

A PRELIMINARY INVESTIGATION OF GROWTH PARAMETERS, OF
ULTRA-VIOLET LIGHT- AND DRUG-SENSITIVITY AND OF MUTANT
ISOLATION OF THE COLONIAL GREEN ALGA, EUDORINA
ELEGANS EHRENBERG.

by

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A Preliminary Investigation of Growth Parameters, of
Ultra-Violet Light- and Drug-Sensitivity and of Mutant
Isolation of the Colonial Green Alga, Eudorina
elegans Ehrenberg.

ABSTRACT

To obtain information for evaluation of the potential use of Eudorina elegans in studies of physiology, morphogenesis and cellular differentiation and the genetic control of these processes, investigations on growth, response to possible mutagens, mutant-enriching agents and ultra-violet light and the possibility of obtaining genetic variants were undertaken.

Growth parameters of the wild-type organism in liquid media were determined by following the increase in colony-forming-units/ml or by increase in absorbance at 560 nm. The colony-forming-abilities on solid minimal and supplemented minimal media were compared to the ability on complete medium.

N-methyl-N'-nitro-N-nitrosoguanidine, a mutagen and ethyl methanesulfonate, a potential mutagen of Eudorina elegans, inactivate Eudorina very rapidly. The drugs, sulfanilamide, streptomycin, 8-azaguanine and penicillin, were tested for their ability to inactivate wild-type in preparation for

for their use in mutant enrichment studies. However none appears to have a great deal of potential for these studies.

The conditions for use of ultra-violet light (UVL) as a mutagen were determined. The response to UVL determined in this investigation was different from published results. Eudorina appears to have little ability to repair radiation-induced lesions under dark conditions, but is very efficient in repairing this damage in visible light.

Five mutants were characterised. All are auxotrophic with two of these having pigment deficiencies and one other being unable to undergo normal coenobial breakdown.

The problems incurred in this study and ideas on future studies using Eudorina elegans are presented.

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CHAPTER I

INTRODUCTION

Some investigations have been made of Eudorina elegans Ehrenberg (Ehrenberg, 1831) in the fields of taxonomy and morphology (Goldstein, 1964), mitotic characteristics (Goldstein, 1964; Rayns and Godward, 1965; Mishra, 1967), life cycle characteristics (Iyenger, 1937; Goldstein, 1964), and electron microscopy (Lang, 1963). These studies contribute information which might be useful in the study of problems of physiology, morphogenesis and cellular differentiation and the genetic control of these processes, with this organism.

However, little work has been done on formal genetic analysis, except for that of Goldstein (1964) and Mishra (1967) who showed that the mating-type is inherited as a single gene difference, and that the zygote pattern is determined by the female organism and inherited in a non-Mendelian manner. Mishra, although unable to obtain auxotrophic, pigment and morphological mutants, did isolate drug-resistant mutants, some having chromosomal inheritance patterns and others inherited as non-chromosomal genes.

Eudorina elegans is a green alga, a member of the order Volvocales and of the family Volvocaceae. It is a colonial flagellate, the colony or coenbium usually con-

sisting of 16, occasionally 32, and rarely 64 chlamydomonad-like cells (Goldstein, 1964), within the coenobial envelope. Each cell of the coenobium has the potential of dividing mitotically four to six times to form a curved plate of cells or a plakea of between 16 and 64 cells (Goldstein, 1964). Following inversion of the plakea [the reversal of the concave and convex sides of the plakea and the subsequent formation of a closed sphere (Frisch, 1956; Goldstein, 1964)], each of these groups of cells becomes a new daughter colony (Goldstein, 1964; Rayns and Godward, 1965).

Eudorina has several features which make it interesting as a tool to study problems of physiology, morphogenesis and cellular differentiation and the genetic control of these processes. The capability of all cells to undergo cell division indefinitely, unless influenced by environmental conditions to form sexual cells, as well as the consistent synchronous nuclear division and number of cleavages which each cell undergoes in the formation of a daughter colony might be studied.

The processes involved in sexual reproduction also make the organism attractive for a study of differentiation. Sexual reproduction requires differentiation of vegetative cells into either eggs or 32-celled sperm packets, depending on the strain (Goldstein, 1964).

Before Eudorina elegans can be useful or even evaluated for use in such studies, an investigation into the parameters of growth, responses to drugs and radiation, and the possibility of isolating and characterising biochemical mutants would seem to be necessary. It is the purpose of this thesis to present some results on these aspects of the biology of Eudorina elegans and to show directions in which future studies might be established.

For ease of discussion the study will be presented as outlined below.

The object of the following chapter (Chapter II) is to describe those materials and methods considered basic to all sections of this investigation. The materials and methods in other chapters describe those procedures which were used only once, or were variations of the general method as outlined in Chapter II.

Chapter III shows the progression of the development of the technique to study growth by use of absorbance measurements. Further, it presents quantitative estimates of the growth characteristics of the wild-type organism in liquid and on solidified media.

