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NAME OF AUTHOR/*NOM DE L'AUTEUR* Anne Louise Toby

TITLE OF THESIS/*TITRE DE LA THÈSE* Enrichment, Isolation and Characterisation of Mutants in *Eudorina elegans*

UNIVERSITY/*UNIVERSITÉ* Simon Fraser University

DEGREE FOR WHICH THESIS WAS PRESENTED/*GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* Doctor of Philosophy

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1979

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. C. L. Kemp, Associate Professor

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ENRICHMENT, ISOLATION AND CHARACTERIZATION OF MUTANTS IN EUDORINA ELEGANS (ERHENBERG)

by

Anne Louise Toby
B.Sc., Simon Fraser University, 1972

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Biological Sciences

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March 1979

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Approval

Name: Anne Louise Toby

Degree: Doctor of Philosophy

Title of Thesis: Enrichment, Isolation and Characterization of Mutants in *Eudorina elegans* (Ehrenberg)

Examining Committee:

Chairman: Dr. M. Mackauer

Dr. C. L. Kemp,
Senior Supervisor

Dr. J. E. Rahe

Dr. A. Beckenbach,
Non-Supervisory Committee Examiner

Dr. G. Kochert,
Professor, University of Georgia
External Examiner

Date approved: April 2, 1979

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Enrichment, Isolation and Characterisation of Mutants in *Eudorina elegans*

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ABSTRACT

An enrichment procedure has been developed that results in at least a 200x increase in the recovery of mutants in the colonial alga, Eudorina elegans (Ehrenberg). A period of nitrogen starvation followed by treatment with 8-azaguanine results in the death of wild type cells and the maintenance of mutants. Optimal conditions of nitrosoguanidine and ultraviolet light mutagenesis prior to enrichment have been established using the reversion frequency of an acetate requiring strain and the induction frequency of sectored colonies. Nitrosoguanidine is more effective in causing the reversion of the acetate requiring strain and inducing auxotrophs. Morphogenetic mutants are more readily induced by ultraviolet light. The effectiveness of ultraviolet light as a mutagen is cell cycle dependent whereas the mutagenic action of nitrosoguanidine is not dependent on the stage in the life cycle at the time of treatment.

As a result of nitosoguanidine treatment and the enrichment procedure, acetate dependent, p-aminobenzoic acid requiring, nicotinamide requiring and mutants requiring a reduced nitrogen source have been isolated. Three independently isolated mutants defective in or lacking nitrate reductase activity have been characterized. Nar-1 is a leaky mutant, deficient in the production of nitrate

reductase activity. Nar-2 and nar-3 both lack the ability to produce nitrate reductase. They differ from each other in that nar-2 grows and nar-3 does not grow when hypoxanthine is the sole nitrogen source. The specific activity of the next enzyme in the pathway, nitrite reductase, is higher in nar-3 than in nar-1, nar-2 and wild type.

dedicated

with affection

to my family

for their enthusiastic interest and support

I extend my sincere appreciation

to Chris McKenzie for her invaluable technical assistance

to Larry Steele and Mike Churchill-Smith for their dedication in performing some experiments pertinent to this thesis

to John Ruffelle and Penny LeCouteur for their encouragement during my undergraduate career

to the participants of the Annual International Volvox Conference for their helpful comments and criticisms of my research

and especially

to Lin, with whom I have learned to accept and to share the frustrations and satisfactions of scientific research.

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Chapter I

INTRODUCTION

Eudorina elegans (Ehrenberg) is a motile, colonial green alga belonging to the family Volvocaceae and the suborder Chlamydomonadineae. This suborder is an interesting one since with its many genera it clearly reveals how a simple cell type typified by *Chlamydomonas* has given rise to increasingly complex colonies of unicells (i.e. *Gonium*, *Pandorina*, *Platydorina*, *Eudorina*, *Pleodorina*, *Volvox*). The evolutionary change within this suborder is marked by an increasing size in the colonies (coenobia) and the number of cells within a colony. As a result of this increase in cell number, highly characteristic morphologies are assumed. For example, *Gonium* is a simple, planar colony whereas *Pandorina* is a ball-like cluster of cells. More complex forms such as *Eudorina*, *Pleodorina* and *Volvox* are spheroids within which constituent cells are arranged as a single layer (Pickett-Heaps 1975).

Inversion is observed when the cells making up the colony are arranged in a spheroid. For example, in *Volvox*, when the colony is first formed the flagella of the constituent cells are on the inside of the sphere. As a result, the colony must invert to be motile. *Eudorina*

undergoes a less complex form of inversion. Instead of forming an 'inside-out' spheroid, Eudorina undergoes a limited number of cell divisions to form a curved plate (plakeal stage). This plakea undergoes a simple inversion process, giving rise to a spheroidal colony.

The evolutionary change within this suborder is characterized by a division of labor between the cells within a colony and a tendency towards complex specialization in reproductive behavior. In Gonium and Pandorina, all the cells of the colony undergo vegetative reproduction *via* cell division, giving rise to daughter colonies. In the colonies of certain species of Eudorina, including the strain used in this study, a similar equivalency of cell potential exists. However, in other species of Eudorina polarity is evident. All of the cells reproduce vegetatively except for some cells that are grouped at the anterior end. In Volvox, only a few of the cells (gonidia) within the colony undergo the cell divisions that lead to vegetative reproduction. The many somatic cells surrounding the gonidia do not divide further and subsequently die.

A feature notable in the development of sexual reproduction within this suborder is the progression from isogamy through anisogamy to oogamy. Isogamy, typical of most species of Chlamydomonas, is characterized

by the gametes being of similar size and morphology. In anisogamous organisms such as _Eudorina_, sperm packets are formed as a result of cell divisions while the cells that function as eggs do not divide and appear morphologically similar to vegetative cells. Oogamous organisms such as _Pleodorina_ and _Volvox_ have gametes of vastly different size and morphology. The female cells, few in number, are large and non-motile whereas the male gametes are numerous, small, and motile.

The species of _Eudorina_ (i.e. _elegans_) used in this study is a relatively 'simple' member of the suborder Chlamydomonadineae, being anisogamous, showing some posterior to anterior polarity in the timing of vegetative division and increasing size of the eyespots in the cells at the anterior end. Each colony or coenobium contains 16 or 32 biflagellated cells arranged peripherally in a spheroidal double-layered gelatinous sheath (Figure I-1). Each cell measures 12μ - 20μ in diameter and each colony, 80μ - 160μ in diameter (Goldstein 1964). All the cells making up the colony are reproductive, each mature cell undergoing 4 or 5 rapid, successive cell divisions. Microspectrometric quantitation has shown that during the division period of these cells one round of DNA replication requires about 13 minutes. This is followed by a mitosis which is completed in

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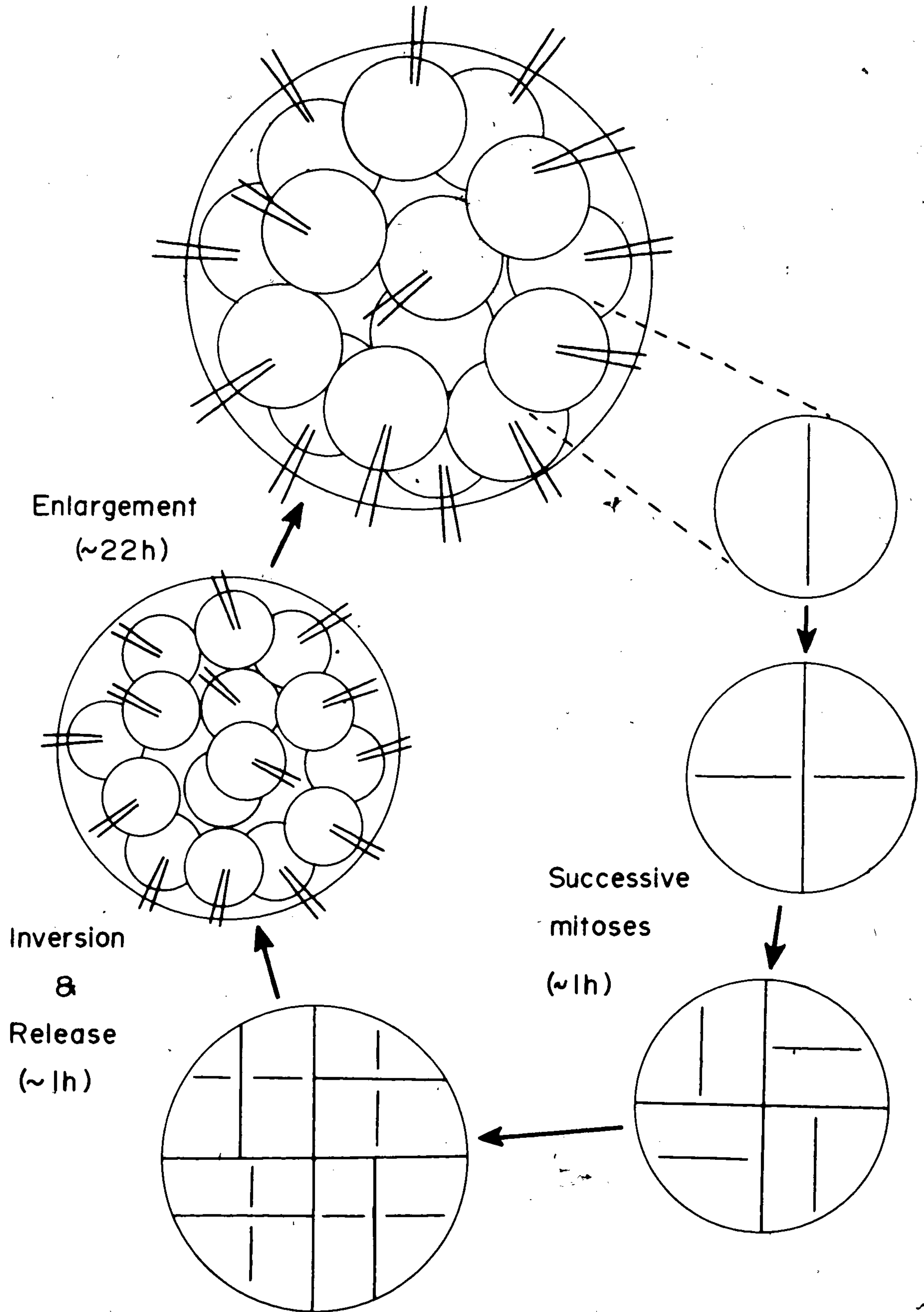
Figure I-1. Vegetative life cycle of *Eudorina elegans*. Each cell of the 16 or 32 celled colony divides 4 or 5 times to form a curved plate of cells or a plakea. Following inversion of the plakea, each of these groups of cells is released as a new daughter colony.


Enlargement
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Successive
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Inversion
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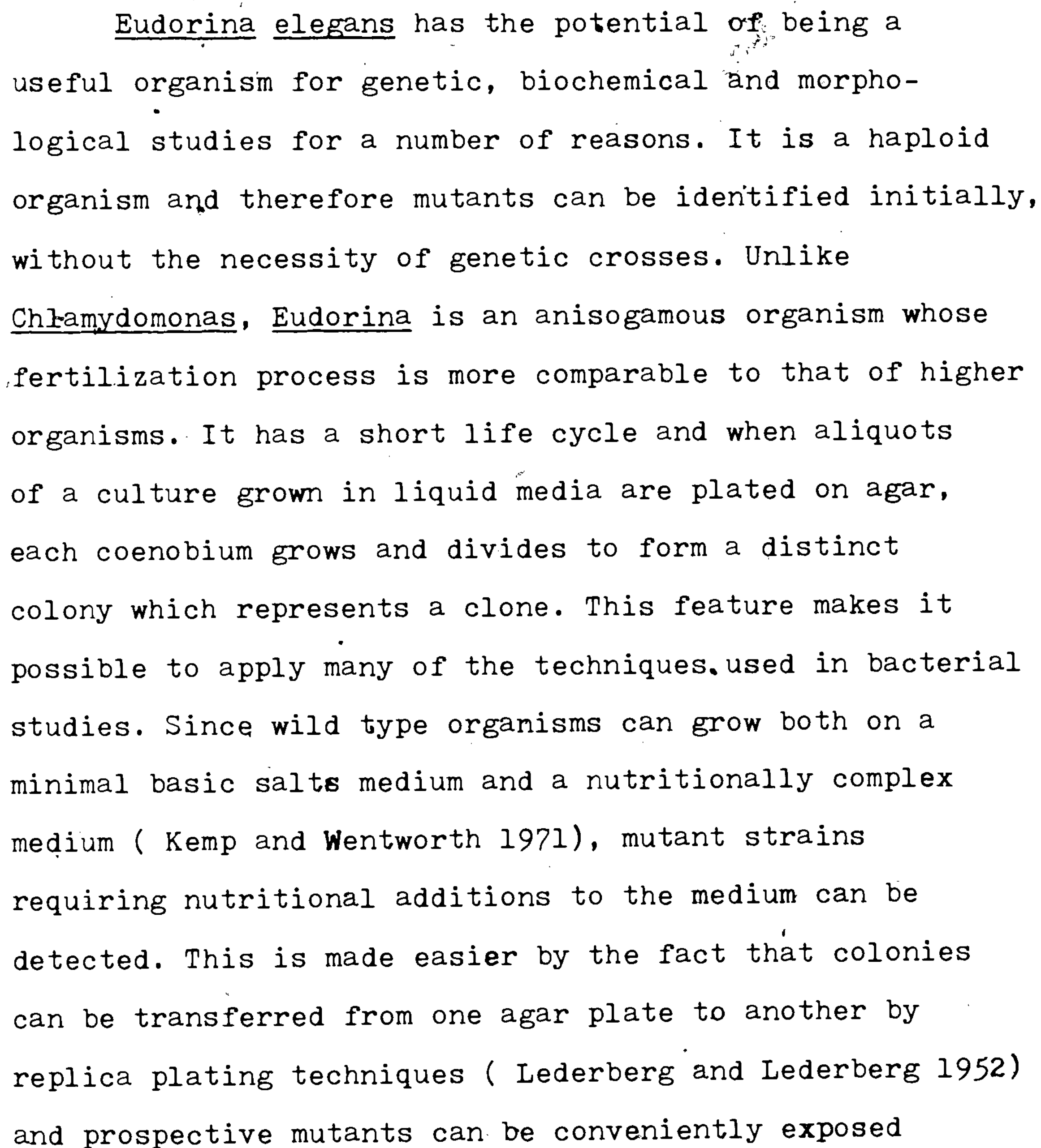

about 2 minutes (Kemp and Lee 1976). The successive divisions in each cell result in the formation of plakea. The colony then undergoes inversion and a short time later the 16 or 32 daughter coenobia are released. Following the 24 h period of enlargement, the cycle is repeated.

Eudorina elegans has the potential of being a useful organism for genetic, biochemical and morphological studies for a number of reasons. It is a haploid organism and therefore mutants can be identified initially, without the necessity of genetic crosses. Unlike Chlamydomonas, Eudorina is an anisogamous organism whose fertilization process is more comparable to that of higher organisms. It has a short life cycle and when aliquots of a culture grown in liquid media are plated on agar, each coenobium grows and divides to form a distinct colony which represents a clone. This feature makes it possible to apply many of the techniques used in bacterial studies. Since wild type organisms can grow both on a minimal basic salts medium and a nutritionally complex medium (Kemp and Wentworth 1971), mutant strains requiring nutritional additions to the medium can be detected. This is made easier by the fact that colonies can be transferred from one agar plate to another by replica plating techniques (Lederberg and Lederberg 1952) and prospective mutants can be conveniently exposed

to different types of selective media.

The division process of this organism provides another area of interest. It is probable that by the use of mutagens, the precise organization and the number of cell divisions may be disrupted. This could lead to an investigation of the control of division in this simple, multicellular system.

Since 1901, when mutation was described by deVries as a functional genetic process, it has been recognized as a source of the variation upon which evolution depends. Mutation has been recognized as such a critical process that modern definitions of life usually include it as a fundamental aspect of living organisms. Because of the obvious importance of mutation 'its analysis has occupied a position close to the center of the geneticist's arena' (Drake 1970). The induction and isolation of mutant individuals has thus become essential to the study of the basic genetic, biochemical and developmental processes of organisms of many phyla and the elucidation of similarities and differences between them. One of the missing links in studies of this nature is the lack of a broadly based understanding of the genetics, biochemistry and metabolism of phototrophic organisms. This lack of information is primarily due to the inability to induce and isolate a varied spectrum of

auxotrophic mutants.

The results of a review done in 1967 (Li et al. 1967) showed that there is a striking difference in mutation spectra following mutagen treatment between chemotrophic bacteria and fungi and the three phototrophic organisms, Chlamydomonas reinhardii, Chlamydomonas eugametos and Arabidopsis thaliana. After exposure to the mutagens nitrosoguanidine, ethyl methanesulfonate, ultraviolet light and x-rays, mutants requiring amino acids and nucleic acid bases were found to be common amongst bacteria and fungi but scarce in the green plants. The obligately phototrophic blue green alga, Anacystis nidulans also fits this pattern (Asato and Folsome 1969). Following treatment with nitrosoguanidine and ultraviolet light many mutants were induced and isolated, none of which was an amino acid auxotroph.

With respect to amino acid mutants in green plants only arginine requiring mutants have been isolated. This type of auxotroph has been found in Chlamydomonas reinhardii (Eversole 1956; Gillham 1956; Loppes 1969) but not in Chlamydomonas eugametos (Gowans 1960), nor in the liverworts Marchantia polymorpha (Miller et al. 1962) and Sphaerocarpus donnellii (Scheider 1976). Carlson in 1969 claimed to have isolated thirty-three auxotrophic gametophytes from the fern Todea barbara

of which he said twenty required amino acids for growth. None of these mutants proved to be genetically stable. In higher green plants, chlorophyll deficient strains do exist that can be corrected by feeding amino acids. These mutants, isolated in barley (Walles 1963,1967; Wijewantha and Stebbins 1964) and Arabidopsis (Redei 1963) are thought to be ' leaky auxotrophs' that grow in the absence of amino acids but grow more efficiently in their presence. Since the amino acids are probably being catabolized by these mutants they may not be true amino acid auxotrophs.

A number of reasons have been postulated to account for the major dichotomy between chemotrophs and phototrophs with respect to mutant spectra. One argument postulated by Li, Redei and Gowans (1967) suggested that there are fundamental metabolic, genetic or other biological differences between phototrophs and chemotrophs. They also suggested that auxotrophs do not arise or cannot be tolerated in phototrophically growing organisms.

