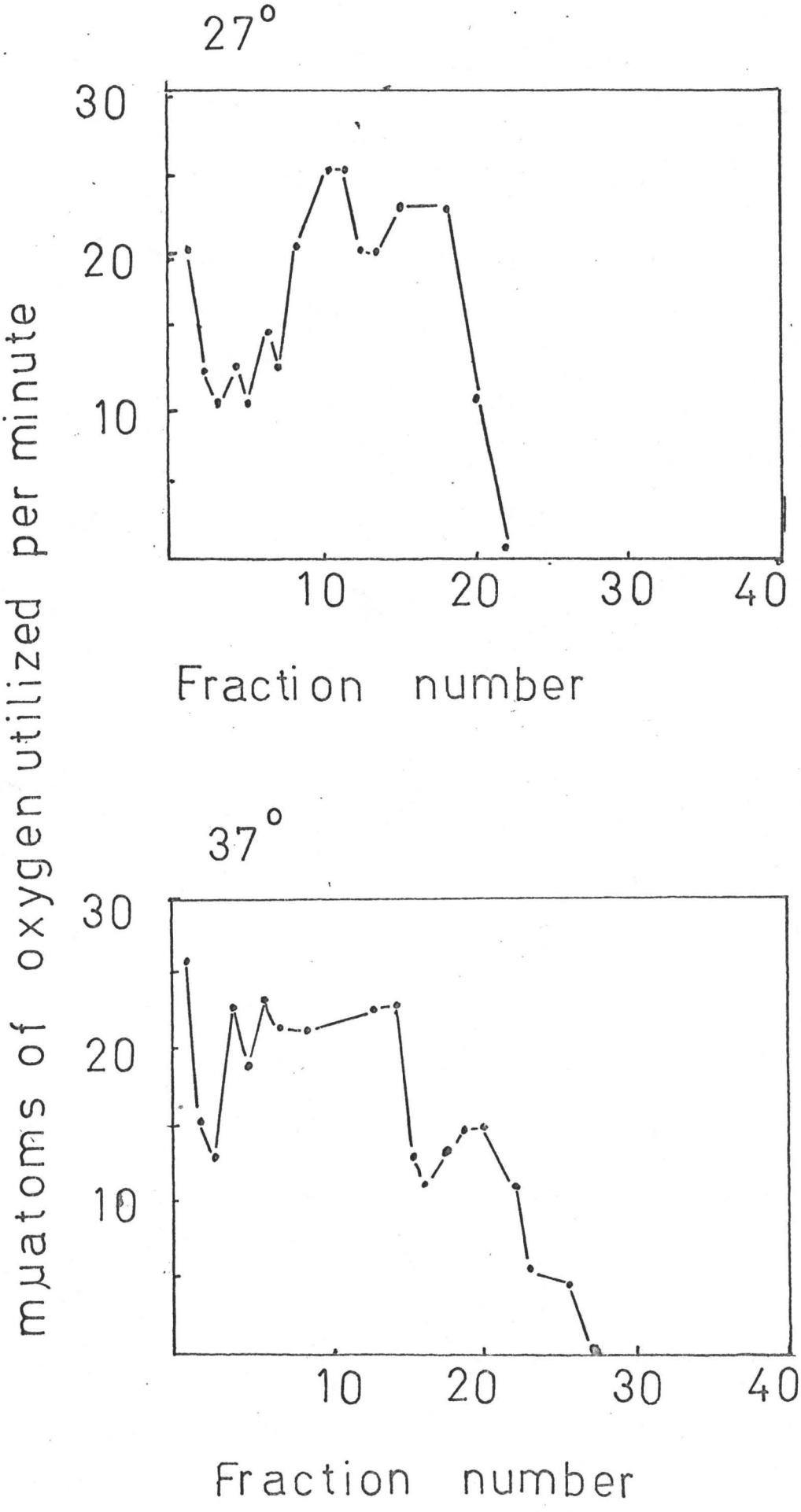

27°
30
20
10
10
20
30
40
Fraction number
37°
30
20
10
10
20
30
40
Fraction number
mμatoms of oxygen utilized per minute

Fig. 33. Profile of mitochondrial respiratory activity in sucrose density gradients through which extracts of wild type mycelia repressed at 27°C (a) and repressed at 37°C (b) have been centrifuged until equilibrium.

One ml of homogenate prepared as described in METHODS was layered on the gradient.