Chapter IV examines briefly the inactivation ability of several potential chemical mutagens and agents which might be useful for enrichment of mutants in treated coenobia.

Chapter V considers the response of Eudorina to ultra-violet radiation and the possibility of repair of the radiation-induced damage.

Chapter VI describes the induction, isolation, and characterisation of several mutants of Eudorina.

Chapter VII presents an overall discussion of this investigation, with particular reference to future studies using Eudorina elegans.

CHAPTER II

MATERIALS AND METHODS

A. The Organism

Axenic cultures of Eudorina elegans, strains 1192 and 1193, were obtained from the Culture Collection of Algae, Indiana University, Bloomington, Indiana, U. S. A. Goldstein (1964) isolated and described these strains, designating them 56m and 56f respectively.

B. Media

The minimal salts and complete media are shown in Tables II-1 and II-2 respectively, and will be designated Bm (Bristol's minimal medium) and Bc (Bristol's complete medium) throughout this work.

Bristol's minimal medium is the same as that given by Starr, (1964), with the single modification that Gaffron's minor element solution (Hughes et al., 1958) supplies the trace elements (Table II-1).

Bc was used in most studies as the complete medium; however, Volvocacean medium (Vm), (Starr, 1964), shown in Table II-2B, was used initially.

The minimal medium supplemented with organic compounds, as shown in Table II-3, was used to test for auxotrophic requirements of presumptive mutants.

Table II-1

Minimal Medium for Growth of Eudorina elegans

Preparation of Medium:

Stock Solution I (Bristol's Stock Solutions A to F).....10.0 ml each

Stock Solution II (Gaffron's Trace Element Solution).... 1.0 ml

Bring to 1000 ml with pyrex-distilled water.

Stock Solutions

I Bristol's Stock Solutions: (in pyrex-distilled water)

| | |
|---|---------------|
| A NaNO_3 | 10.0 g/400 ml |
| B CaCl_2 | 1.0 g/400 ml |
| C $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 3.0 g/400 ml |
| D K_2HPO_4 | 3.0 g/400 ml |
| E KH_2PO_4 | 7.0 g/400 ml |
| F NaCl | 1.0 g/400 ml |

II Gaffron's Trace Element Solution:

| | |
|---|---------|
| H_3BO_3 | 3.100 g |
| $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ | 2.230 g |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.287 g |
| $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ | 0.088 g |
| $\text{Co}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ | 0.146 g |
| $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ | 0.033 g |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | 0.125 g |
| KBr..... | 0.119 g |

Table II-1 (cont'd.)

| | |
|---|---------|
| KI..... | 0.083 g |
| Cd(NO ₃) ₂ ·4H ₂ O..... | 0.154 g |
| NiSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O..... | 0.198 g |
| VO SO ₄ ·2H ₂ O..... | 0.020 g |
| Al ₂ (SO ₄) ₃ K ₂ SO ₄ ·24H ₂ O..... | 0.474 g |
| Cr(NO ₃) ₃ ·7H ₂ O..... | 0.037 g |

Add salts to 1000 ml pyrex-distilled water. Adjust the pH to approximately 7.5 after autoclaving, with HCl.

Table II-2

Complete Media for Growth of Eudorina elegans

A. Bristol's Complete Medium

Preparation of Medium:

| | |
|---|--------|
| Minimal Medium (Table II-1)..... | 800 ml |
| Stock Solution III (Modified Euglena Medium)..... | 200 ml |

Stock Solutions

III Modified Euglena Medium (After Starr, 1964):

| | |
|---|--------|
| Yeast extract (Difco)..... | 2 g |
| Sodium acetate..... | 1 g |
| Beef extract..... | 1 g |
| Stock Solution IV (Casamino Acid Solution)..... | 20 ml |
| CaCl ₂ | 0.01 g |
| Pyrex-distilled water..... | 980 ml |

IV Casamino Acid Solution:

| | |
|---|--------|
| Casamino acid hydrolysate (Difco vitamin free)..... | 100 g |
| Pyrex-distilled water..... | 1000 g |

Add a spoonful of charcoal to decolorize. Filter;

Millipore filter.

Table II-2 (cont'd.)

B. Volvocacean Medium

Preparation of Medium:

| | |
|---|--------|
| Stock Solution V (Waris Solution)..... | 800 ml |
| Stock Solution VI (Euglena Medium)..... | 200 ml |

Stock Solutions

V Waris Solution:

| | |
|---|------|
| 10% KNO_3 | 1 ml |
| 2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 1 ml |
| 2% $(\text{NH}_4) \cdot \text{HPO}_4$ | 1 ml |
| 5% CaSO_4 | 1 ml |
| Stock Solution VII (Iron Sequestrine Solution)..... | 1 ml |

Bring to 1000 ml with pyrex-distilled water.