Another reason postulated by Asato and Folsome (1969) is that chemical mutagens such as nitrosoguanidine do not penetrate the cell and that physical agents such as ultraviolet light are absorbed by carotenoids which protect the DNA from damage. However, the isolation of

nuclear mutations controlling pigment levels and morphology tends to reduce the validity of this argument.

One popular idea is that of extensive duplication of genetic material in phototrophs. An increase in DNA amounts through the course of evolution has been attributed to longitudinal replication. Cytological examination of hybrids between Allium species that differ markedly in their DNA content indicates that such replication may be regional rather than general (Rees and Jones 1967). If the replicated region involves genes with important functions, the kind of restricted mutation found in prototrophs could result. However, this idea may lose favor since reassociation kinetics of Chlamydomonas reinhardii DNA have recently indicated that the nuclear genome is comprised almost entirely of unique, single copy DNA (Howell and Walker 1976). It is also possible that nuclear genes are duplicated by genes carried in the chloroplast (Sager and Ramanis 1970). The divergent base composition of nuclear and chloroplast DNA in Chlamydomonas (Sueoka et al. 1967) suggests that this may not be an acceptable possibility. However, recent evidence (Howell et al. 1977) suggests that the genetic information of the nucleus and chloroplast of Chlamydomonas may be interacting. As a result, a multimeric enzyme may be composed of some parts coded by mRNA

of the chloroplast and some by mRNA of the nucleus.

Another possibility is that the medium used for the isolation of mutants determines which amino acid auxotrophs are found (Lein et al. 1948; Haas et al. 1952; Grenson et al. 1966). It has been observed in yeasts and Chlamydomonas that active transport systems for some amino acids can be disturbed by the presence of ammonia so that specific amino acid requiring mutants cannot be isolated (Loppes 1969,1970). When ammonia is replaced by another nitrogen source, arginine requiring mutants have been isolated.

One possibility that apparently has not commanded much attention is the idea that auxotrophs may be induced under appropriate conditions at very low frequencies in phototrophic organisms. As a result, these mutants would usually not be isolated unless enrichment procedures are employed. Such enrichment procedures have been successfully applied to a variety of prokaryotic and eukaryotic systems. An outline of these procedures and some recent modifications will now be presented.

An enrichment technique causes the death of metabolizing cells yet allows cells that are not metabolizing to survive. The first example of such a method was the penicillin technique developed by Davis(1949). The success of this procedure is based on

the fact that penicillin causes the sterilization of multiplying bacteria by interfering with the formation of mucopeptide in the cell wall (Parker and Johnson 1949). Therefore, cells that are placed in a medium where they cannot metabolize will be immune to the action of penicillin. The initial studies were done using a tryptophan requiring mutant of the bacterium, Escherichia coli. If this mutant is placed in a minimal medium which lacks tryptophan, preventing growth, penicillin has no effect. As a result, when a mixture of tryptophan requiring cells and wild type cells are exposed to penicillin, the wild type cells metabolize and die and the mutants are maintained. The procedure was then adapted to screen for auxotrophs in a mutagenized culture and resulted in the isolation of mutants requiring various nutrients.

The original penicillin technique has been adapted in many ways to suit the organism being studied and to permit the isolation of specific classes of mutants. These modifications include the use of drugs other than penicillin, mutagenization of organisms exhibiting 'unbalanced growth', tritium suicide, cryobiological and differential heat selection methods and the use of base analogs.

Since some organisms are resistant to the action

of penicillin, other drugs have been used as enriching agents. For example, isoniootinic acid hydrazide has been used to enrich for mutants of another bacterium, _Mycobacterium smegmatis_ (Holland and Ratlidge 1971). Drugs commonly used to isolate mutants of yeasts and fungi are the antifungal agents nystatin, amphotericin and endomycin (Moat et al. 1959; Stanley and English 1965; Snow 1968; Stromnaes and Mortimer 1968; Thomulka and Moat 1968; McDonald 1969; Ditchburn and McDonald 1971). Nystatin has recently been used, in conjunction with a medium that inhibits the growth of mutants requiring tyrosine, to selectively enrich for tyrosine auxotrophs in the fungus _Hanensula polymorpha_ (Sanchez et al. 1978). Tyrosine auxotrophs in this organism have the peculiar property of being able to grow on a minmal basic salts medium supplemented with tyrosine but not on a nutritionally enriched medium containing tyrosine. If a wild type culture is mutagenized, grown on an enriched medium that allows the growth of prototrophs and auxotrophs not requiring tyrosine, then treated with nystatin prior to plating on a basic minimal salts medium containing tyrosine, all the mutants that survive the nystatin treatment require tyrosine for growth. This procedure demonstrates that it is possible to obtain specific auxotrophs by modifying the growth and selective

conditions.

Netropsin has also been used instead of penicillin to aid in the isolation of mutants of the mycomycete Physarum polycephalum (Gorman and Dove 1974) and the yeast Saccharomyces cerevisiae (Young et al..1976). Netropsin is a basic oligopeptide produced by Streptomyces netropsis that binds specifically to A-T pairs in duplex DNA, inhibiting DNA and RNA synthesis. The use of netropsin in these organisms is beneficial since it requires no special media to be effective, it kills metabolizing cells quickly and it does not result in the dying cells breaking open causing the release of nutrients which may allow mutants to metabolize and thus be killed by the drug.

Although the penicillin technique and the modifications discussed so far have led to the isolation of a wide spectrum of mutants in many organisms, certain classes of mutants elude isolation. These mutants include those that require thymine (Bauman and Davis 1957), inositol (Lester and Gross 1959) or certain fatty acids (Henry and Herowitz 1975) for growth. These mutant classes have subsequently been found to be difficult to isolate because they die quickly in the absence of their growth requirement. As a result, when the penicillin technique is employed they die before they can be recovered. This is thought to be

due to ' unbalanced growth ' where death results because of vigorous growth with respect to cytoplasmic function and deficiency with respect to DNA synthesis (Cohen and Barner 1954,1956).

In 1960 a method was discovered whereby thymine requiring mutants of Escherichia coli can be produced with frequencies in the order of 50% (Okada et al. 1960). This method involves the principle of differential growth rates. The growth of both thymine requiring and non-requiring strains is interfered with by aminopterin, an inhibitor of nucleic acid synthesis. Only the growth of the thymine requiring strains can resume following the addition of thymine to the medium.

The properties of thymine requiring mutants have been used to enrich for doubly auxotrophic mutants. Experiments done with Bacillus megaterium (Wachsman and Hogg 1964) and yeasts (Barclay and Little 1974) have resulted in mutants requiring both thymine and an amino acid. Any mutant of this kind will survive in a medium lacking thymine and the required amino acid since they are not subject to thymineless death.

Another method developed as a substitute for the penicillin technique is tritium suicide. By this method death of cells is caused by the decay of tritium incorporated into the macromolecular components. In

the 1950's it was known that growing cells could incorporate radioactive thymidine into their DNA in a form that is unable to diffuse out during cold storage. The isotope then decays during cold storage, causing death of the cell. When a culture of Escherichia coli was mutagenized, grown in a minimal medium containing radioactive thymidine and then put into cold storage, a mutant frequency of 10% of the survivors resulted (Lubin 1959). This method has been modified to suit the fungus Aspergillus flavus (Donkersloot and Mateles 1968) and mammalian cells (Drew and Painter 1959; Whitmore and Gulyas 1966; Thompson et al. 1970).

An advantage of this procedure is that it can be made quite specific by the use of different tritiated precursors. Such modifications have been successful in specifically enriching for mutants of Escherichia coli with an altered acyl CoA synthetase (Hill and Angelmaier 1972) and mutants with defective phospholipids (Cronan et al. 1970) by the use of tritiated oleate. By labelling with tritiated amino acids at 40°C temperature sensitive mutants of Escherichia coli have been isolated that are defective in protein synthesis (Tocchini-Valentini and Mattoccia 1968). Suicide experiments involving tritiated uridine have been shown to be effective in enriching for mutants

defective in RNA metabolism (Reid 1971; Littlewood and Davies 1973).

Cryobiological methods and methods involving differential heat selection have recently been employed as enrichment techniques for specific kinds of organisms. With filamentous fungi (Leef and Gaertner 1975; Peters and Sypherd 1978) cryobiological methods have been successful because metabolically active conidia are much more sensitive to freezing than inactive ones. As a result, when mutagenized conidia are incubated on a medium that allows only growth of wild type cells and the medium is then frozen and thawed, auxotrophs preferentially survive since their germination is blocked by the ommission of a growth supplement. This method has resulted in a 400x - 700x enrichment of fungal mutants.

A method dependent on the fact that the spores and mycelia of <u>Phycomyces</u> <u>blakesleeanus</u> are heat sensitive has resulted in the isolation of auxotrophs in high frequency (Brunke et al. 1977). Mycelia and germinating spores die at temperatures exceeding 35°C whereas mutants whose spores have not germinated can withstand these temperatures for extended periods.

Another variation of the original penicillin technique is the use of base analogs as selective

agents (Cohen and Barner 1956; Zamenof and Griboff 1954; Wachsman and Mangalo 1962). For example, 5-bromodeoxyuridine, which quantitatively replaces thymine in DNA (Dunn and Smith 1954), has been used to isolate proline deficient mutants in mammalian cells (Kao and Puck 1967). This method involves the use of 5-bromodeoxyuridine in conjunction with near visible light. If 5-bromodeoxyuridine is added to a culture of growing and non-growing cells, those that are able to synthesize DNA will incorporate 5-bromodeoxyuridine. Upon exposure to visible light, these cells die.

Recently, fluorouracil has been used to isolate asparagine requiring Chinese hamster cells (Goldfarb et al. 1977). This compound is readily incorporated into mRNA and acts by producing nonheritable uracil to cytosine transitions in mRNA during transcription.

The limited spectrum of mutants isolated in phototrophic organisms suggests that if other auxotrophic mutants are being induced in a population, these mutants must occur at low frequencies. If this is true, the only way in which they could be isolated would be to utilize a mutant enrichment procedure. None of the methods discussed above have been reported as being applied, let alone successful, in isolating mutants in phototrophic

systems. In *Eudorina elegans*, penicillin and other drugs have not been successful enriching agents because of a high level of drug resistance exhibited by this organism. Radioactive suicide techniques are probably not practical, as indicated by a limited uptake of thymidine (Kemp et al. 1972). Many of the other procedures previously discussed have been reported as the current study was nearing completion and thus have not been tried with *Eudorina*.

As a thesis project I attempted to develop an enrichment procedure which would increase the probability of isolating auxotrophs in the colonial alga, *Eudorina elegans*. Once this goal was achieved it was possible to explore a variety of questions. I chose to focus my attention on the optimization of mutagen treatment and to examine more fully the characteristics of nitrogen defective mutants isolated during the course of my studies.

The thesis is comprised of five chapters. In addition to this introductory chapter and a concluding chapter, three chapters (II, III and IV) are presented that report the results of the main focus of my research. Since they have or will appear in the scientific literature, they are written in a format acceptable for publication. Chapter II deals with

the establishment and parameters of the mutant enrichment technique and is published in Genetics 81, 243 - 251 (1975). Chapter III discusses the optimal conditions of nitrosoguanidine and ultraviolet light treatment prior to enrichment and has been submitted for publication to the Journal of Phycology. Chapter IV discusses the characteristics of mutants lacking or deficient in the production of nitrate reductase and is published in the Journal of Phycology 13, 368-372 (1977).

CHAPTER II

MUTANT ENRICHMENT IN THE COLONIAL ALGA, EUDORINA ELEGANS

INTRODUCTION

Unlike chemotrophic organisms, a broad spectrum of auxotrophic mutants have proven to be difficult to isolate in phototrophic organisms (Li et al. 1967). A variety of possibilities has been suggested to account for this observation. Such possibilities include genome redundancy (cf. Auerbach and Kilbey 1971), permeability and/or metabolic insufficiency, and a lack of transcriptional control (cf. Neilson and Lewin 1974). The lack of appropriate enrichment procedures may also have contributed to the failure to isolate some classes of mutants in algae. In order to examine this latter possibility, we have initiated studies using the colonial, phototrophic alga, *Eudorina elegans*. This heterothallic organism is amenable to basic genetic analysis (Mishra and Threlkeld 1968; Herbst and Kemp 1974), and as a simple, multicellular organism offers the opportunity to study morphogenetic processes involving cell-cell interactions.

Enrichment procedures that result in the death of wild type organisms and the maintenance of mutants have not been reported for algal systems. With many bacteria, fungi and mammalian cells, the original penicillin technique (Davis 1949; Lederberg and Zinder 1948;

Gorini and Kaufman 1960) has been modified to suit the organism being studied and often the type of mutants required. Such modifications include the use of other antibiotics or antifungal agents such as nystatin (Stanley and English 1965; Snow 1966; MacDonald 1968; Cook 1974), amphotericin (Moat et al. 1966; Thomulka and Moat 1968) and endomycin (Moat et al. 1959; Moat et al. 1966), the base analog 8-azaguanine (Wachsman and Mangalo 1962), the isolation of double auxotrophs in conjunction with thymineless death (Wachsman and Hogg 1964), tritium suicide (Lubin 1959; Whitmore and Gulyas 1966; Donkersloot and Mateles 1968) and base analog in conjunction with visible light (Kao and Puck 1967). In Eudorina elegans, although drug resistant mutants have been isolated (Mishra and Threlkeld 1968), only one auxotroph, a nicotinamide requiring mutant, has been found (Herbst and Kemp 1974). The use of penicillin and other antibiotics has not been successful due to a high level of resistance exhibited by this organism. Radioactive suicide procedures are probably not practical, as indicated by a limited uptake of thymidine (Kemp et al. 1972).

This report shows that nitrogen starvation followed by exposure to 8-azaguanine may be useful as a mutant enrichment system for Eudorina elegans.

MATERIALS AND METHODS

Eudorina elegans strain 1201 was originally obtained from the Culture Collection of Algae, Indiana University (Starr 1964). The nicotinamide requiring strain, *nic-1*, was isolated in our laboratory by replica plating following treatment with ultraviolet light.

Media

The minimal medium (BM) was a Bristol's basic salt solution (Cain 1965) modified by replacing H-B and H-S solutions with Gaffron's trace elements solution (Hughes et al. 1958). The minimal medium lacking nitrogen (Bm-N) was the BM medium not containing sodium nitrate. The complete medium (BC) was an enriched medium consisting of 800 ml of BM and 200 ml of modified *Euglena* medium (Kemp and Wentworth 1971).

The defined complete medium consisted of BM medium to which was added White's amino acid mixture (Reinhart and White 1956), a vitamin mixture containing nicotinamide, nicotinic acid, pyridoxine, riboflavin, thiamine (each 1 µg/ml), p-aminobenzoic acid (0.5 µg/ml), folic acid (0.1 µg/ml), pantothenic acid (0.01 µg/ml), cobalamine and biotin (each 0.015 µg/ml), and a mixture of nucleic acid bases (each 10 µg/ml).

Drugs

8-azaguanine (8AG) was obtained from CalBioChem and N-methyl-N'-nitro N-nitrosoguanidine (MNNG) was obtained from Aldrich Chemical Company. Solutions were made up in potassium phosphate buffer (1.7 x 10^{-3} M) and filter sterilized. These were diluted into the culture medium at the concentrations indicated in the text.

Culture Conditions

Unless otherwise stated, the cultures were grown and treated when concentrations reached about 10^4 viable organisms/ml in 50 - 75 ml volumes. They were maintained as shaking cultures at 32°C under cool-white fluorescent lights (1000 ft-c incident light) on a 16:8 L:D cycle. The organisms were plated using an agar overlay technique (Adams 1959). Each sample was plated in duplicate and colonies were counted after 5 days on BC plates and 10 - 12 days on BM plates. The average of the duplicate counts was taken.

RESULTS

When 8AG was added to a culture of *Eudorina elegans* growing in BM medium no inhibition of growth occurred. If a nitrogen starved culture was subjected to 8AG in the absence of nitrogen, the titre remained constant. However, when the culture was starved of nitrogen and put into BM medium containing 8AG, extensive inactivation occurred (Figure II-1).

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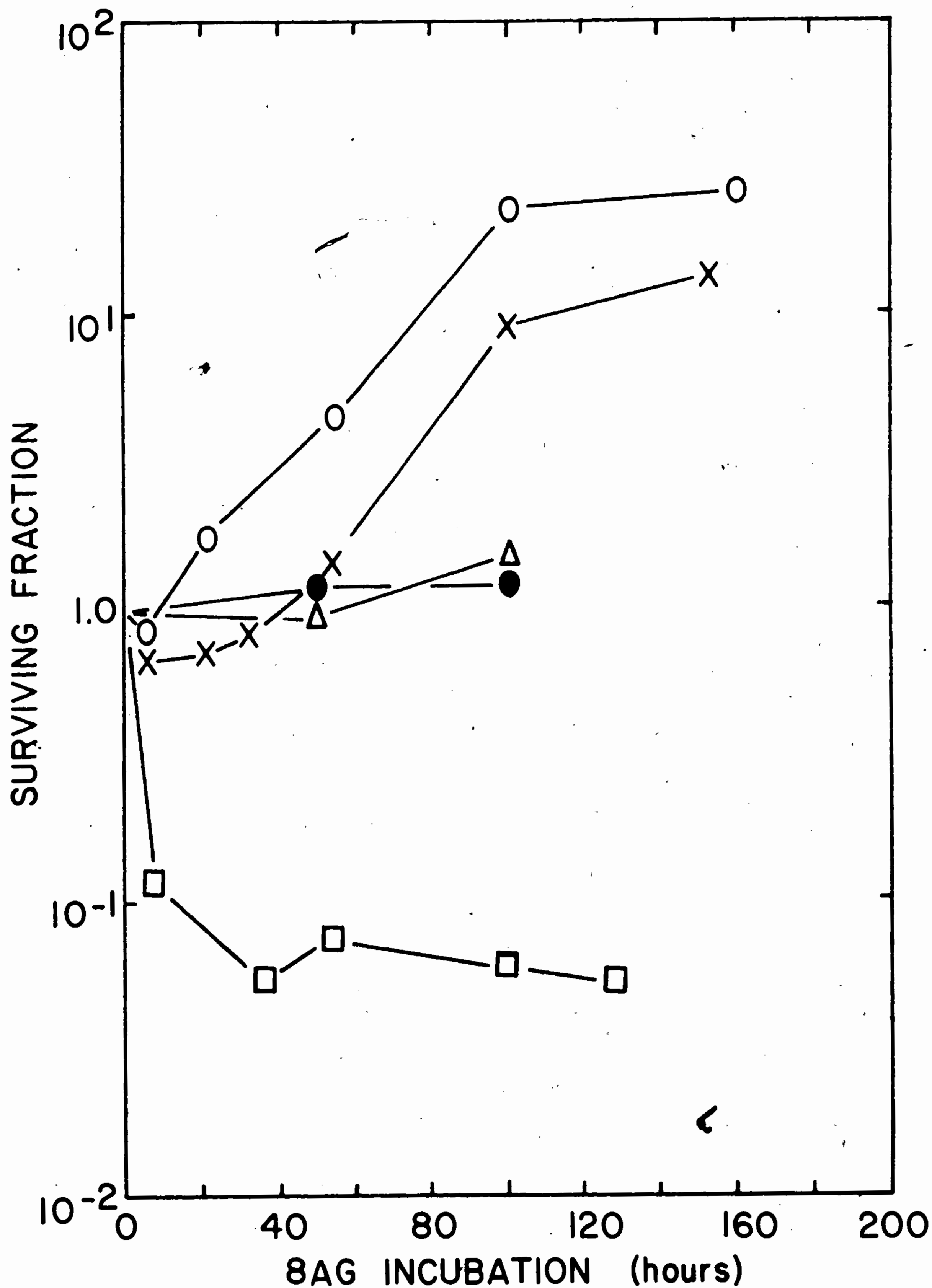
Figure II-1. The effect of 8AG (60 μg/ml) on the growth of <u>Eudorina</u> <u>elegans</u> (strain 1201) in various media prior to and during treatment. Cultures were incubated in BM or BM-N as shaking cultures for 24 h at 32°C. Aliquots were then transferred from BM to BM + 8AG (x—x), and from BM-N into BM-N (●—●), BM (o—o), BM-N+8AG (▲—▲) or BM+8AG (□—□).