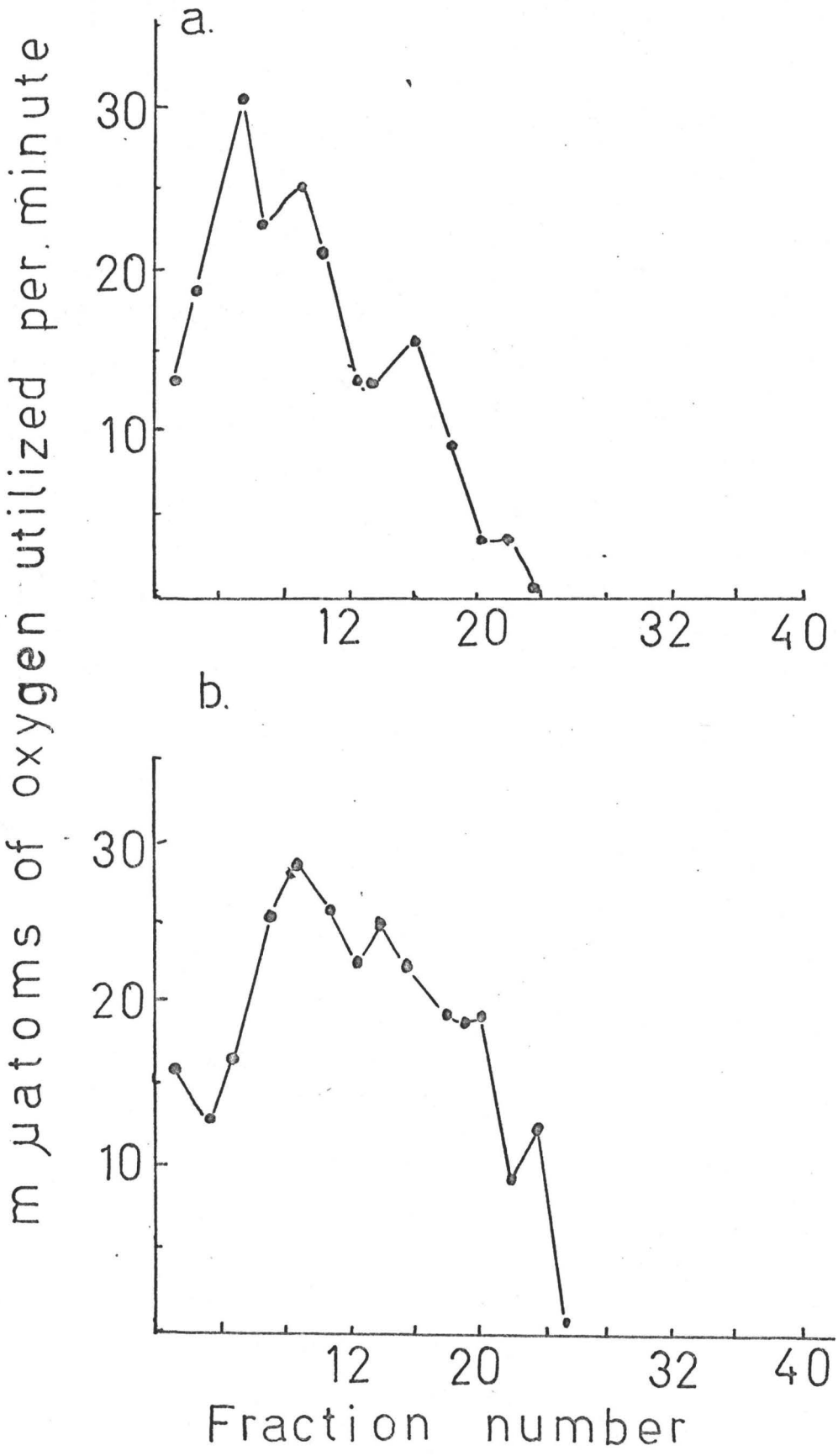

a.
30
20
10
12
20
32
40
b.
30
20
10
12
20
32
40
m μatoms of oxygen utilized per minute
Fraction number

Fig. 34. Profile of mitochondrial respiratory activity in sucrose density gradient through which extracts of mutant T_{23} mycelia derepressed at 37°C (a) and of mutant 'O' derepressed at 27°C (b) have been centrifuged until equilibrium.

One ml of the homogenate prepared as described in METHODS was layered on the gradient.