10^2
10^1
1.0
10^{-1}
10^{-2}
SURVIVING FRACTION
0
40
80
120
160
200
8AG INCUBATION (hours)

Conditions under which nitrogen starvation took place affected survival upon subsequent exposure to 8AG. At 32°C, about 24 h of starvation was required before 8AG had any effect on survival, while at 22°C, 48 h of starvation was required before 8AG would kill. Starvation of nitrogen in the dark up to three days resulted in no 8AG effect (Table II-1).

8AG inactivation of nitrogen starved cultures of Eudorina was initially rapid to some maximum, followed by a period of up to 5 days where no further loss of colony-forming ability occurred (Figure II-1). Eventually, growth of the culture resumed. A concentration effect of 8AG (40 - 300 µg/ml) was not evident when aliquots of the same nitrogen starved culture were treated (Figure II-2). However, when 1201 cultures at different stages of the life cycle were starved of nitrogen and exposed to the same concentration of 8AG, marked differences in the amount of inactivation were found (Figure II-3).

Nitrogen starvation followed by 8AG treatment was the only combination found that resulted in inactivation. Starvation of other constituents of the BM medium and the use of the purine analogs mercaptopurine and 2-amino purine, the azide containing compounds azathymine and aza-uracil, as well as fluorouracil, bromouracil and deoxy-glucose resulted in growth rather than death. Growth also

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Table II-1. The effect of different conditions of nitrogen starvation on 8-azaguanine effect in <u>Eudorina elegans</u> (strain 1201). The values are presented as the ratio (N/No) of colony forming units/ml following 48 h incubation in BM + 60 µg/ml 8AG (N) to the colony forming units/ml following starvation (No). ND = not done.

Table II-1

	Time of starvation		
BM-N	1 day	2 days	3 days
$32^{o}C$	6.98×10^{-2}	ND	5.04×10^{-2}
$22^{o}C$	1.01	5.26×10^{-2}	9.6×10^{-3}
$32^{o}C$ dark	1.16	1.88	1.47

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Figure II-2. The effect of different concentrations of 8AG on a culture of <u>Eudorina</u> <u>elegans</u> (strain 1201) resuspended in BM+8AG after nitrogen starvation. The culture, grown in BC, was starved for 24 h and then aliquots were resuspended in BM + 40 μg/ml 8AG (x —— x), BM + 100 μg/ml 8AG (o —— o), BM + 200 μg/ml 8AG (● —— ●) and BM + 300 μg/ml 8AG (□ —— □).

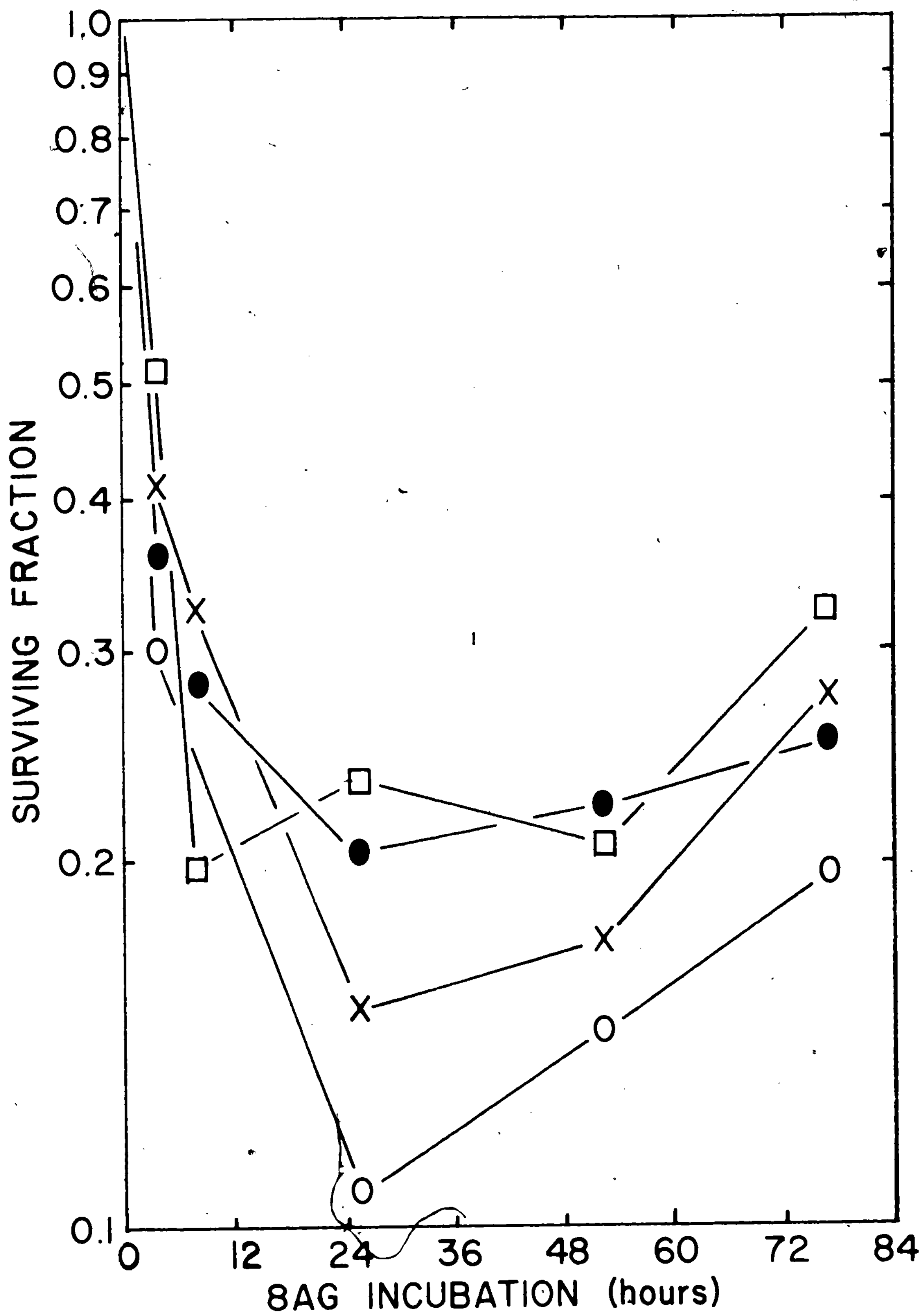
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
SURVIVING FRACTION
0
12
24
36
48
60
72
84
8AG INCUBATION (hours)

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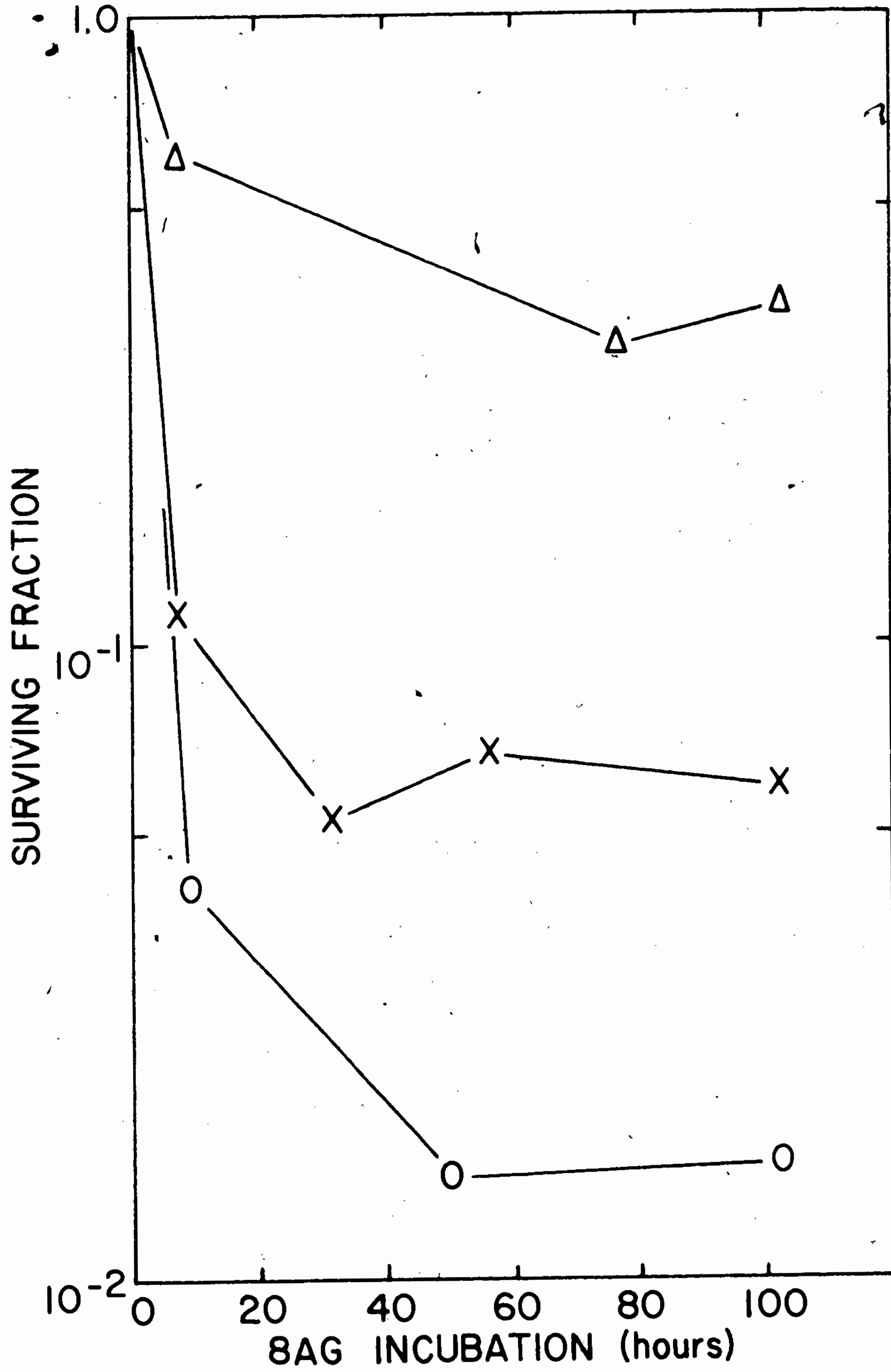
Figure II-3. The effect of BM+8AG (60 µg/ml) on different cultures of _Eudorina elegans_ (strain 1201) after nitrogen starvation. Three different cultures (Δ—— Δ), (x——x) and (o——o) were exposed to nitrogen starvation and 8AG treatment under the same conditions.


1.0
10^{-1}
10^{-2}
SURVIVING FRACTION
0
20
40
60
80
100
8AG INCUBATION (hours)

resulted when a culture was starved of nitrogen and exposed to 8AG in a defined complete medium. The constituents of this medium capable of blocking the lethal effects of 8AG were the purines, adenine and guanine (Figure II-4). However, their respective nucleosides (10 μg/ml) did not interfere with the 8AG inactivation of nitrogen starved cultures.

The above data suggest that cultures of Eudorina may be enriched for mutants if metabolism is reduced by starvation and their regrowth in the presence of 8AG inhibited due to lack of nutrients. In order to evaluate this possibility, a series of experiments was done using the nicotinamide requiring strain nic-1. Starvation of nic-1 in BM-N, with or without added nicotinamide, for 24 h at 32°C followed by 8AG treatment was lethal only if nicotinamide was also included in the post-starvation medium (Figure II-5). No organic additions were required to observe the lethal effects with wild type cells. Enrichment for nic-1 from a population containing both nic-1 and wild type organisms was achieved by subjecting the mixture to several cycles of nitrogen starvation and 8AG exposure in the absence of nicotinamide (Table II-2). Regrowth in BC medium for 24 h followed exposure to 8AG. The starvation, 8AG treatment, and regrowth of a culture

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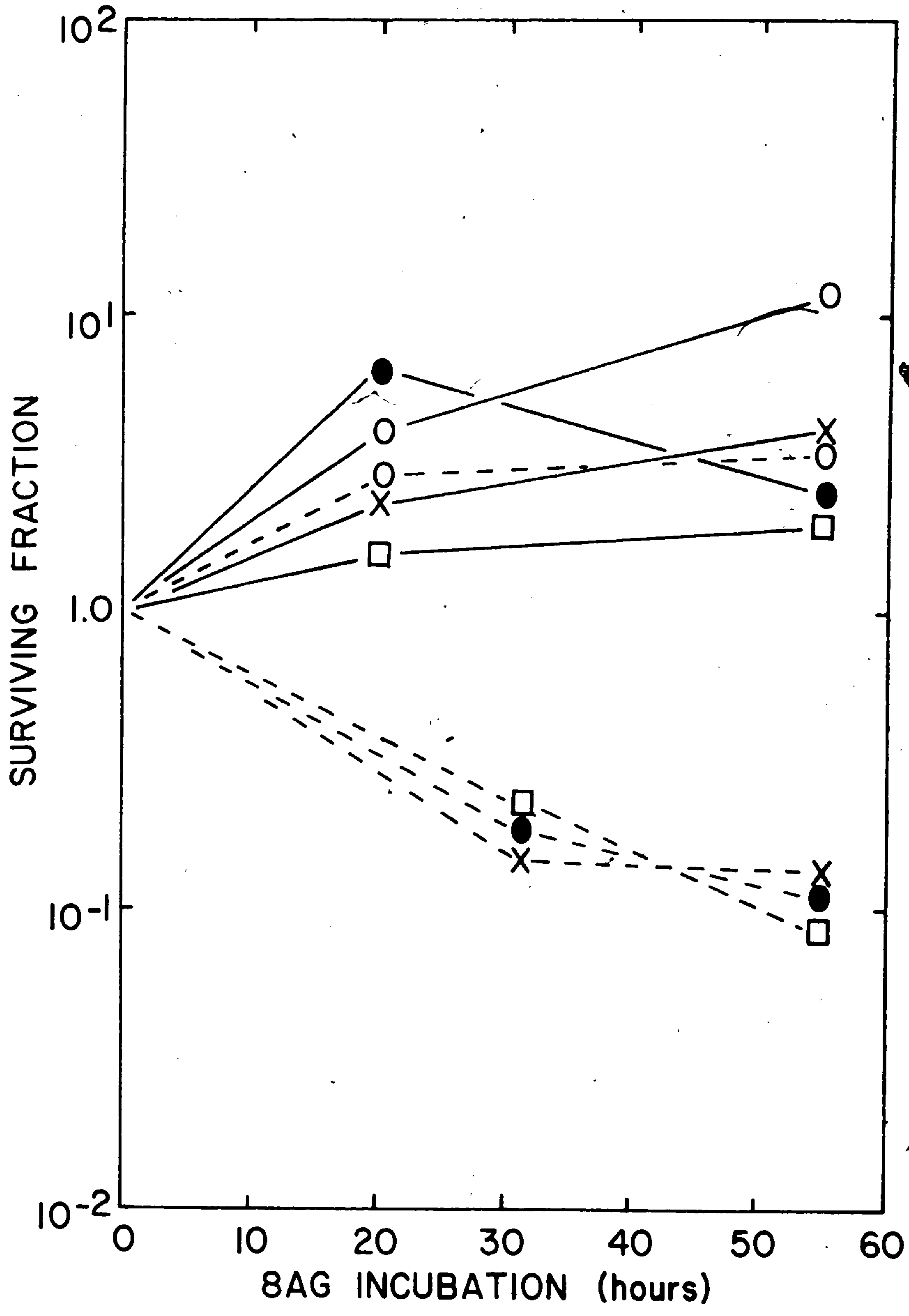
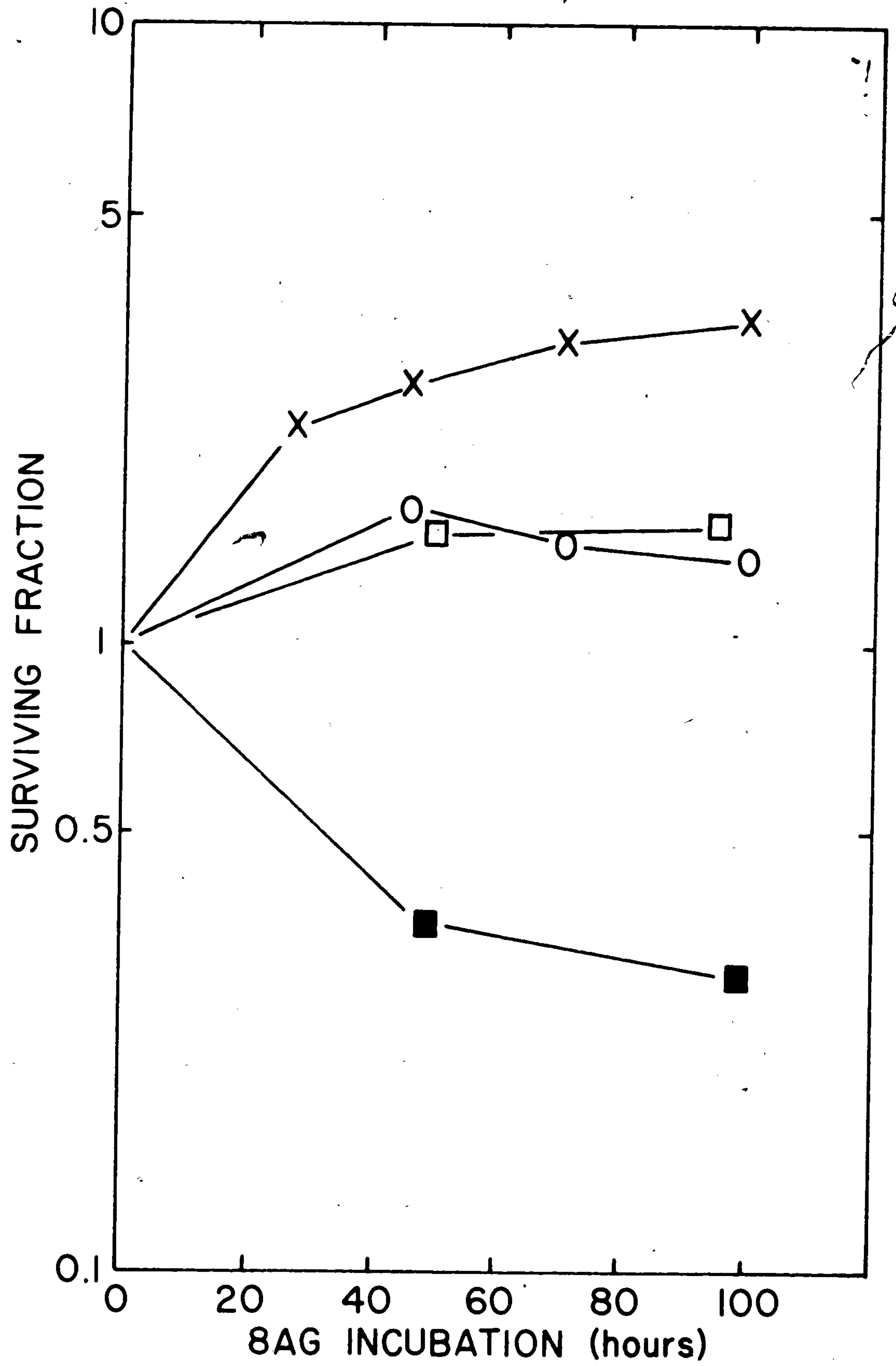
Figure II-4. The effect of nutritional additions to the BM+8AG medium after nitrogen starvation. The culture was starved of nitrogen for 24 h at 32°C and aliquots were then transferred to BM (x——x), BC (o——o), BM+8AG (x---x), BC+8AG (o——o), BM + adenine + 8AG (□——□), BM + guanine + 8AG (●——●), BM + adenosine + 8AG (□---□), and BM + guanosine + 8AG (●---●).



10^2
10^1
1.0
10^{-1}
10^{-2}
SURVIVING FRACTION
0
10
20
30
40
50
60
8AG INCUBATION (hours)

LEAF 31 OMITTED IN PAGE NUMBERING.

Figure II-5. Growth patterns of *Eudorina elegans* (*nic-1*) in different media conditions. Two aliquots of a *nic-1* culture growing in BC were resuspended in BM (o——o) and BM + nicotinamide (x——x). Another aliquot was put in BM-N for 24 h and then was exposed to BM+8AG (□——□), and another was put into BM-N+nicotinamide for 24 h and then was exposed to BM + nicotinamide + 8AG (■——■).



10
5
1
0.5
0.1
SURVIVING FRACTION
0
20
40
60
80
100
8AG INCUBATION (hours)

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Table II-2. Enrichment of *Eudorina elegans* for *nic-1*. A mixed population (total of about 10^4 viable organisms/ml) of wild type and nicotinamide requiring (*nic-1*) *Eudorina* was subjected to a series of three mutant enrichment cycles. The titres of each type were obtained before and after each cycle by plating aliquots on BM and BC medium. The number of *nic-1* was the difference between the number of colonies on BC and the number on BM, and their proportion was the number of *nic-1* / number of 1201 + the number of *nic-1*.

Table II-2

cycle #	1		2		3	
	prior 8AG cfu/ml	after 8AG cfu/ml	prior 8AG cfu/ml	after 8AG cfu/ml	prior 8AG cfu/ml	after 8AG cfu/ml
1201	5.6×10^3	6.5×10^2	7.9×10^3	3.0×10^1	9.0×10^1	1.5×10^1
nic-1	3.6×10^3	3.8×10^3	1.1×10^4	5.9×10^3	2.3×10^3	2.4×10^3
nic-1 / total	0.39	0.85	0.58	0.95	0.96	0.99

constituted an enrichment cycle.

The isolation of MNNG-induced mutants using the enrichment procedure was then attempted. A culture was exposed to MNNG (1 µg/ml) for 2 h and allowed to grow in BC for 24 h or until the increase in titre indicated that the parental coenobia had broken down, releasing the daughter organisms. The culture was then cycled through the enrichment procedure twice. The treated culture was plated on BC plates and individual colonies surviving the treatment were transferred to BM and BC plates _via_ a sterile set of points arranged in a fixed array (Roberts 1959). The growth factor(s) of the colonies growing on BC but not on BM were determined by plating aliquots on plates containing different known concentrations of organic supplements added to the BM medium (Holliday 1956). Seventy-five auxotrophs were found in a total of 4726 colonies. Two of these required p-aminobenzoic acid, one required a reduced nitrogen source and the rest required acetate. A non-mutagenized culture, also cycled twice through the enrichment procedure, yielded 3 acetate requiring mutants among 11,089 colonies examined. Genetic analysis of these mutants is currently in progress. In contrast, a culture treated with a mutagen but not cycled through the enrichment procedure following the 24 h of growth

in BC failed to yield mutants in a total of 14,000 colonies studied.

DISCUSSION

A procedure has been developed which results in the enrichment of auxotrophic mutants of *Eudorina elegans*. The procedure depends upon the ability of the purine analog, 8AG, to kill organisms recovering from nitrogen starvation. Because the recovery medium is a simple salt solution, any organisms requiring organic supplements presumably do not initiate recovery and are immune to the lethal effects of 8AG.

Nitrogen starvation prior to the exposure to antibiotics and the antifungal agent, nystatin, has led to the successful isolation of mutants in fungi (Snow 1966; Cook 1974). 8AG has been useful in the isolation of mutants of *Bacillus megaterium* (Wachsman and Mangalo 1962). To our knowledge, 8AG has never been used in conjunction with nitrogen starvation as an enrichment procedure.

Conditions at the time of nitrogen starvation and at the time of 8AG treatment greatly influence the lethal effects of 8AG. The age and growth stage of a culture undoubtedly contribute to the different sensitivities of *Eudorina* to 8AG (Figure II-3). However, strain 1201 has proven difficult to synchronize

and consequently comparative studies were done using aliquots of the same culture. The increased time required for nitrogen starvation at 22°C and the failure of nitrogen starvation in the dark suggest that some cellular processes are necessary during the starvation period for the subsequent 8AG treatment to be effective. 8AG killing does not occur if either or both of the purines, adenine and guanine, are present in the medium at the time of 8AG treatment. In other organisms (Mandel 1957; Chantrenne and Devreux 1960; Mangalo and Wachsman 1964) the purine nucleosides as well as the purines show this effect. The purine nucleosides are ineffective in Eudorina.

If the enrichment factor is defined as the frequency of MNNG-induced mutants after enrichment divided by the frequency of MNNG-induced mutants before enrichment ($1.6 \times 10^{-2} / < 7.0 \times 10^{-5}$), then at least a 200x increase in mutant frequency results from the enrichment procedure. The data indicate that the frequency of spontaneous mutants present in the population of this organism is low. Since the frequency of spontaneous mutations present after enrichment is 2.7×10^{-4} and the enrichment procedure appears to cause at least a 200x increase in the frequency of mutants, the frequency of spontaneous mutants in an untreated population is less than 5.4×10^{-6}. The results also indicate

the powerful nature of the mutagen, MNNG. Treating at low concentrations of MNNG (1 μg/ml) for 2 hours causes at least a 60x increase in the mutant frequency. This is determined by dividing the frequency of MNNG-induced mutants after enrichment by the frequency of mutants of a non-treated culture after enrichment (1.6×10^{-2} / 2.7×10^{-4}).

A problem is encountered with the enrichment procedure. The degree of amplification of a mutant as a result of growth during the enrichment procedure is unknown. In the case of the mutants isolated in Eudorina, differences in the morphology and growth patterns of the p-aminobenzoic acid requiring and of some of the acetate requiring mutants suggest that they may have originated from separate mutational events. Genetic studies are underway to determine the inheritance patterns of these mutants.

CHAPTER III

NITROSOGUANIDINE AND ULTRAVIOLET LIGHT MUTAGENESIS IN *EUDORINA ELEGANS*

INTRODUCTION

The induction and isolation of auxotrophic individuals has become essential to the understanding of basic genetic processes of organisms of many different phyla and have aided in the elucidation of similarities and differences between them. The lack of a large spectrum of auxotrophs in phototrophic organisms (Li et al. 1967) has resulted in a missing link in studies of this nature. This lack of mutants has been attributed to a number of different causes. Two possibilities experimentally difficult to study are permeability problems resulting in metabolic insufficiency and genome redundancy (Auerbach and Kilbey 1971). However, an equally plausible possibilty that is experimentally more easily dealt with is a lack of enrichment procedures that have proven useful in isolating auxotrophs that occur infrequently in other systems. As a result, the choice of mutagen and conditions of mutagen treatment that are most likely to yield maximum auxotroph induction in phototrophs have not been established.

Chapter II described a mutant enrichment procedure

for the colonial alga, Eudorina elegans. Mutants estimated to occur at a frequency at least as low as 10^{-5} in an initial nitrosoguanidine mutagenized population were isolated at a frequency of 10^{-2} following enrichment.

This chapter deals with the determination of conditions of N-methyl-N'-nitro N-nitrosoguanidine (MNNG) and ultraviolet light (UV) treatment that are most conducive to the induction of auxotrophs prior to exposure to the enrichment technique. The two parameters used to establish these conditions are reversion frequency of a previously isolated acetate requiring mutant (ac8) and formation of pigment sectored colonies.

MATERIALS AND METHODS

The organisms used in this study were Eudorina elegans (Ehrenberg) UTEX 1201 obtained from the Culture Collection of Algae at the University of Texas and an acetate requiring strain (ac8) isolated after treatment with MNNG and subsequent enrichment.

Media

Minimal medium (BM) was modified basic salts solution (Cain 1965) with Gaffron's trace elements solution (Hughes et al. 1958) replacing the H-B and H-S solutions. The complete medium (BC) consisted of

800 ml of BM and 200 ml of modified *Euglena* medium (Kemp and Wentworth 1971).

Culture Conditions

Cultures used for determining sectoring frequencies were grown in 50 - 75 ml volumes in 250 ml flasks as shaking cultures. They were incubated at 32°C under cool-white fluorescent lights (*ca* 1000 ft-c) on a 16:8 L:D cycle. Aliquots of the surviving organisms were plated in duplicate by the agar overlay technique. The ratio of sectored colonies to the total number of surviving organisms was scored after 5 days of incubation.

Cultures used for reversion studies were grown in 5 liter vessels in BC medium to a concentration of 5×10^4 colony forming units/ml. Following mutagen treatment, 1 liter aliquots were removed and collected by centrifugation. The resulting pellets were washed twice in phosphate buffer (1.7×10^{-3}M, pH 7.0) and finally resuspended in 10 ml of the buffer. Aliquots, plated on BM plates, were incubated for 14 days before revertant colonies were scored.

Mutagen treatment

Ultraviolet light was obtained from G15T8 germicidal lamps at a dose of 2 J / m^2s. The cultures, suspended in phosphate buffer, were irradiated in open petri dishes. The depth of the cultures during irradiation

did not exceed 1 mm and they were constantly agitated during exposure. Photoreactivation was prevented by plating the treated organisms under yellow light (Jagger 1967) and incubating the plated algae for 3 days in the dark before light incubation.

MNNG was obtained from the Aldrich Chemical Company. Stock solutions were made up fresh in water (pH 5.5), filter sterilized and diluted to the appropriate concentration in the culture.

Sectored Colonies

A sectored colony resulted when one or more of the cells making up the coenobium were morphologically changed by the mutagen to cause a change in pigmentation. When plated on agar these cells replicated. This resulted in a defined segment of the colony showing a different pigmentation than the rest of the colony. Many of the sectored colonies isolated were tested to determine whether the morphological characteristic was stable. In 85% of the colonies examined, the differentially pigmented area of the colony could be recovered when the colony was disrupted in buffer and replated.

RESULTS

When a culture was treated with 1 µg/ml MNNG for 48 hours (Figure III-1), neither an increase nor

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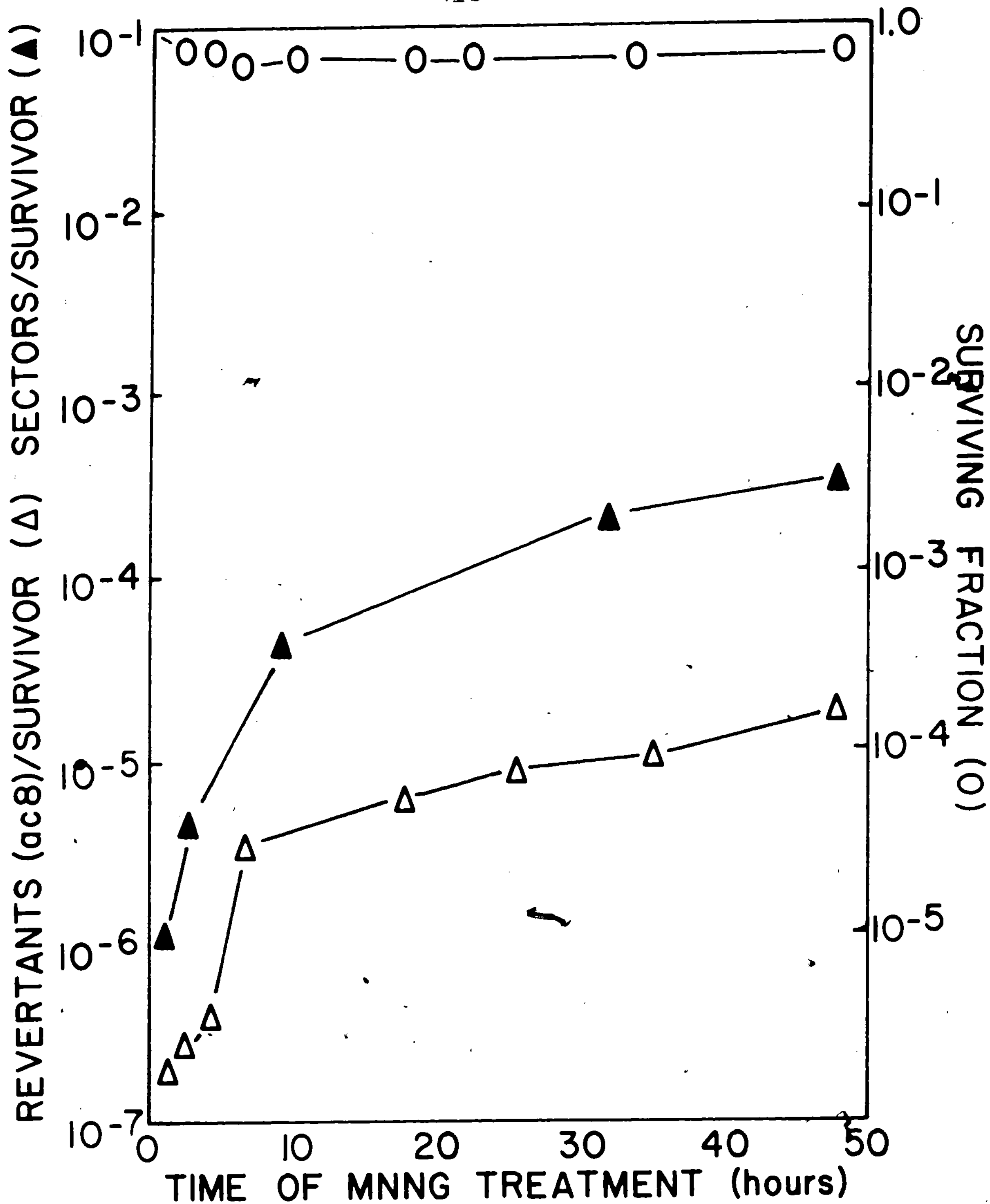
Figure III-1. Cell survival (o—o), revertant frequency of <u>ac8</u>/survivor (△—△) and sectoring frequency/survivor (▲—▲) of cultures of <u>Eudorina elegans</u> exposed to 1 μg/ml MNNG. At 0 h the revertant frequency of <u>ac8</u>/survivor was $<1.25 \times 10^{-8}$ and the sectoring frequency/survivor was $<2.3 \times 10^{-7}$. Each plot is a representative of at least 2 experiments done when the majority of cells were in the growth stage.


REVERTANTS (ac8)/SURVIVOR (Δ) SECTORS/SURVIVOR (▲)
SURVIVING FRACTION (O)
TIME OF MNNG TREATMENT (hours)
10-1
10-2
10-3
10-4
10-5
10-6
10-7
1.0
10-1
10-2
10-3
10-4
10-5
0
10
20
30
40
50

a decrease in viable organisms occurred. The revertant frequency/survivor increased within the first 30 minutes of exposure to the mutagen and then remained stable for about 2 hours before increasing about 10x over the next 2 - 3 hours. An additional 10x increase occurred gradually over the next 43 hours to about 2×10^{-5} revertants/survivor. The frequency of sectored colonies/survivor of 1201 followed the same general trend as the reversion frequency of ac8 although the initial 2 - 3 hour plateau was not observed. The frequency of sectored colonies was about 10x greater than the revertant frequency during exposure to the mutagen.

Exposure to 10 µg/ml MNNG caused cell death. Only about 2% of the organisms survived a 2 hour exposure to the mutagen (Figure III-2). Despite this toxicity, revertants of ac8 and differentially pigmented sectors were induced to maximum values after 60 minutes of exposure. The maximum reversion frequency obtained using MNNG was 1.5×10^{-4} and the maximum sectoring frequency was 1×10^{-3}.