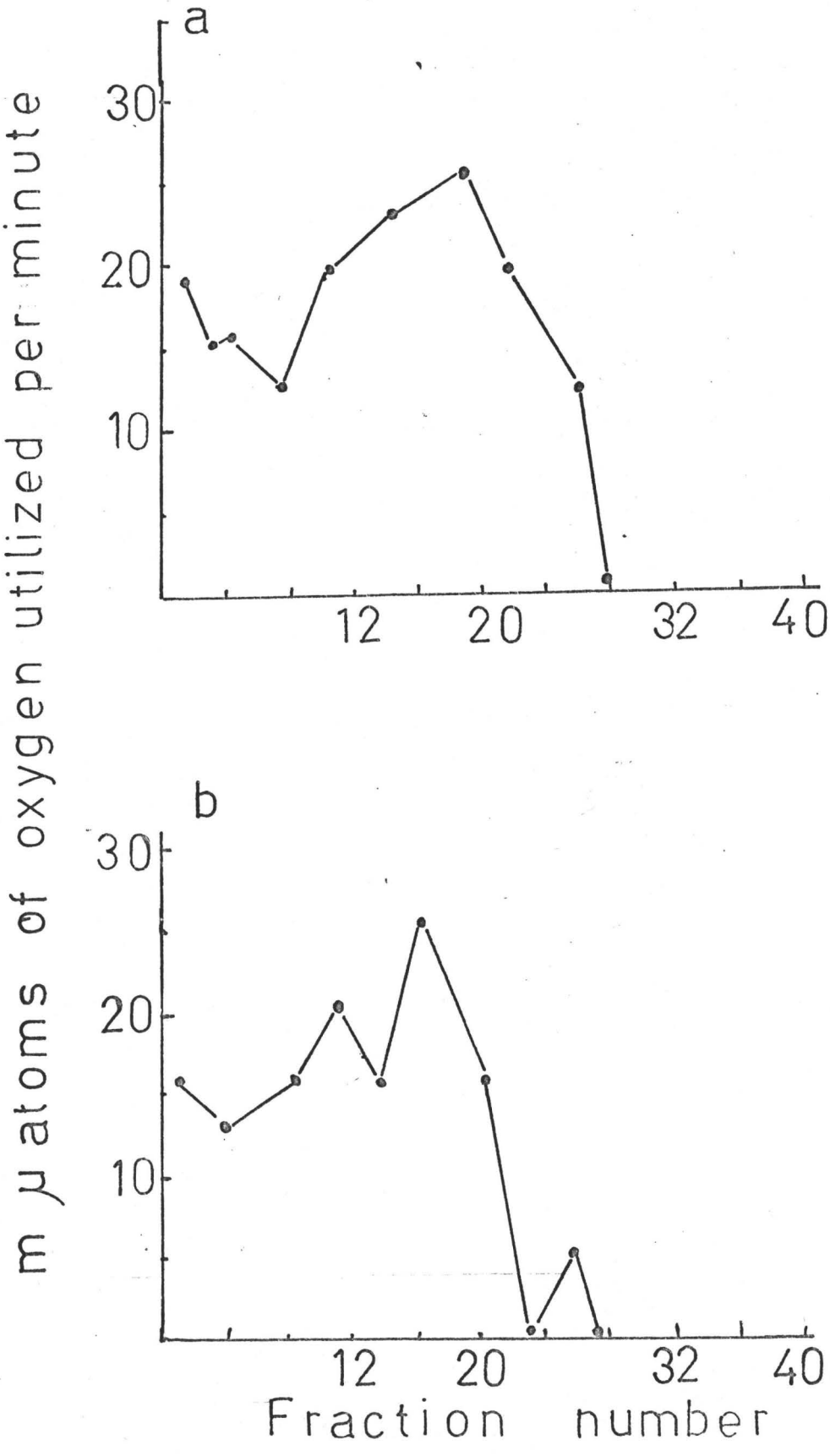
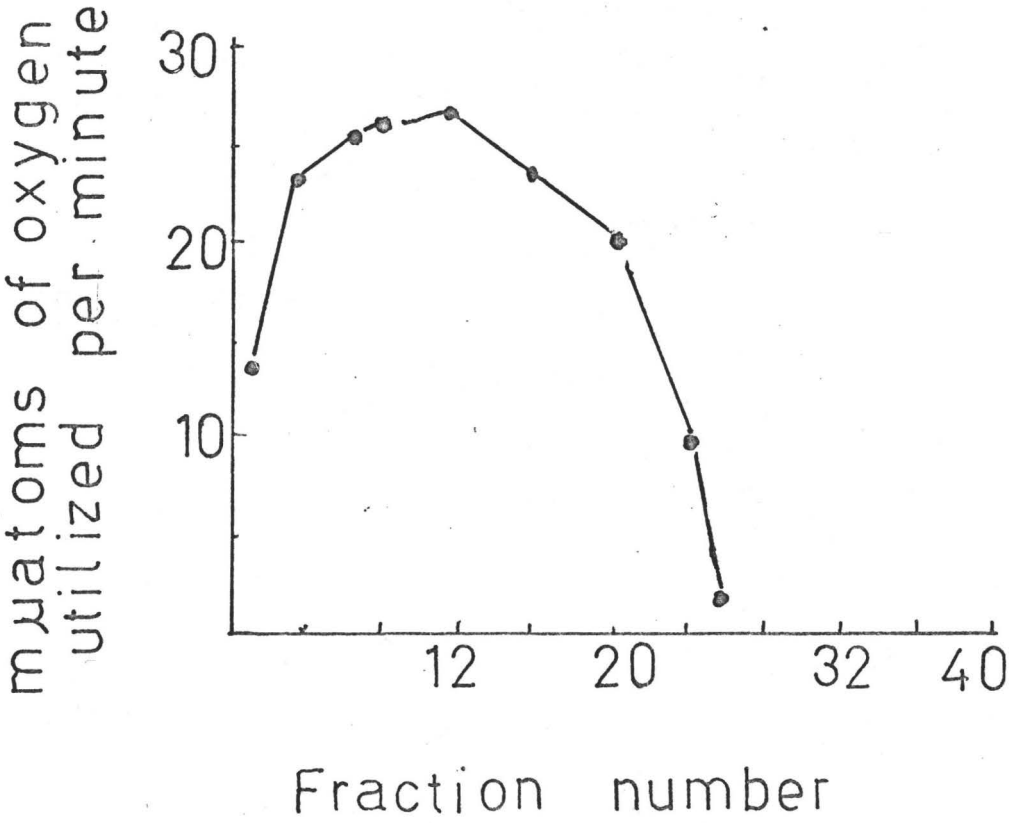