When the cultures were exposed to UV, the reversion frequency/survivor increased to a maximum at a dose of 300 J/m^2s and then decreased again (Figure III-3). The maximum revertant frequency/survivor obtained using

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Figure III-2. Cell survival (o—o), revertant frequency of ac8/survivor (△—△) and sectoring frequency/survivor (▲—▲) of cultures of *Eudorina elegans* exposed to 10 µg/ml MNNG. At 0 h the revertant frequency of ac8/survivor was $<5.0 \times 10^{-8}$ and the sectoring frequency/survivor was $<1.2 \times 10^{-5}$. Each plot is a representative of at least 2 experiments done when the majority of the cells were in the growth stage.

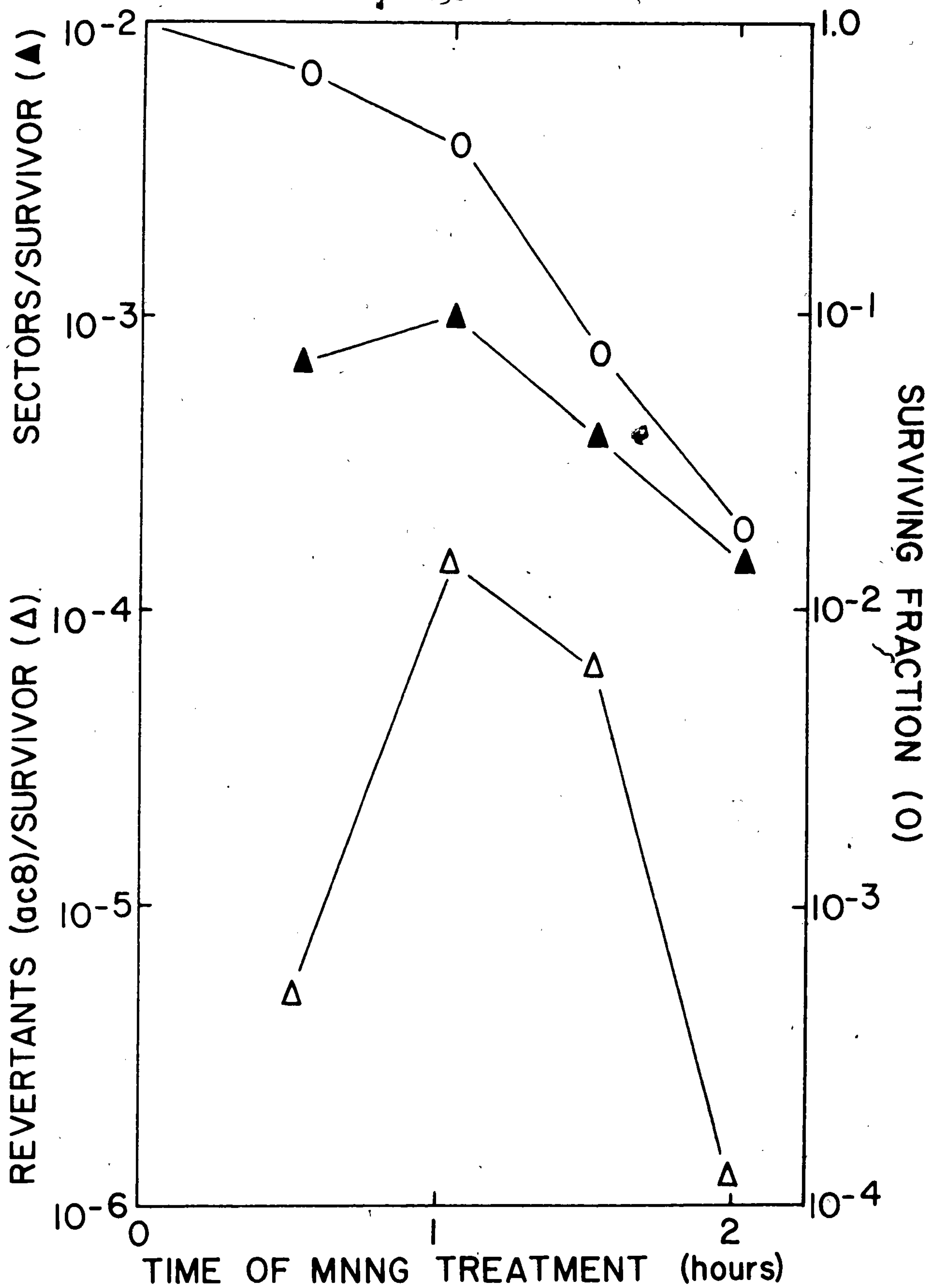
SECTORS/SURVIVOR (▲)
REVERTANTS (ac8)/SURVIVOR (Δ)
SURVIVING FRACTION (O)
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
1.0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
1
2
TIME OF MNNG TREATMENT (hours)

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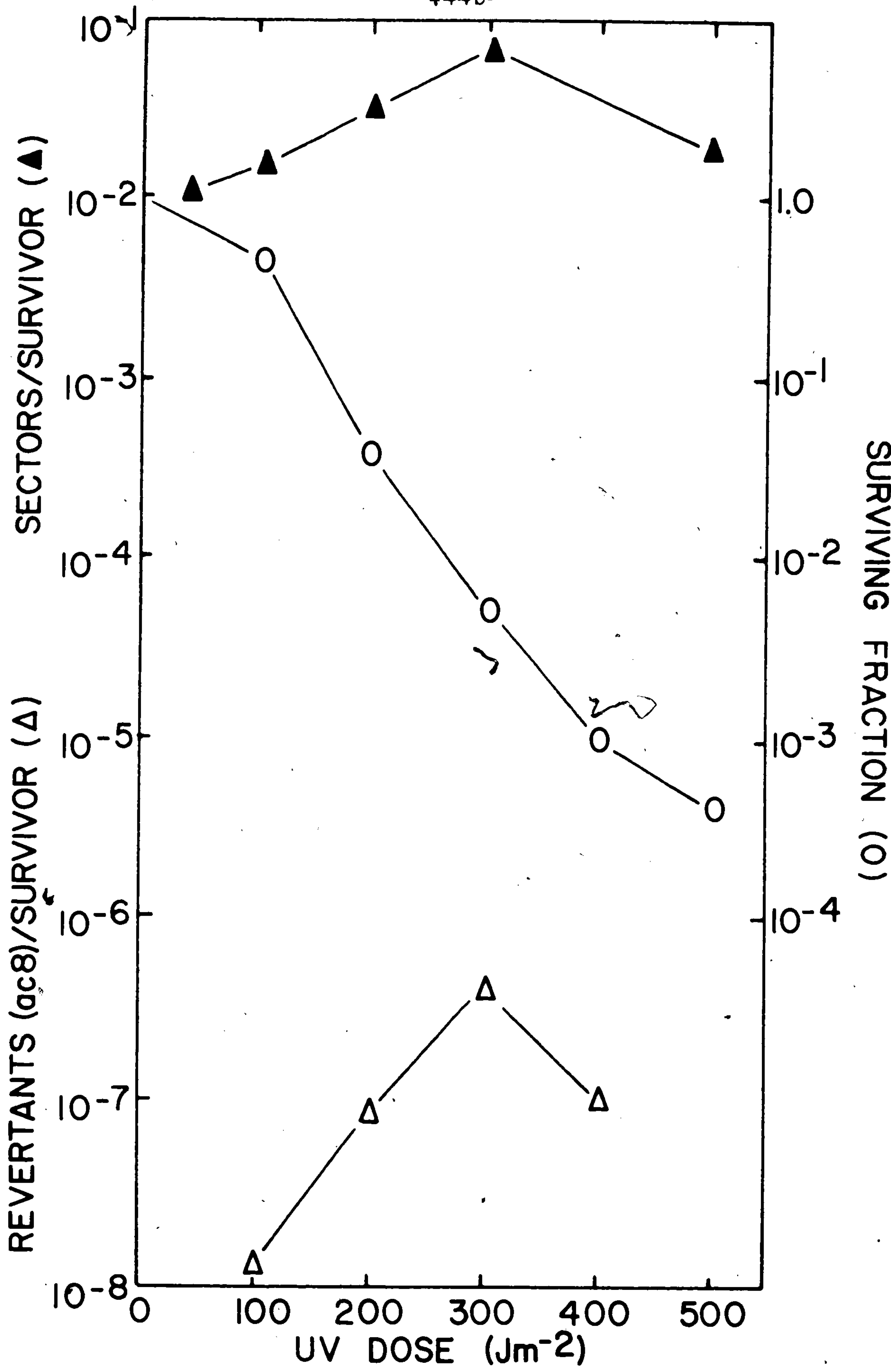
Figure III-3. Cell survival (o——o), revertant frequency of _ac8_ / survivor (△——△) and sectoring frequency/survivor (▲——▲) of cultures of _Eudorina elegans_ exposed to UV. At 0 dose the revertant frequency of _ac8_ / survivor was $< 6.4 \times 10^{-9}$ and the sectoring frequency/survivor was $< 2.8 \times 10^{-4}$. Each plot is a representative of at least 2 experiments done when the majority of the cells were in the growth stage.


SECTORS/SURVIVOR (▲)
REVERTANTS (ac8)/SURVIVOR (Δ)
SURVIVING FRACTION (O)
UV DOSE (Jm-2)
10-1
10-2
10-3
10-4
10-5
10-6
10-7
10-8
1.0
10-1
10-2
10-3
10-4
0
100
200
300
400
500

UV was 4.5×10^{-6}. The sectoring frequency/survivor followed the same trend, reaching a maximum of 6.5×10^{-2} after exposure to 300 J/m^2.

The optimal conditions of MNNG treatment (10 µg/ml for one hour) and UV (300 J/m^2) were used to determine the effect of stage in the life cycle on mutagen action (Tables III-1 and III-2). Cultures at the post release stage (small), the growth stage (medium) and the stage in the life cycle just prior to division (large) were relatively easy to obtain in a synchronous state and therefore were used as representative stages of the life cycle. Lethality and reversion of <u>ac8</u> induced by MNNG were not markedly influenced by life cycle stage (Table III-1).

The stage in the life cycle at the time of exposure to UV was critical. As the organisms grew and approached the division process, their resistance to UV became greater. Exposure to 300 J/m^2 resulted in about a 100x greater surviving fraction among large celled organisms compared to newly released organisms. The revertant frequency of <u>ac8</u>/survivor was greatest at the large cell stage. Revertants were not found when cells that were just released from the parental coenobium were exposed to 300 J/m^2. This was due to their sensitivity to UV and, as a result, not

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Table III-1. The effect of stage in the life cycle of *Eudorina elegans* on survival, reversion frequency of *ac-8* and sectoring frequency, following MNNG treatment (10 μg/ml, 1 h). The MNNG surviving fraction is presented as the ratio (N/No) of the number of colony forming units/ml after treatment (N) to colony forming units/ml prior to treatment (No).

Table III-1

	Experiment #	Stage in the life cycle		
		small	medium	large
MNNG surviving fraction	1	4.5×10^{-1}	2.4×10^{-1}	5.0×10^{-1}
	2	3.0×10^{-1}	3.8×10^{-1}	3.1×10^{-1}
reversion frequency / survivor	1	1.6×10^{-4}	1.2×10^{-4}	1.4×10^{-4}
	2	2.2×10^{-4}	1.5×10^{-4}	2.0×10^{-4}
sectoring frequency / survivor	1	7.5×10^{-3}	1.0×10^{-3}	2.5×10^{-2}
	2	8.4×10^{-3}	1.2×10^{-3}	4.8×10^{-2}

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Table III-2. The effect of stage in the life cycle of _Eudorina elegans_ on survival following UV treatment (300 J/m^2), reversion frequency/survivor of _ac-8_, and sectoring frequency/survivor. The UV surviving fraction is presented as the ratio (N/No) of the number of colony forming units/ml after treatment (N) to colony forming units/ml prior to treatment (No).

Table III-2

	Experiment #	Stage in the life cycle: small	medium	large
UV surviving fraction	1	5.0×10^{-4}	5.7×10^{-3}	4.0×10^{-2}
	2	3.4×10^{-4}	5.3×10^{-3}	1.2×10^{-2}
reversion frequency / survivor	1	$< 5.0 \times 10^{-5}$	7.9×10^{-7}	3.5×10^{-6}
	2	$< 1.9 \times 10^{-5}$	4.4×10^{-7}	6.6×10^{-6}
sectoring frequency / survivor	1	6.6×10^{-2}	6.6×10^{-2}	4.8×10^{-2}
	2	6.5×10^{-2}	4.8×10^{-2}	4.8×10^{-2}

enough organisms survived to isolate revertants. However, when these cultures were treated with lower doses of UV, where the percentage of surviving cells suggested that revertants could be recovered, none were found. The UV-induced sectoring frequency/survivor was not stage-dependent.

The exposure of cultures to the optimal mutagen conditions established by the reversion and sectoring studies led to the isolation of mutants at high frequencies following enrichment (Table III-3). The maximum mutant frequency/survivor resulted after treatment with MNNG at a concentration of 10 μg/ml for one hour. Although organisms that were dependent on acetate for growth comprised the majority of mutants isolated, p-aminobenzoic acid requiring mutants and mutants requiring a reduced nitrogen source were also isolated. When cultures were treated with MNNG at less than optimal conditions, the mutant frequency was lower. When a culture was treated with 1 μg/ml for 5 hours, the mutant frequency was 5x lower than when a culture was treated with 10 μg/ml for one hour. All the mutants isolated required acetate for growth. When the time period that the culture was exposed to 1 μg/ml was extended to 48 hours, the total population survived and the mutant frequency

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Table III-3. The effect of conditions of treatment with MNNG and UV on the frequency of isolation of mutants following enrichment. * - colony forming units/ml. ** - N/No - surviving fraction.

Table III-3

Treatment		UV 300 J/m^2	UV 300 J/m^2	MNNG 1 ug/ml 5 h	MNNG 1 ug/ml 48 h	MNNG 10 ug/ml 1 h
prior to mutagen treatment	cfu/ml*	1.5×10^4	1.7×10^4	5.2×10^4	2.6×10^4	6.7×10^4
	N/No**	1.0	1.0	1.0	1.0	1.0
after mutagen treatment	cfu/ml	3.6×10^2	3.0×10^3	7.4×10^4	5.2×10^4	-
	N/No	2.5×10^{-2}	1.7×10^{-1}	1.0	2.0	-
prior to 8AG	cfu/ml	7.1×10^4	5.1×10^4	2.3×10^4	1.5×10^4	1.8×10^4
	N/No	1.0	1.0	1.0	1.0	1.0
after 8AG	cfu/ml	1.8×10^3	1.5×10^3	1.0×10^3	1.5×10^2	1.3×10^3
	N/No	2.5×10^{-2}	3.0×10^{-2}	4.3×10^{-2}	1.0×10^{-2}	7.2×10^{-2}
mutant frequency		1/4800 $= 2.1 \times 10^{-4}$	3/2502 $= 1.2 \times 10^{-3}$	6/1097 $= 5.5 \times 10^{-3}$	5/569 $= 8.8 \times 10^{-3}$	25/2420 $= 1.8 \times 10^{-2}$
spectrum of mutants isolated		none	acetate requiring mutants	acetate requiring mutants	3 acetate requiring mutants 2 multiple mutants	19 acetate requiring mutants 2 p-amino acid requiring mutants 4 nitrate reductase mutants

was only 2x lower than that obtained with optimal conditions of MNNG treatment. However, a major disadvantage encountered with this treatment condition was the isolation of mutants having more than one nutritional requirement.

Treatment of synchronous populations with UV at 300 J/m^2 showed maximum mutant induction when the culture was at the large cell stage. However, this frequency was 10x less than the maximum frequency obtained when MNNG was used as the mutagen. No mutants were isolated when a culture of newly released organisms was treated.

DISCUSSION

The optimal conditions of MNNG and UV treatment for mutant induction in _Eudorina elegans_ have been determined by examining the maximum revertant frequency/survivor and the sectoring frequency/survivor under a variety of conditions. UV is a more effective mutagen in producing morphological changes (i.e. sectors) and MNNG is more effective in producing physiological changes (i.e. revertants and auxotrophs). MNNG has also been recognized as a powerful auxotroph inducing mutagen in many systems (Alderberg et al. 1965, Singer and Frankel-Conrat 1967, Mandel and Greenberg 1963) and has been found to cause a higher rate of

mutagenesis than UV in baoteria and yeasts (Laprieno and Clark 1965).

A further disadvantage of using UV as a mutagen in Eudorina elegans is that the stage in the life cycle at the time of treatment is critical. The closer the organisms are to division the more resistant they are to UV since Eudorina has processes operating during division to repair UV induced damage (Kemp 1972, Kemp and Malloy 1975, Kemp and Wentworth 1971). If the organisms are temporally far removed from division, the damage caused by UV is rendered lethal to the organism before the time of repair is reached. Although the organisms that are irradiated close to division are more likely to be exposed to the repair mechanisms they are also more likely to be physiologically changed. This is because the repair processes are error prone (Witkin 1966).

The use of MNNG as a mutagen is advantageous since the stage in the life cycle at the time of treatment is not critical. Although it has been found in Chlamydomonas reinhardii that the lethal and mutagenic effects of MNNG are maximal during the nuclear S-phase (Lee and Jones 1976), and in Volvox carteri just prior to cleavage (Korn et al. 1978; Huskey, pers. comm.), this stage dependency is not seen in Eudorina.

Although synchronous cultures of dividing organisms are difficult to obtain in Eudorina, many of the cells that are exposed to MNNG at the large cell stage enter division during the time of treatment. The lethal and mutagenic effects of MNNG do not increase at this stage.

The isolation of auxotrophic mutants in addition to acetate dependent mutants following optimal MNNG treatment and enrichment suggests that the lack of an extensive spectrum of mutant phenotypes in green plants may be due both to less than optimal mutagenic procedures and to the lack of appropriate enrichment techniques.

CHAPTER IV

NITRATE REDUCTASE MUTANTS IN EUDORINA ELEGANS

INTRODUCTION

The metabolism of nitrate to nitrite and then to ammonia has been extensively studied in recent years. In particular, the initial enzyme of the pathway, nitrate reductase, has been studied in bacteria (Forget 1974), fungi (Choudary and Rao 1975; Sorger and Davies 1973), algae (Barea et al. 1976; Morris and Syrett 1963; Piotórious et al. 1976) and higher plants (Beevers and Hageman 1969). In some of these organisms investigations of physiological, regulatory and genetic aspects of nitrate metabolism have been aided by the isolation of mutants that are unable to utilize nitrate as a nitrogen source.