a
b
30
20
10
12
20
32
40
m μ atoms of oxygen utilized per minute
Fraction number

Fig. 35: Profile of mitochondrial respiratory activity in a sucrose density gradient through which an extract of mutant T_{13} mycelia derepressed at 37°C has been centrifuged until equilibrium.

One ml of homogenate prepared as described in METHODS was layered on the gradient.


mμatoms of oxygen utilized per minute
30
20
10
12
20
32
40
Fraction number

(2) that mitochondria are present in fractions which contain nitrite reductase activity (Species I, II, III and part of IV).

If binding of nitrite reductase to a particle is necessary for activity, the species of activity absent in sucrose density gradient profiles of the mutants could be due either to missing nitrite reductase enzymes or to missing or defective particles to which the nitrite reductase molecule normally binds. The following experiment was carried out to try and resolve this question. Repressed wild type strain should have all the particles necessary for growth on Casamino Acids because it can grow normally on this medium. If non specific binding of nitrite reductase to particles is necessary for activity, the species of activity missing in the mutant nitrite reductase activity profile should be regenerated when mixtures of extracts of wild type mycelia repressed at 27°C and extracts of derepressed mutant mycelia are analysed by sucrose density gradient centrifugation. Mixtures (1:1) of extracts of wild type mycelia repressed and 27°C and of extracts of mutant 'O' mycelia derepressed at 27°C or of mutant T_{13} derepressed at 37°C were subjected to equilibrium sucrose density gradient centrifugation. Similar nitrite reductase activity profiles were obtained whether the mutant extracts were centrifuged alone or in mixtures with repressed wild type extract (Fig. 36). This experiment shows that nitrite reductase does not bind

loosely and non-specifically to particles. The data are also compatable with the hypothesis that nitrite reductase is tightly bound to different types of particles.

Fig. 36. Profiles of nitrite reductase activity in sucrose density gradients through which mixtures of extracts of repressed wild type mycelia and of derepressed mutant 'O' or of mutant T_{13} mycelia have been centrifuged until equilibrium.