In *Eudorina elegans*, mutants deficient or lacking in the production of nitrate reductase have been isolated. These types of mutants have not been previously characterized in sexual algae. This paper discusses how these mutants (*nar* -1,-2,-3) differ from wild type and each other in their growth responses to different nitrogen sources, in their production of the enzymes nitrate and nitrite reductase, and in their response to potassium chlorate.

MATERIALS AND METHODS

The wild type isolate used in the experiments was *Eudorina elegans* UTEX 1201 (Culture Collection of Algae, University of Texas). The *nar* mutants are derivatives of 1201, each isolated after exposure to N-methyl-N'-nitro N-nitrosoguanidine and subsequent enrichment (Chapter II).

The basic minimal medium (BM) was Bristol's basic salt solution (Cain 1965) containing sodium nitrate as nitrogen source. In some experiments described here, sodium nitrate was replaced by urea (BM-U), uric acid (BM-UA), ammonium nitrate (BM-NH4), sodium nitrite (BM-NO2) or hypoxanthine (BM-HX) in the same molar concentration as sodium nitrate (i.e. 3×10^{-3} M). Trace elements were supplied by Gaffron's solution (Hughes et al. 1958). The enriched medium (BC) was made up of 800 ml of BM and 200 ml of modified *Euglena* medium (Kemp and Wentworth 1971).

Cultures were maintained at 30°C under cool-white fluorescent lights (10 J / m^2s) on a 16 : 8 L : D cycle. For the growth experiments the cultures were grown as shaking cultures in 125 ml erlenmeyer flasks containing 50 ml of medium. The strains, growing in BC, were washed and inoculated at low titre (ca. 100 organisms/ ml) into minimal medium containing the nitrogen source being studied. Numbers of viable organisms were determined

daily by their ability to form visible colonies on nutrient agar plates. These data are presented as colony forming units (cfu/ml). For the enzyme assays, the cultures were grown in 800 ml cylindrical vessels to which were attached entrance ports for three reservoirs of sterile media. The vessels also had an inoculation port, an outlet from which media could be removed, and a port through which a gas mixture of 3% CO2 in air was bubbled.

Enzyme extracts were prepared from algae collected by centrifugation (2,500 rpm, 5 min). The organisms were washed three times in cold ($4^{o}C$) extraction buffer (0.1 M potassium phosphate buffer, pH 7.5, for the nitrate reductase assay and 0.5 M Tris-HCl buffer, pH 8.0, for the nitrite reductase assay). Following resuspension in buffer, the cells were sonicated on ice at 90 intensity (Bronwill Biosonic, Bronwill Scientific, Rochester, New York) for 5 second bursts repeated 5 times every 55 seconds. The sonicated material was used directly for the nitrite reductase assay. For the nitrate reductase assay, the sonicate was centrifuged in the cold at 13,000 rpm for 20 minutes to precipitate the particulate material. The supernatant was then used for the analysis of nitrate reductase activity.

Nitrate reductase was assayed by a modification of the procedure of Wray and Filner (1970). The assay

mixture contained 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.5; 0.1 ml of 0.1 M KNO_3; 0.1 ml of 1 mM NADH and 0.5 ml of algal extract. This was incubated for 15 min at 30°C, then 1 ml of sulfanilamide in 3M HCl and 1 ml of 0.02% N-(1 naphthyl) ethylenediamine dihydrochloride was added to stop the reaction and assay for nitrite. The tube contents were clarified by centrifugation and after 5 min at room temperature the absorbance was measured at 540 nm. These values were converted into amounts of nitrite by reference to a standard curve.

The nitrite reductase assay mixture (Ramirez et al. 1966) contained 0.3 ml of 0.5 M Tris-HCl buffer, pH 8.0; 0.2 ml of 20 nM $NaNO_2$; 0.3 ml of 5 mM methyl viologen; 0.9 ml of algal extract; 0.3 ml of sodium dithionite solution (25 mg in 1 ml of 0.29 M $NaHCO_3$). This mixture was incubated for 10 min at 30°C. The reaction was stopped by oxidizing the mixture by vigorously shaking on a Vortex mixer until the dye became colorless. The tube contents were then diluted 100 times and nitrite determined on a 2 ml aliquot.

Protein in the algal extracts was determined using bovine serum albumin as standard (Lowry et al. 1951).

RESULTS

When ammonia (BM-NH_4) was provided as sole

nitrogen source, wild type and the nar mutants all grew well (Figure IV-1). Similar results were observed when the strains were grown in BM-U, BM-UA and BM-NO2. In BM, wild type grew as well as in BM-NH4 whereas nar-1 grew at a slower rate, reaching maximum titre 2 days later than wild type (Figure IV-2). After an initial rise in titre during the first 3 days after inoculation, the titre of nar-2 and nar-3 did not change for 7 days. In BM-HX, wild type and nar-2 grew well whereas nar-1 and nar-3 did not (Figure IV-3). The initial rise in titre exhibited by nar-2 and nar-3 in BM and by nar-1 and nar-3 in BM-HX may have been due to coenobial breakdown and/or a low level of usable nitrogen in the medium or the algal cells.

Nitrate reductase activity was measured in wild type and the nar mutants at intervals following transfer from BM-NO2 to BM which contained sodium nitrate as nitrogen source (Figure IV-4). In wild type, nitrate reductase increased markedly for 2.5 h, decreased slightly during the next 2 h and then increased again. Nitrate reductase activity of nar-1 was not detected until 7 h after induction, and in nar-2 and nar-3 nitrate reductase activity was not found during a 25 h induction period. To examine the possibility that an inhibitor of nitrate reductase was present in nar-2 and nar-3 , a

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Figure IV-1. Growth of <u>Eudorina elegans</u> wild type (o—o), <u>nar-1</u> (●—●), <u>nar-2</u> (△—△) and <u>nar-3</u> (▲—▲) in BM-NH4. --- = aliquot transferred to fresh medium.

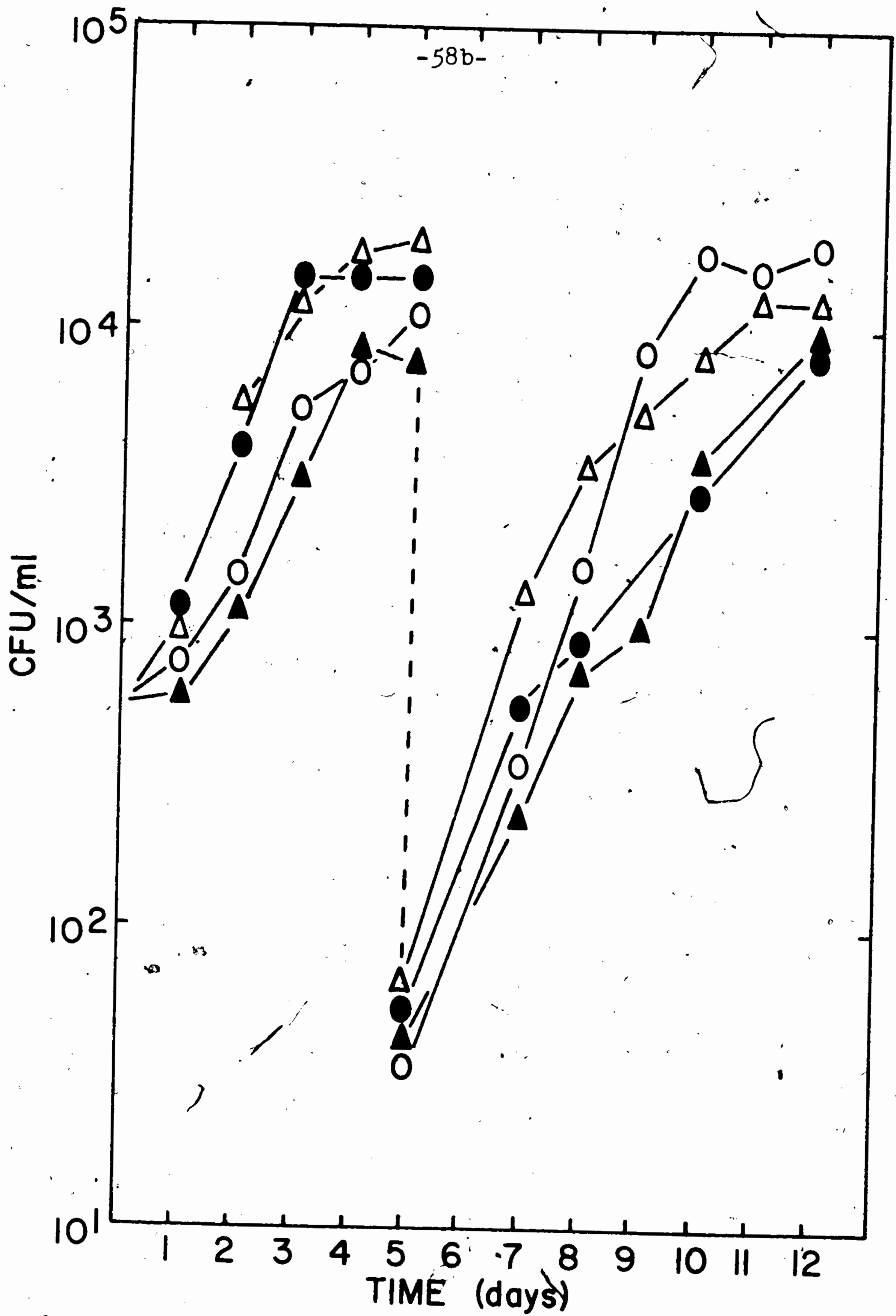
10^5
10^4
10^3
10^2
10^1
CFU/ml
1 2 3 4 5 6 7 8 9 10 11 12
TIME (days)

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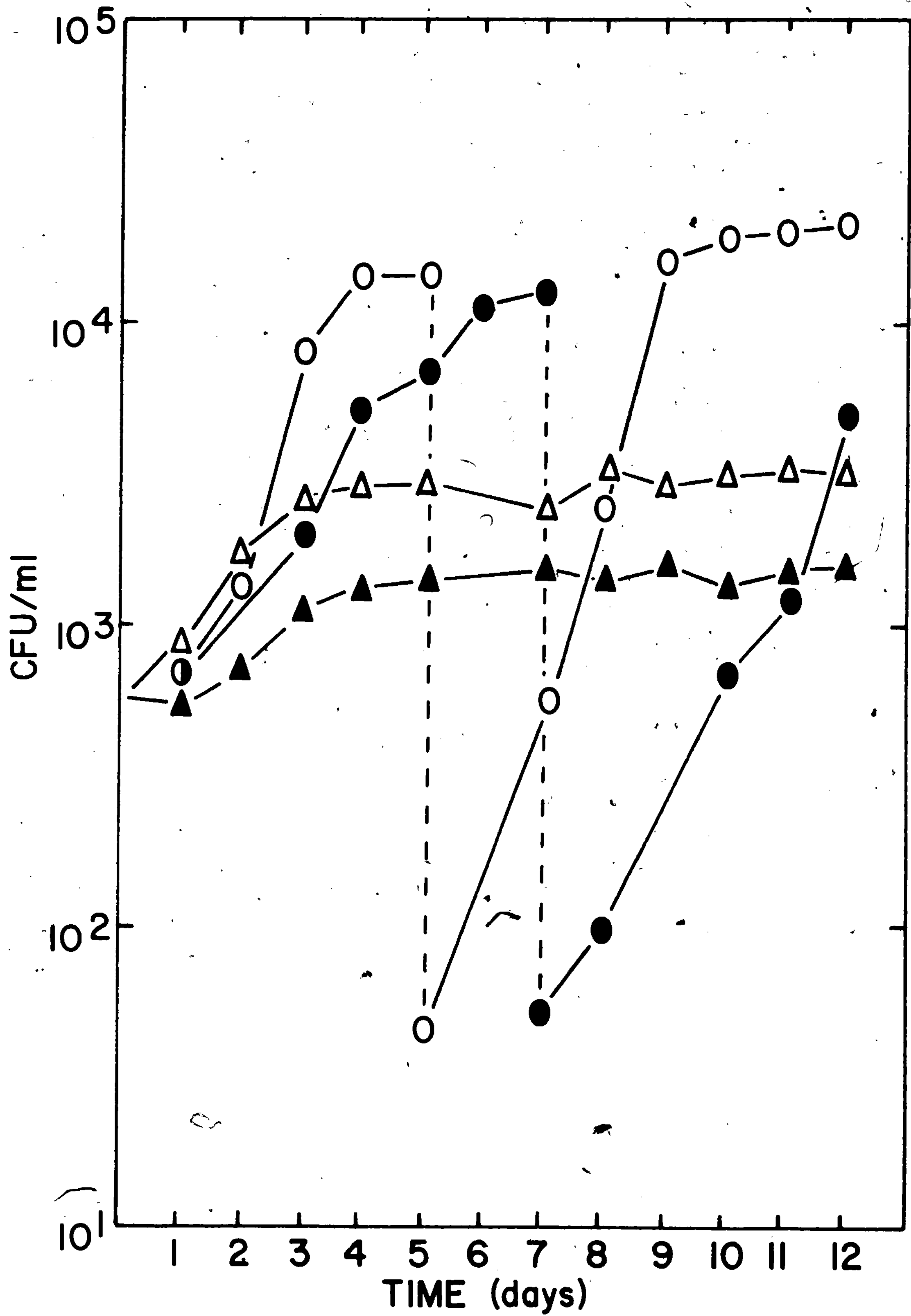
Figure IV-2. Growth of _Eudorina_ _elegans_ wild type (o—o), _nar-1_ (●—●), _nar-2_ (△—△), and _nar-3_ (▲—▲) in BM. --- = aliquot transferred to fresh medium.



10^5
10^4
10^3
10^2
10^1
CFU/ml
1 2 3 4 5 6 7 8 9 10 11 12
TIME (days)

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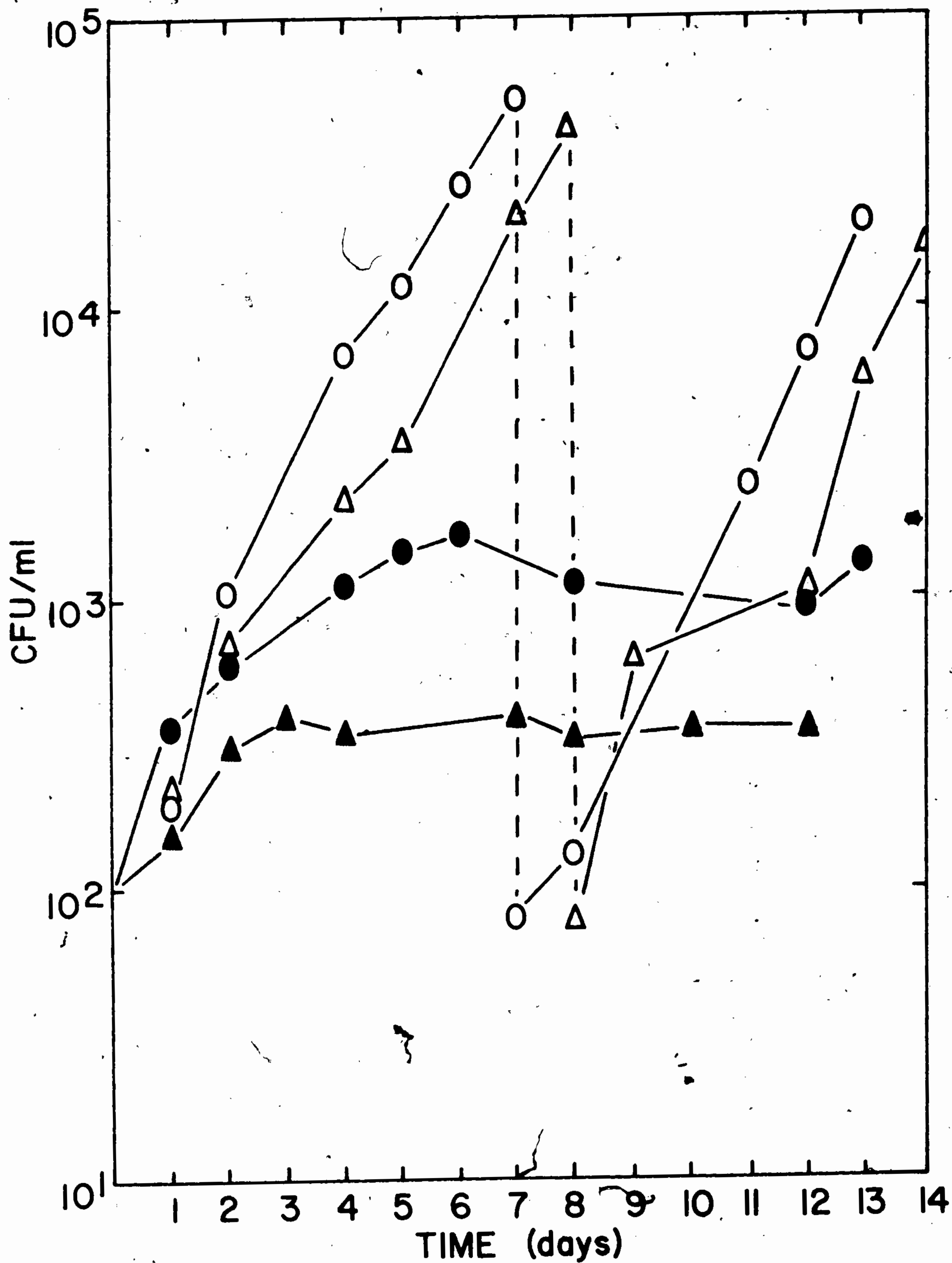
Figure IV-3. Growth of Eudorina elegans wild type (o— o), nar-1 (●—●), nar-2 (△—△) and nar-3 (▲—▲) in BM-HX. ---- = aliquot transferred to fresh medium.