(a) Profiles of nitrite reductase activity of extracts of T_{13} mycelia derepressed at 37°C (Δ--Δ), of wild type mycelia repressed at 27°C (•—•) and of a mixture (1:1) of extracts of wild type mycelia repressed at 27°C and of mutant T_{13} mycelia derepressed at 37°C (□....□). Units of nitrite reductase activity layered on the gradients and percent recovery of activity from the gradients respectively: extract of T_{13} mycelia derepressed at 37°C, 42 units and 78%; extract of wild type repressed at 27°C, 37 units and 79%; mixture of extracts of T_{13} mycelia derepressed at 37°C and of wild type repressed at 27°C, 36 units and 61%.

(b) Profiles of nitrite reductase activity of extracts of mutant 'O' mycelia derepressed at 27°C (▲--▲) and of wild type repressed at 27°C (•—•). Units of nitrite reductase activity layered on the gradients and recovery of activity from the gradients respectively: extracts of mutant 'O' mycelia derepressed at 27°C, 50 units and 61%; extract of wild type mycelia repressed at 27°C, 37 units and 79%.

(c) Profiles of nitrite reductase activity in sucrose density gradients through which a mixture (1:1) of extracts of wild type mycelia repressed at 27°C and of mutant 'O' mycelia

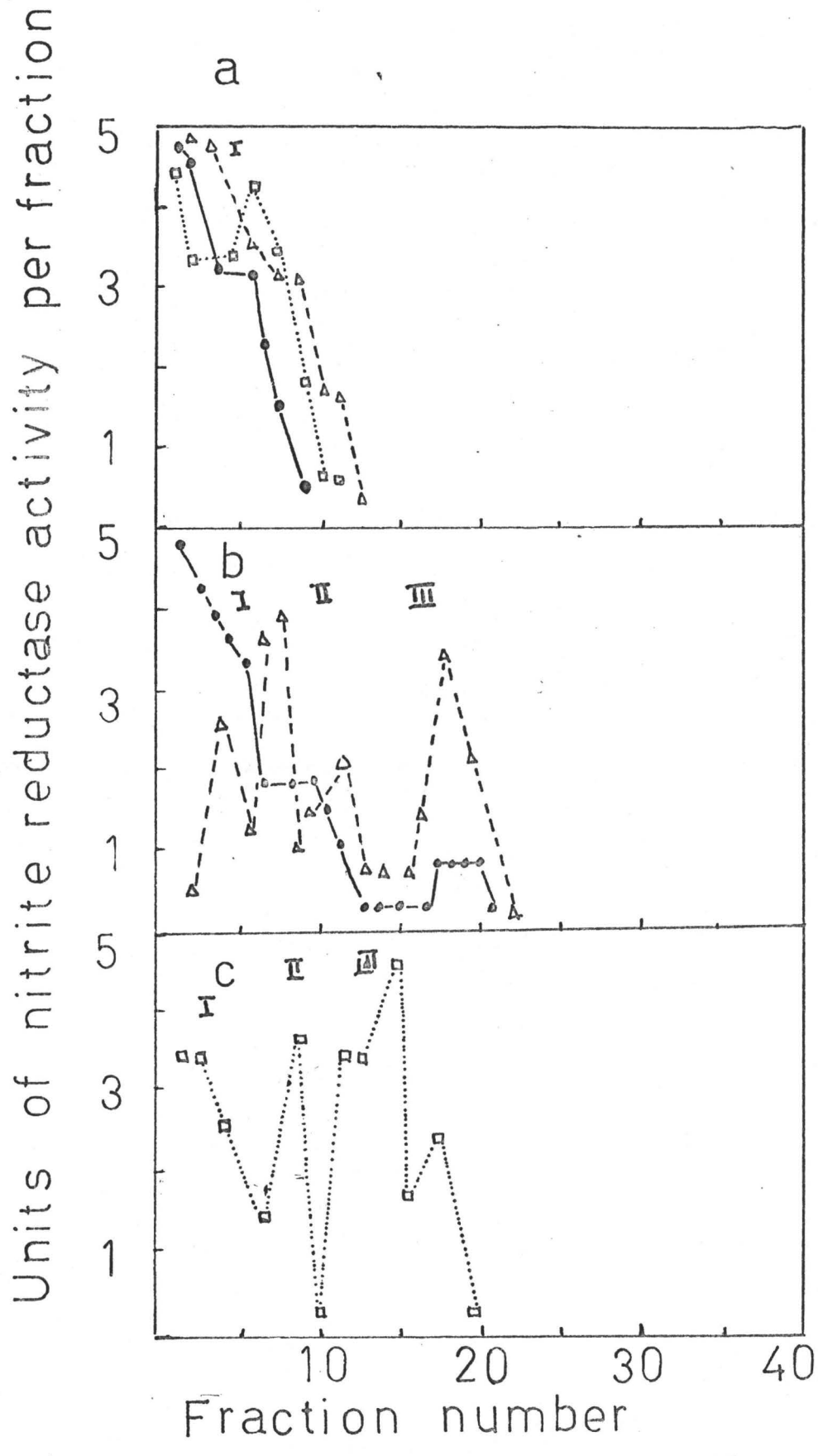

a
b
c
I
II
III
Units of nitrite reductase activity per fraction
Fraction number
1
3
5
10
20
30
40

Fig. 36 (continued)

repressed at 27°C were centrifuged until equilibrium (▫·····▫). Units of activity layered on the gradient and percent recovery of activity from the gradient respectively: 50 units and 75%.

DISCUSSION AND CONCLUSIONS

The following conclusions can be made from the studies reported here:

1. It appears that at least three and probably four genes and at least three and probably four cistrons are involved in the control of nitrite reduction. The genes are located on two linkage groups and are not allelic to the genes controlling nitrate reductase.
2. Nitrite reductase activity is present in more than one differential centrifugal fraction of extracts of derepressed wild type mycelia. Nitrite reductase activity is present in fractions from differential centrifugation and from equilibrium sucrose density gradient centrifugation which also contain mitochondria.
3. The sucrose density gradient studies suggest that species IV of nitrite reductase activity which is present in extracts of mycelia of strains able to grow on nitrite but absent from extracts of mycelia of mutants unable to grow on nitrite, may be the activity necessary for the *in vivo* reduction of nitrite.

The conclusions from the genetic studies are made with the reservation that the very low germination obtained in crosses could represent differential germination

of phenotypes. The recombination data from the crosses was, however, in agreement with the complementation studies.

The nitrite-non-utilizing mutants obtained in this study had nitrite reductase activity (4). This finding could be explained in the following three ways:

(a) The assay system used (4,5) does not measure an *in vivo* activity,

(b) The assay system used measures more than one activity,

(c) Nitrite reductase is localized within a particle.

A permease barrier in the mutants could prevent nitrite, which has resulted from nitrate reduction in the cytoplasm, from entering the particle and being further reduced. Such particles could have been disrupted during the extraction procedure (4,5) and the enzyme thus exposed to its substrate. Evidence is presented that suggests hypotheses (b) and (c) may be involved.

The nitrite-non-utilizing mutants of Silver and McElroy (58) also had nitrite reductase activity. However, the nitrite reductase assay used by these workers is now known to be doubtful. No attempts were made to determine how the nitrite reductase activity of the mutants differed from that of the wild type strain. In this study extracts of mutants were found to differ from extracts of the wild type strain in the presence of certain species of nitrite reductase when analysed by sucrose density gradient

centrifugation.

Nitrite reductase in higher plants is reported to be localized within an organelle (2,28,36,46,51,52,72). The studies with differential centrifugation and equilibrium sucrose density gradients reported here also suggest an association of nitrite reductase activity with subcellular particles. These studies also suggest that there is more than one kind of nitrite reductase in Neurospora crassa.

The nitrite reductase activities represented by species I at 27°C and by species I* and species II* at 37°C may be constitutive. It is possible that some of the species I activity is associated with whole cells which were not disrupted during the extraction procedure. The activity in these species was approximately one fifth of the total nitrite reductase activity recovered from a gradient through which extracts of derepressed wild type mycelia had been centrifuged until equilibrium. The finding of a possible constitutive nitrite reductase species is in agreement with the report of Cook (4,5) that casamino acids (repressing medium) only partially repressed nitrite reductase. The level of specific activity of nitrite reductase in extracts of repressed mycelia was one third to one quarter of the specific activity obtained in extracts of derepressed mycelia.

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* cf. RESULTS, page 94

The analysis of nitrite-non-utilizing mutants by equilibrium sucrose density gradient centrifugation, suggests that of the five nitrite reductase species observed, species IV* may be necessary for the reduction of nitrite *in vivo*. This species (IV) is present in strains which can grow on nitrite but absent in the two mutants which are unable to grow on nitrite. Much variation was observed in the position in the gradients of the various species of nitrite reductase activity. These studies should therefore be continued and should be further substantiated with the analysis of other mutants that are unable to grow on nitrite.