10^5
10^4
10^3
10^2
10^1
CFU/ml
1 2 3 4 5 6 7 8 9 10 11 12 13 14
TIME (days)

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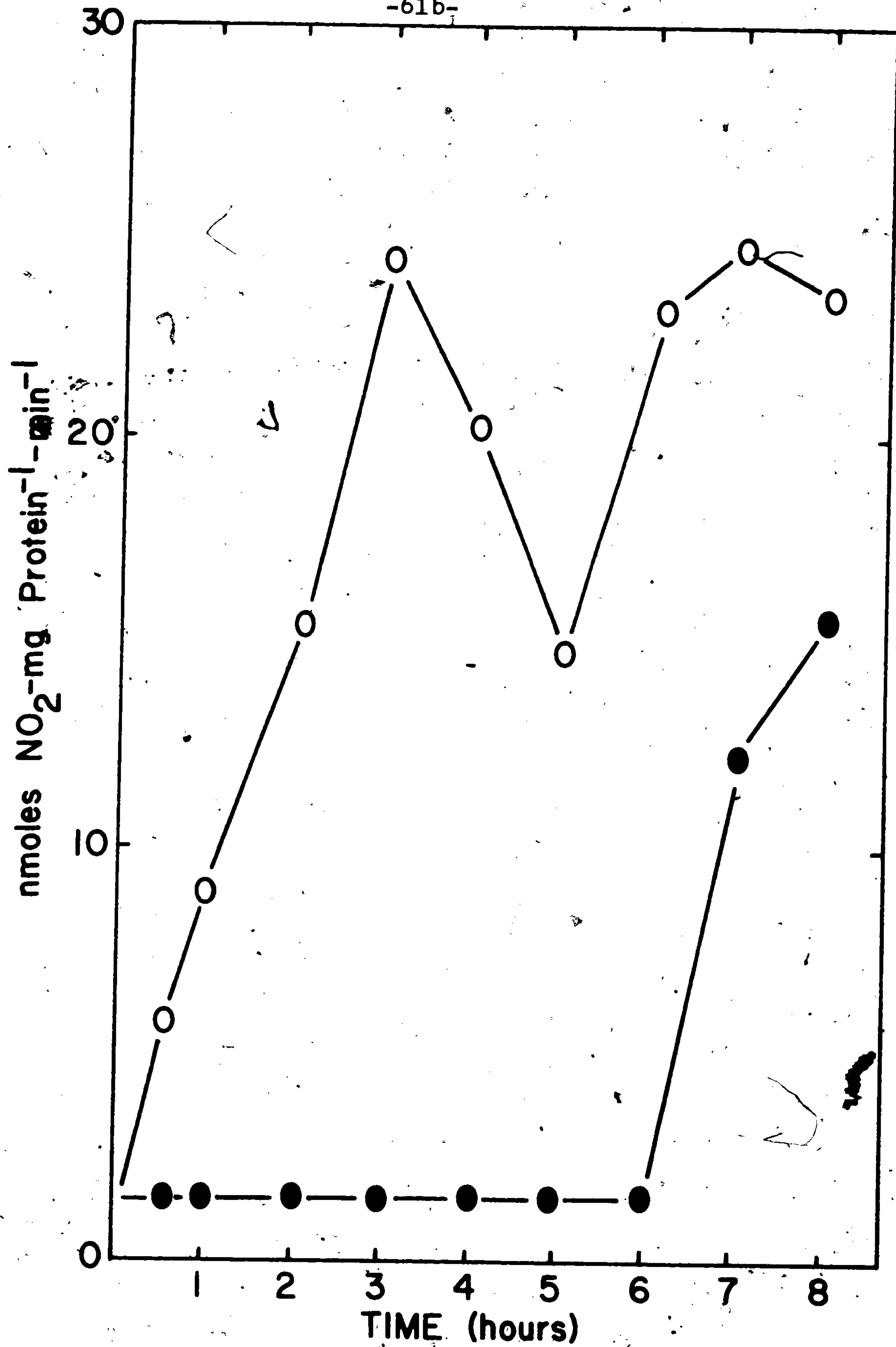
Figure IV-4. Induction of nitrate reductase activity in Eudorina elegans wild type (o—o) and nar-1 (●—●) cultures grown in BM-NO2 and then transferred to BM at 0 time.


30
20
10
0
nmoles NO_2-mg Protein^{-1}-min^{-1}
1
2
3
4
5
6
7
8
TIME (hours)

crude enzyme fraction from each mutant was mixed with a equal volume of an enzyme fraction from wild type. In both mutants the activity obtained in the mixture was similar to that expected if the wild type enzyme alone was active (Table IV-1).

The specific activity of nitrite reductase was also found to vary among the four strains (Table IV-2). Since the specific activity of the enzyme has been found to vary with the stage in the life cycle, at the time of the enzyme assay (unpublished) the experimental results in Table IV-2 were obtained when the cultures were at comparable stages, i.e. when $>$90% of the population were newly released from the parental coenobia. Compared with the wild type, nar-1 showed a 1.[illegible]x increase, nar-2 a 2.5x increase and nar-3 a 7.6x increase in the specific activity of nitrite reductase.

Wild type and the nar mutants also exhibited varying levels of resistance to potassium chlorate (Figure IV-5). Wild type showed minimal chlorate tolerance. The growth increment declined at 0.1 mM chlorate until no survivors were apparent at 10 mM chlorate. Nar-1 was more resistant to chlorate than wild type. A decline in the growth increment was observed at 1 mM chlorate and no viable organisms remained at 50 mM chlorate. Nar-2 and nar-3 showed the greatest resistance

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Table IV-1. The effect of extracts of *nar-2* and *nar-3* on the activity of nitrate reductase from wild type *Eudorina elegans*. Values are expresses as μ moles NO2 formed /mg protein/minute. Sonicates of *nar-2* and *nar-3* were mixed 1:1 with sonicates of wild type *Eudorina* (mixture). Expected value = wild type value/2.

C

Table IV-1

	Experiment number 1	Experiment number 2
wild type	2.3×10^{-2}	2.6×10^{-2}
nar-2	0	0
mixture	1.1×10^{-2}	1.1×10^{-2}
expected value	1.15×10^{-2}	1.3×10^{-2}

	Experiment number 1	Experiment number 2
wild type	3.8×10^{-2}	2.6×10^{-2}
nar-3	0	0
mixture	1.7×10^{-2}	1.4×10^{-2}
expected value	1.7×10^{-2}	1.3×10^{-2}

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Table IV-2. Nitrite reductase activity (μmoles nitrite removed/mg protein/minute) in wild type and the nar mutants of Eudorina elegans. The values represent the mean ± the standard error of three experiments.

Table IV-2

wild type	0.38 ± 0.1
nar-1	0.57 ± 0.04
nar-2	0.97 ± 0.03
nar-3	2.9 ± 0.1

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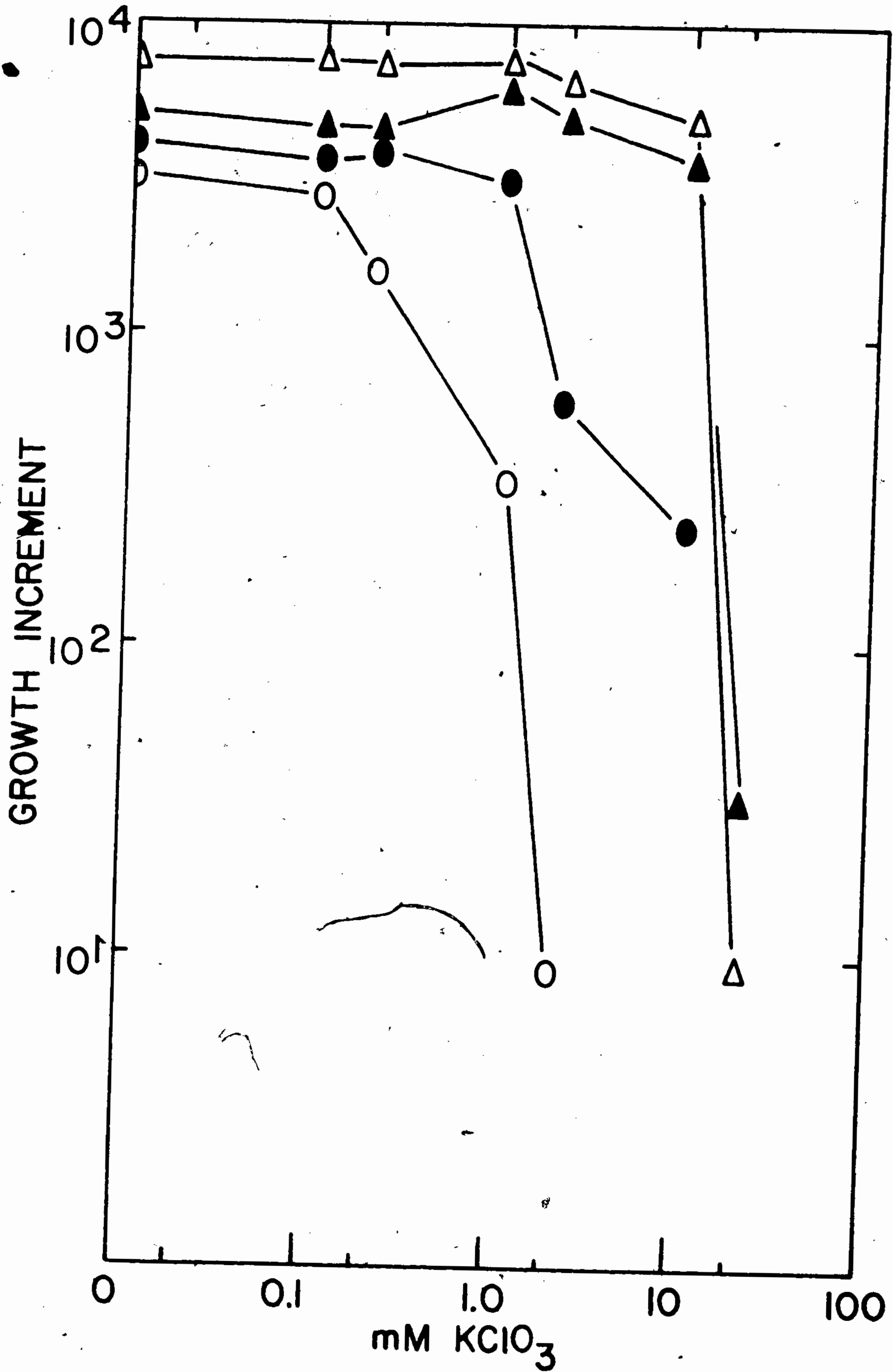
Figure IV-5. Effect of potassium chlorate on growth of <u>Eudorina</u> <u>elegans</u> wild type (o—o), <u>nar-1</u> (●—●), <u>nar-2</u> (△—△) and <u>nar-3</u> (▲—▲). The growth increment was determined after 6 days in each concentration.


GROWTH INCREMENT
10^4
10^3
10^2
10^1
0
0.1
1.0
10
100
mM KClO3

to chlorate with a decline in the growth increment observed at a chlorate concentration of 50 mM. At 100 mM chlorate concentration no viable organisms remained.

DISCUSSION

Analyses of growth in various nitrogen sources, the activity of nitrate and nitrite reductase, and the response to potassium chlorate indicate that nar-1 is a leaky mutant deficient in nitrate reductase activity and that nar-2 and nar-3, although different from each other, lack the ability to produce this enzyme. Due to an insufficient production of nitrate reductase, nar-1 grows in BM at a reduced rate when compared with wild type. Nar-2 and nar-3 do not produce nitrate reductase, but they differ from each other in that nar-2 grows in BM-HX whereas nar-3 does not. The inability of nar-1 to grow well in BM-HX suggests that this strain may be a leaky mutant of the nar-3 variety.

Based solely on their growth responses to different nitrogen sources, nar-2 and nar-3 can be compared to mutants isolated in Neurospora crassa (Coddington 1976) and Aspergillus nidulans (Pateman et al. 1964). Nar-2 grows in the same nitrogen sources as nit-3 of Neurospora and the nia D mutants of Aspergillus. Nit-3 mutants have been shown to lack nitrate reductase and

cytochrome c reductase but to maintain benzyl viologen nitrate reductase activity (Sorger 1966). The nia D gene has been shown to be the structural gene for the nitrate reductase apoenzyme (MacDonald and Cove 1974; Pateman et al. 1964).

Nar-3 responds to the same nitrogen sources as the cnx mutants of Aspergillus. These genes direct the synthesis of a co-factor shared by nitrate reductase and xanthine dehydrogenases 1 and 2 (Pateman et al. 1964). Nar-3 can also be compared to the nit-1 mutant of Neurospora which lacks NADPH nitrate reductase but retains cytochrome c reductase. It is suggested that the conversion of cytochrome c reductase to nitrate reductase in this Neurospora mutant is dependent on a labile molecule that requires added molybdenum for its effect (Lee et al. 1974). It is postulated that this molybdenum co-factor acts as an electron carrier and also links the protein subunits of nit-1 together. In Eudorina elegans, a concentration of molybdate greater than 2.97 mM results in the death of wild type cells. When nar-3 is exposed to molybdate concentrations ranging from the normal concentration found in BM (2.97×10^{-3} mM), to 10^{4} times this amount (29.7 mM) no effect on their response to nitrate is observed.

Induction of nitrate reductase in wild type

Eudorina elegans occurs rapidly when the organisms are transferred from nitrite to nitrate. The drop in activity apparent after the rapid rise in activity during the first 2.5 h could be due to repression caused by a buildup in the ammonia concentration. Subsequent removal of ammonia by further amino acid synthesis would cause a resumption of nitrate reductase activity (Coddington 1976). Alternatively, nitrate reductase, when not in a complex form with nitrate, could act as its own repressor or as a co-repressor of its own synthesis (Cove 1967). As a result, when the enzyme concentration rises to such a level that the intracellular nitrate concentration is no longer sufficient to saturate it, repression occurs. This situation is remedied when the nitrate concentration increases again due to the fall-off of nitrate reductase content.

Induction of nitrate reductase in *nar-1* occurs after a 7 h delay, but the eventual rate of increase in activity is similar to wild type. However, the maximal nitrate reductase activity on *nar-1* is only about half that of wild type, accounting for the slower growth rate of *nar-1*.

There are two theories to account for the action of chlorate. One suggests that chlorate itself is not toxic, but is rendered toxic by the conversion of

chlorate to chlorite as the result of the catalytic action of nitrate reductase (Cove 1976b). The second theory is that chlorate is toxic because it mimics nitrate in mediating, _via_ nitrate reductase, a shut down of nitrogen metabolism (Cove 1976a). Nitrogen starvation follows because chlorate cannot act as a nitrogen source. Although the nitrate reductase mutants of _Eudorina elegans_ were not isolated on the basis of their chlorate resistance they exhibit varying levels of resistance to potassium chlorate. The mechanism of chlorate toxicity is not known but it is apparently related to the ability to form nitrate reductase. Wild type produces nitrate reductase and is susceptible to chlorate toxicity. _Nar-1_, producing a lower level of nitrate reductase than wild type, shows some resistance to chlorate, whereas _nar-2_ and _nar-3_ being deficient in nitrate reductase activity are much more resistant to chlorate.

Nar-1 and _nar-3_ exhibit a Mendelian pattern of inheritance (Kemp, pers. comm.). However, crosses of _nar-2_ and intercrosses of _nar-1_ and _nar-3_ have not been successful and therefore the genetic relationship between the _nar_ mutants has not yet been established.

The isolation of these algal mutants and the establishment of their properties could aid in investi-

gations of the regulation of nitrogen metabolism in photosynthetic systems.

CHAPTER V

CONCLUSION

The first enrichment procedure reported for a phototrophic organism designed to isolate auxotrophic mutants has been developed using the colonial alga, Eudorina elegans. This method has the potential of being a powerful tool to examine the possibility of increasing the mutant spectrum of phototrophic systems and, as a result, to expand the biochemical and genetic knowledge of this large group of organisms. The procedure, to this point, has resulted in a recovery of mutants at a frequency of 1.6×10^{-2} and has yielded mutants never before seen in Eudorina elegans.

In the following pages I will present some of my thoughts concerning the use of this technique to aid in present and future research using Eudorina elegans. These include selective enrichment, conditional mutant isolation, expansion of the screening procedure of an enriched population and progress in the understanding of nitrogen metabolism.

Although amino acid auxotrophs have not yet been isolated in Eudorina, modifications of the enrichment technique are actively being pursued in order to increase the possibility of isolating these mutants. One such modification is the use of different mutagens. Since it

has been demonstrated that the effectiveness and optimal conditions of mutagen treatment can be conveniently ascertained by reversion and sectoring studies (Chapter III), a large variety of mutagens can be screened relatively easily. Initially, however, studies will be limited to mutagens that have been shown to have some effect on algae. The mutagens include methyl methanesulfonate (MMS), an alkylating agent which has been extensively studied in Chlamydomonas reinhardii (Loppes 1966), and ethyl methanesulfonate (EMS) which has been shown to cause a high reversion frequency of an arginine requiring strain of Chlamydomonas reinhardii (Loppes 1968). Hydroxyurea, an inhibitor of ribonucleotide reductase, will also be examined as it has been shown to increase the frequency of streptomycin resistant mutants in Chlamydomonas reinhardii (Adams and Warr 1976). The effect of 4-nitroquinoline 1-oxide, a carcinogen capable of causing single strand scissions of DNA will also be studied since preliminary experiments have determined that damage induced by this drug is not repairable by an excision-resynthesis-repair system in Eudorina elegans (Kemp and Malloy 1973).

Another variation in the enrichment procedure involves the selective enrichment of particular classes of mutants that may be present at extremely low

frequencies in the mutagenized population. These modifications include increasing the number of cells being mutagenized by increasing the culture volume, recycling the survivors of the 8-azaguanine treatment through the enrichment procedure several times, and regrowing the surviving cells in a defined medium supplemented with the nutritional requirements of the specific mutants being selected for prior to each starvation step.

Temperature sensitive mutants are also being isolated by modifying the enrichment technique. This is being tried because many of the mutations induced may be lethal if they affect enzymes that are essential for growth. This problem has been circumvented in many systems by isolating conditional mutants in which the mutant gene product can function under permissive conditions (i.e. lower temperature) but not under restrictive conditions (i.e. higher temperature). The conditional mutants will also be useful in examining the life cycle-dependent timing of metabolic and/or morphological events since the time at which a gene product is acting can be determined by shifting the organisms to restrictive conditions at various times during development (Suzuki 1970).

Recently the mutant enrichment procedure has

been adapted to isolate temperature sensitive mutants in Eudorina by mutagenizing, regrowing and starving the organisms of nitrogen at the permissive temperature (25°C) and then refeeding nitrogen and 8-azaguanine at the restrictive temperature (35°C). Those mutants that cannot grow at the higher temperature are unaffected by 8-azaguanine. The surviving cells are plated on an enriched agar medium, grown at 25°C and then screened for their ability to survive at both temperatures on minimal and enriched media. Three different classes of temperature sensitive mutants have been isolated. The majority of them are temperature sensitive lethal mutants that are able to grow on minimal and enriched media at the permissive temperature but that are unable to grow on either medium at the restrictive temperature. The second class is comprised of auxotrophic, non-lethal temperature sensitive mutants that fail to grow only on minimal medium at the restrictive temperature. The third class is characterized by the ability to grow only on enriched medium at the permissive temperature. All of these mutants are in the process of being further characterized.