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APPENDIX

TABLE 8

Phenotypic Characteristics of Presumptive Mutants

Strain	Growth of Mycelium on Various Nitrogen Sources % of dry weight on casamino acids				Accumulation of Nitrite mμ moles/ml of media/mg dry weight at 70 hrs
	Amino Acids	NH_3	NO_3^-	NO_2^-	
A	230	39	11	28	0.14
B	260	40	8	14	0.14
C	193	56	27	10	0.14
D	170	53	23	27	2.2
F	80	85	25	38	0.16
I	260	38	16	5	0.13
J	100	60	6	25	1.03
M	100	42	8	10	1.07
N	150	43	8	11	1.11
O	230	44	2	2	9.52
Q	150	30	5	15	0.64
R	115	5	4	4	12.36
T	20	-	-	-	-
U	175	57	28	3	0.53
V	8	-	-	-	-
W	212	56	92	6	3.98
X	210	48	12	6	0.95
Y	215	47	30	2	3.76
Z	140	54	4	2	3.54
A-1	260	15	2	4	3.00
A-2	160	48	1	9	0.06
A-5	180	-	-	-	
A-6	185	51	46	13	0.05
A-7	40	60	13	23	0.16
wild type	220	43	55	48	0.11
nit-1	265	43	1	12	0.01

Each strain was tested semiquantitatively for growth on various nitrogen sources and for accumulation of nitrite at 27°C as described in METHODS.

TABLE 9

Phenotypic Characteristics of Presumptive Mutants

Strain	Growth of Mycelium on Various Nitrogen Sources % of dry weight on casamino acids						Accumulation of Nitrite mμ moles/ml of media/mg dry weight at 70 hrs	
	Amino Acids		Nitrate		Nitrite			
	27°	37°	27°	37°	27°	37°	27°	37°
T-1		233		1		3	0	0.09
T-2	47	352	34	28		18	12.9	17.18
T-3		41		3		10	0.01	-
T-4	257	20	9	17		10	0.06	0.31
T-5	140	160	7	2		23	0	0.56
T-6	145	118	3	3		8	0.02	0.95
T-7	220	195	3	3		16	0	0.16
T-8	243	200	15	34		10	0.04	0.81
T-9	197	200	18	35		20	0	4.90
T-10	200	185	5	6		12	0.25	1.24
T-11		264		15		10	0.06	0.40
T-12							0.34	13.04
T-13	153	187	11	1	21	4	0.88	9.39
T-14	160	190	56	46		6	0.05	2.09
T-15	173		5				-	-
T-16	175	320	20	25		13	0.17	0.77
T-17	172	175	28	24		24	1.21	0.82
T-18	223	195	29	26		14	3.22	0.51
T-19	175	195	47	17		11	0.31	0.59
T-20	100	133	72	35		69	3.44	7.47
T-21		225		49		-	0.26	0.27
T-22		221		67		-	0.15	0.20
T-23	222	309	74	27	44	39	0.11	0.94
T-24		255		48			0.15	0.19
T-25		337		36			0.15	0.61
T-26		230		28			0.33	0.24
T-27		236		29			0.21	0.21
T-28		288		63			0.15	0.83
T-29		305		39			0.18	-
T-30		221		15			0.33	0.32
T-31		232		76			0.45	0.09
T-32		210		49			0.16	0.66
T-33		225		31			0.14	0.33
T-34		100		70			2.15	4.41
T-35		277		27			0.30	0.26
T-36		61		59			0.39	1.01

TABLE 9 (continued)

Strain	Growth of Mycelium on Various Nitrogen Sources % of dry weight on casamino acids						Accumulation of Nitrite mμ moles/ml of media/mg dry weight at 70 hrs	
	Amino Acids		Nitrate		Nitrite			
	27°	37°	27°	37°	27°	37°	27°	37°
T-37		151		46			0.19	0.24
T-38		213		75	44	37	3.74	0.41
T-39		202		49			0.40	2.01
							0.16	0.88
wild type	226	192	24	55	48	18	0.04	0.14
nit	265	213	1	1	12	12	0	0

Each strain was tested for nitrite accumulation and for growth on various nitrogen sources at 27°C and at 37°C as described in METHODS.