The ability to replica plate colonies of Eudorina elegans allows for the rapid screening of potential mutants for auxotrophic properties. This

screening procedure may be expanded to isolate certain classes of mutants defective in morphological or other non-auxotrophic metabolic processes by using the appropriate selective media and growth conditions. The enrichment procedure is expected to select for these classes of mutants only if their metabolic processes are absent or reduced by the mutation event and they escape the action of 8-azaguanine.

In many systems the only means of detecting cell division mutants is to microscopically scan mutagenized organisms for division abnormalities (Simchen 1978). This is, however, a tedious screening method that can be improved upon when using _Eudorina_. The natural, fixed pattern and number of cell divisions produces a characteristic morphology of a colony on a agar surface. Thus, any colony with an abnormal morphology is a putative cell division mutant. Recently, a mutant was isolated in our laboratory in a temperature sensitive screen because of the abnormal appearance of the replicated colony at the restrictive temperature. It was subsequently found that the cells of this mutant strain divide the normal four or five times at the permissive temperature but appear to divide anywhere from one to five times at the restrictive temperature. My studies of this mutant are continuing.

However, other mutants may be induced that are

not phenotypically distinctive, but whose division processes are abnormal. This type of mutant may be identified by the fact that it grows on a medium containing a specific inhibitor of cell division. The effectiveness of colchicine as a selective agent to isolate cell division mutants has been shown for *Chlamydomonas reinhardii* (Adams and Warr 1971; Sato 1976). Colchicine disrupts microtubular formation by binding to the microtubular protein, tubulin. The temperature sensitive class of cell division mutants of *Chlamydomonas* has an altered microtubular structure and was initially selected due to its resistance to colchicine at the restrictive temperature.

As an analog of arginine that is incorporated into protein, canavanine could provide a means of screening for mutants defective in protein synthesis. This possibility has been examined in *Chlamydomonas reinhardii* and mutants conditionally resistant to canavanine were shown to be defective in protein synthesis (Mahon 1971). The disadvantage of canavanine selection may be that it selects for only a subset of all possible mutants of protein synthesis because of the peculiarities of its mode of action. Cycloheximide is also a possible selective agent for mutants defective in protein synthesis. It is an inhibitor of the

translation step of protein synthesis. Therefore, mutants selected against the inhibitor are expected to have a more efficient translational apparatus. This compound may, however, be disadvantageous since mutants of *Chlorella vulgaris* that are conditionally resistant to this compound also show a decrease in carotenoid and RNA content (Passera and Ferrari 1973).

The most useful selective agent for mutants with defective protein synthesis will probably be 6-methyl-purine. This compound preferentially interferes with the transcriptional step of protein synthesis and also causes multiple collateral effects on cellular metabolism, particularly the inhibition of ATP synthesis. Mutants resistant to this drug should therefore have a more efficient transcriptional apparatus and higher ATP synthesizing power (Passera and Ferrari 1973). Although protein synthesis-defective mutants of *Chlamydomonas reinhardii* and *Chlorella vulgaris* isolated so far are not overproducers of specific proteins it is possible that , if isolated, overproducing mutants may be commercially profitable as a source of protein. This is because their selective characteristics may result in a faster growth rate, quantitative and qualitative amelioration of algal protein and a higher content of vitamins (Passera and Ferrari 1973).

Simple color reactions, extremely useful for isolating mutants that cannot be easily detected by phenotypic characteristics, can be incorporated into the mutant screen. This method has been extremely useful in detecting mutants defective in the production of certain enzymes. For example, a simple visualization procedure has been employed to isolate mutants with defective phosphatase activity in bacteria (Messer and Vielmetter 1965), fungi (Dorn 1965), yeast (Cabib and Duran 1975) and Chlamydomonas (Loppes and Matagne 1973). Those organisms that liberate alkaline phosphatase are detected by the appearance of a yellow halo around the colonies due to the production of p-nitrophenyl from p-nitrophenyl phosphate through the action of alkaline phosphatase.

A color reaction has also been used to detect urease activity. This reaction is based on the liberation of ammonia when the test organism is cultivated with urea as substrate (Paliwal and Randhava 1977). Ammonia release can be detected because its production causes a rise in pH which leads to a red to pink color change in the phenol red indicator.

A powerful method has recently been developed that could be most useful in screening mutagenized Eudorina colonies for the ability to produce $^{14}CO2$ from labelled

metabolites (Tabor et al. 1976). When organisms are grown in ^{14}C labelled metabolites and are capable of producing $^{14}CO_2$, $Ba^{14}CO_3$ is formed when the colony is covered with filter paper saturated with BaOH. Since $Ba^{14}CO_3$ is insoluble, $^{14}CO_2$ is fixed immediately above the colony. The presence of ^{14}C is revealed by using x-ray film radioautography. Colonies lacking the metabolic ability to convert the specific substrate to CO_2 are identified because of a lack of radioactivity corresponding to their position on the plate.

The detection of antibiotic resistant mutants can be incorporated into a general screen of the mutagenized, enriched population by replica plating the organisms on to a medium containing the minimum concentration of the drug that is lethal to wild type organisms (Mottley and Griffiths 1977). Not only are these mutants of use in reversion studies but also as genetic markers. Antibiotic resistant mutants of Chlamydomonas reinhardii have also been used to study the genetic control of chloroplast ribosome biosynthesis. Erythromycin and streptomycin resistant mutants have altered protein in the large chloroplast ribosomal subunit (Mets and Bogorad 1972; Davidson and Hanson 1974). Mutants resistant to streptomycin and erythromycin have recently been isolated in

Eudorina elegans by the replica plating technique. All the streptomycin resistant mutants tested have shown a uniparental pattern of inheritance (Kemp, pers. comm.). The erythromycin resistant mutants have only recently been isolated and their inheritance characteristics are in the process of being determined.

The application of the mutant enrichment procedure has resulted in the isolation and characterization of a number of mutant phenotypes. The major class of mutants is dependent on acetate for growth. It has retained the obligate requirement for light and cannot grow in the dark in supplemented medium. The photosynthetic system of these mutants examined to date appears unaffected as indicated by normal Hill reactions and ribulose phosphate carboxylase activites (Anderson and Kemp, pers. comm.).

Additional mutants defective in nitrogen metabolism have recently been isolated. They are nir-1 which cannot utilize nitrate or nitrite and HX-1 which grows in all tested nitrogen sources but hypoxanthine. They will be used to further expand our understanding of nitrate reduction.Enzymes of the nitrate reducing system of wild type organisms of Chlamydomonas reinhardii (Herrera et al. 1972; Barea and Cardenas 1975; Barea, Maldonado and Cardenas 1976; Barea, Sosa, and Cardenas

1976) and Chlorella vulgaris (Losada et al. 1970) are well characterized. Wild type organisms of Chlamydomonas reinhardii have been used to determine that the nitrate reductase enzyme of this organism has a molecular weight of 350000 - 500000, that it contains flavine adenine dinucleotide, molybdenum and a heme iron, that the enzyme consists of a protein complex with two activities acting sequentially and that it is repressed by ammonia (Barea and Cardenas 1975; Bare, Maldonado and Cardenas 1976). However, mutants defective in their nitrate assimilation capacity have only recently been employed to expand our knowledge of the regulation and mechanism of action of nitrate reductase in Volvox carteri (Huskey et al. 1979) and Chlamydomonas reinhardii (Nichols et al. 1978; Nichols and Syrett 1978; Sosa et al. 1978).

Recent studies of wild type organisms and the nar mutants of Eudorina have revealed significant differences between the algal reduction system and that in fungi. A series of preliminary experiments have been undertaken to examine cytochrome c reductase in Eudorina since this enzyme and nitrate reductase have been found to be associated with each other in Neurospora crassa (Sorger 1963). I have established that a basal level of cytochrome c reductase exists in urea grown cultures of

wild type and the nar mutants. Upon transfer to nitrate or nitrate and urea media the level of cytochrome c reductase increases in wild type, nar-1 and nar-3 (Table V-1). In contrast to the situation in Neurospora (Sorger 1963,1965), where the induced form of cytochrome c reductase differs from the basal form in molecular size as determined by sucrose gradient centrifugation, the cytochrome c reductase in Eudorina sediments identically from both induced and uninduced strains. Nar-2 differs from wild type and the nar mutants in that if urea is present in addition to nitrate, cytochrome c reductase activity is not induced. This inhibition of induction by urea in nar-2 appears to be unique to this mutant strain of Eudorina elegans.

An examination of the effect of ammonia and a lack of nitrogen on the stability of nitrate reductase has begun. These experiments involve switching a maximally induced culture of wild type organisms to medium containing nitrate, ammonia, ammonia and nitrate or a medium lacking nitrogen, and following nitrate reductase activity (Figure V-1). Unlike the situation in fungi (Subramanian and Sorger 1972), where both a lack of nitrogen and the presence of ammonia result in a rapid loss of nitrate reductase activity, only ammonia produces a marked loss of nitrate reductase activity in Eudorina elegans. A lack of nitrogen has much less effect. Following an initial decrease in nitrate

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Table V-1. Cytochrome c reductase activities (ΔOD/mg protein/min) in wild type and the *nar* mutants of *Eudorina elegans* at time intervals following transfer from BM(U) to BM(NO3) and BM(NO3 + U).

TABLE V-1

	BM (U)	BM (NO_3) 45 min	BM (NO_3) 3 h	BM (NO_3+U) 45 min	BM (NO_3+U) 3 h
1201	0.61	0.83	1.03	0.81	1.02
nar-1	0.62	0.87	1.04	0.83	1.07
nar-2	0.63	0.85	1.07	0.64	0.71
nar-3	0.66	0.88	1.32	0.82	1.30

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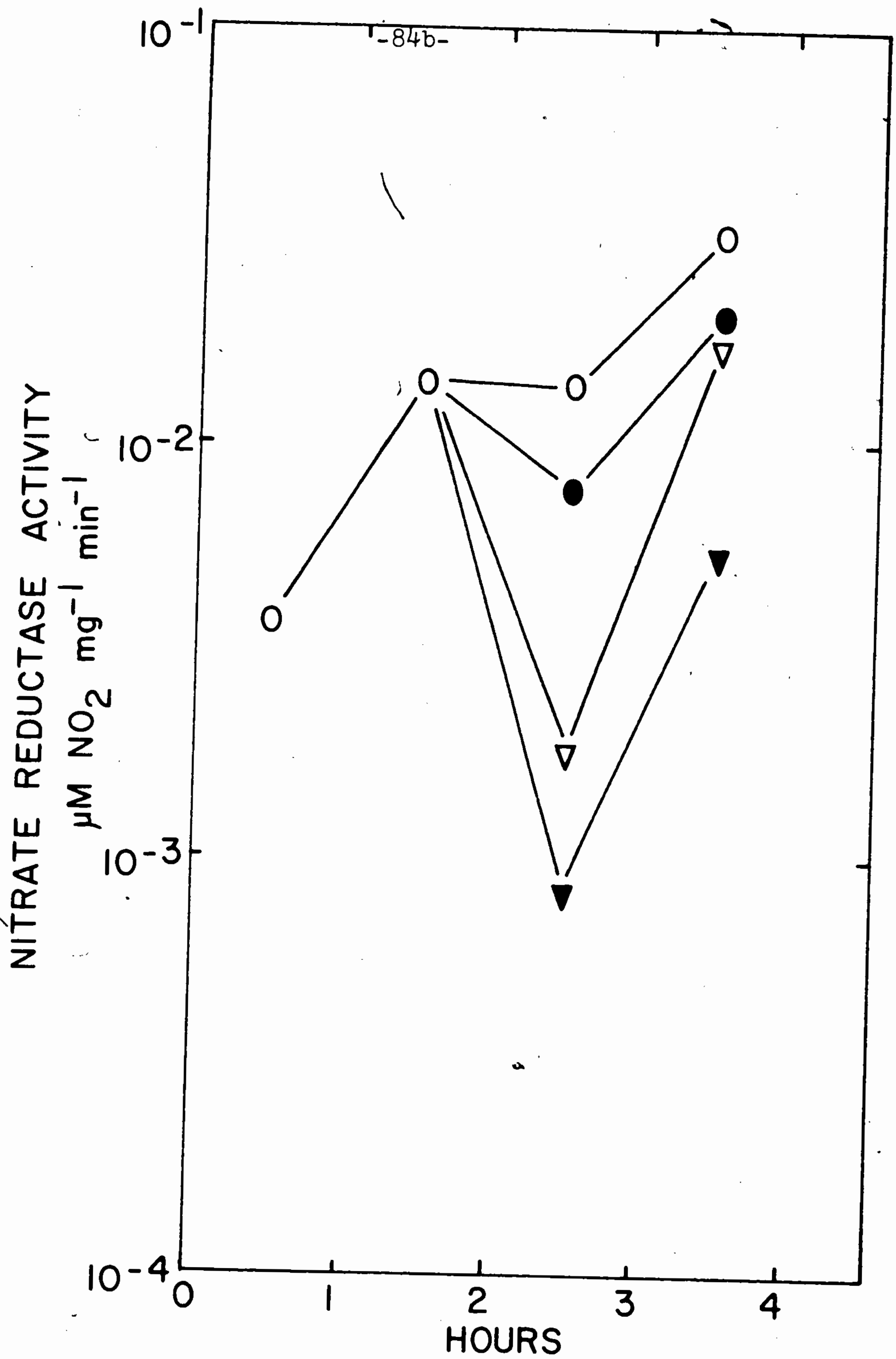
Figure V-1. The effect of different nitrogen sources on nitrate reductase activity (μM NO2/mg protein/min) of *Eudorina elegans*. A culture of wild type organisms growing in BM(U) was washed and resuspended in BM(NO3) (o—o) for 90 minutes. Aliquots of the culture were then transferred to BM(NO3) (o—o), BM(-N) (●—●), BM(NH4+NO3) (▽—▽), and BM(NH4) (▼—▼). Nitrate reductase activity was followed at intervals throughout the experiment. At 0 h, the nitrate reductase activity was $< 10^{-4}$.


NITRATE REDUCTASE ACTIVITY
μM NO2 mg-1 min-1
10-1
10-2
10-3
10-4
0
1
2
3
4
HOURS

reductase activity in media containing ammonia, ammonia and nitrate and a medium lacking nitrogen, nitrate reductase activity increases again. This phenomenon also has not been observed in fungi.

Recent studies of the nit-2 mutant of *Neurospora* (Pendyala and Wellman 1978) suggest that the inability of this mutant to utilize hypoxanthine as a nitrogen source may be a consequence of defective purine metabolism. This possibility has not yet been examined in HX-1 but it is interesting to note that the *Eudorina* mutant is different from nit-2 in that it can utilize nitrate, nitrite and urea as nitrogen sources whereas nit-2 cannot (Coddington 1976).

The above discussion focuses attention on the preliminary, fundamental differences in nitrogen metabolism I observed in *Eudorina elegans* compared to the fungi. This may be expected, but I feel it emphasizes the necessity to conduct investigations with organisms from a variety of genera in order to gain insight into evolutionary differences and similarities.

Genetic studies of the mutants defective in nitrogen metabolism in *Eudorina* have recently indicated that the phenotypically similar nar-1 and nar-3 are not alleles and that they segregate in Mendelian fashion. Nar-1 and nir-1 are not linked but nir-1 appears to be

loosely linked to nar-3. Preliminary experiments suggest that the nir-nar double mutant and the nar-1-nar-3 double mutant may be lethal. Nar-2 is phenotypically different from the other mutants and has not yet been successfully crossed. The genetic studies of these mutants as well as the other mutants isolated following enrichment have resulted in a preliminary genetic map for Eudorina elegans (Kemp, pers. comm.).

The original purpose of this thesis project, to develop a procedure to enrich for auxotrophs in a phototrophic system, has been achieved and surpassed. It is my hope that the mutants that have and will be isolated as a result of this procedure will be used not only to gain insight into why auxotrophs occur at such low frequency, but also to elucidate biochemical pathways and increase our genetic knowledge of this vast group of organisms previously neglected in studies of this nature.

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Curriculum Vitae

Name:	Anne Louise Toby
Place and Year of Birth:	Vancouver, British Columbia, Canada. 1950.
Education :	Capilano College 1968 - 1970 BSc. Simon Fraser University 1972 Graduate Studies, Simon Fraser University 1973 - 1979
Teaching Experience:	<u>Teaching Assistant</u> Biology 101, 202, 302 Simon Fraser University 1975 - 1978 <u>Instructor</u> Biology 335 - Principles of Genetics University of British Columbia 1978
Research Activities:	<u>Technical Experience</u> Research Assistant and Technician Algal Physiology and Genetics Simon Fraser University January 1973 - August 1973 <u>Publications</u> 1975 Toby, A.L. and C.L. Kemp. Mutant enrichment in the colonial alga, <u>Eudorina elegans</u>. Genetics <u>81</u>: 243 -251. 1977 Toby, A.L. and C.L. Kemp. Nitrate reductase mutants in <u>Eudorina elegans</u> (Chlorophyceae). J. of Phycol., <u>13</u> : 368 - 371. 1979 Toby, A.L. and C.L. Kemp. Nitrosoguanidine and ultraviolet light mutagenesis in <u>Eudorina elegans</u>. (submitted) J. of Phycol.

External Professional Activities
(papers presented, abstracts)

1975 Toby, A.L. A mutant enrichment procedure for the colonial alga, *Eudorina elegans*. Canadian Journal of Genetics and Cytology, 17 : 468.

1976 Toby, A.L. and C.L. Kemp. *Eudorina elegans* - an overview. Volvox Newsletter.

1977 Toby, A.L. Nitrate reductase mutants in *Eudorina elegans*. Volvox Newsletter.

1977 Toby, A.L. Mutagenesis studies in *Eudorina elegans*. Volvox Newsletter.

1977 Toby, A.L. Mutant enrichment in *Eudorina elegans*. J. of Phycol., 13 : 68.

1978 Toby, A.L. and C.L. Kemp. Nitrogen metabolism in *Eudorina elegans*. J. of Phycol., 14 : 37.

1978 Toby, A.L. Mutagenesis in *Eudorina elegans*. J. of Phycol., 14 : 21.

Awards: Graduate Student Stipend
Simon Fraser University
1978

Community Services: Organized and ran a Red Cross summer swimming program for children
1967 - 1972

Awards
1967 Swimming Award of Merit
1968 Swimming Award of Distinction
1968 Red Cross and Royal Life Saving Instructors Certificate
1972 Recertification