VI Euglena Medium:

Same as Modified Euglena Medium (Stock Solution III)
except that Stock Solution IV (Casamino Acid Solution)
is replaced with

| | |
|-----------------------|-----|
| Tryptone (Difco)..... | 2 g |
|-----------------------|-----|

Table II-2 (cont'd).

VII Iron Sequestrine Solution:

| | |
|--|--------|
| EDTA or Sequestrine AA (Nutritional Biochem. Co.)... | 2.61 g |
| FeSO ₄ ·7H ₂ O..... | 2.49 g |
| KOH (1 N)..... | 27 ml |
| Pyrex-distilled water..... | 500 ml |

Table II-3

Supplemented Bristol's Minimal Media for Growth of
Eudorina elegans

Original test series: (for the classification of mutants into vitamin,
nucleic acid, or amino acid auxotrophs)

1. Minimal Medium (Table II-1)
2. Complete Medium (Table II-2A)
3. Minimal + Sodium Acetate (1g/l)
4. Minimal + Stock Solution IV (Casamino Acid Solution) (25 ml/l)
5. Minimal + Yeast Extract (2.0 g/l)
6. Minimal + Stock Solution VIII (Nucleic Acid Hydrolysate) (1 ml/l)

Stock Solution

VIII Nucleic Acid Hydrolysate (Eversole, 1956):

| | |
|---|-------|
| Yeast nucleic acid (Eastman Organic Chemical Co.) | 10 g |
| 2.5% NH_4OH | 25 ml |

Autoclave at 15 p.s.i. for one hour. Filter and dilute to
100 ml with sterile pyrex-distilled water.

Vitamin Test Series (After Eversole, 1956):

To 100 ml Bm add one ml of one of the following vitamin stocks,
made in 95% ethanol.

Table II-3 (cont'd).

| | |
|---|---|
| Thiamine (100 $\mu\text{g}/\text{ml}$) | Folic acid (1 $\mu\text{g}/\text{ml}$) |
| Pyridoxine (75 $\mu\text{g}/\text{m}$) | Biotin (0.05 $\mu\text{g}/\text{ml}$) |
| Ca-Pantothenate (200 $\mu\text{g}/\text{ml}$) | Vitamin B ₁₂ (0.05 $\mu\text{g}/\text{ml}$) |
| <u>para</u> -Aminobenzoic acid (5 $\mu\text{g}/\text{ml}$) | Inositol (1000 $\mu\text{g}/\text{ml}$) |
| Nicotinamide (75 $\mu\text{g}/\text{ml}$) | |
| Choline HCl (300 $\mu\text{g}/\text{ml}$) | |

When required, solid medium for plates and for maintenance cultures was made by adding 1.5% Difco agar to the liquid medium. Agar for the pour-plating technique was prepared by addition of 0.6% Difco agar to the medium.

All minimal, supplemented minimal and complete media were autoclaved at 15 p.s.i. for 20 minutes. Organic supplements, if denatured by autoclaving, were sterilized by Millipore filtering, prior to addition to autoclaved minimal medium.

C. Chemicals

The inorganic compounds added to the media were reagent grade chemicals. The sources of the organic supplements are given in Table II-2 and II-3.

Stock solutions of streptomycin sulfate (K. and K. Laboratories Inc., Plainview, N. Y.) at 2.0 mg/ml, sulfanilamide (Aldrich Chem. Co., Milwaukee, Wisc.) at 25 mg/ml and 8-azaguanine (Calbiochem., Los Angeles, Calif.) at 1.52 mg/ml were prepared in Bm; stock solutions of penicillin G (Potassium salt; Sigma Chemical., St. Louis, Missouri; 1590 units/mg) at 15 mg/ml were prepared using pyrex-distilled water. Streptomycin and penicillin were sterilized by Millipore filtering; sterilization of 8-azaguanine and sulfanilamide was considered unnecessary in view of high toxicity. These stocks were stored at 5°C and

were not used after 4-5 weeks of storage.

Ethyl methanesulfonate (EMS), obtained at 8 M from Eastman Organic Chemical, was added directly to the coenobial suspension, to give the desired concentration. N-methyl-N'-nitro-N-nitrosoguanidine (NTG), from Aldrich Chemical Company, Milwaukee, Wisc., was dissolved in 95% ethanol at 3 mg/ml for experiments discussed here.

D. Growth Conditions

Before initiation of this work, and at numerous other times during the study, the strains were cloned: that is, they were streaked several times on Bc plates, and then the colonies arising from single coenobia were tested for ability to grow on both Bm and Bc. This procedure reduced some genetic heterogeneity with respect to growth ability, but did not consider sensitivity to drugs and irradiation.

All light intensities given in the following sections were calibrated by an ISCO spectroradiometer.

D. (i). Stock Culture Maintenance

Stock cultures were maintained on Bc agar slants in cotton-stoppered test tubes (25 x 180 mm) at temperatures of 15°C and 22°C. Illumination by two 40-watt cool-white fluorescent tubes (16 hours light - 8 hours dark) gave an intensity of 500 ft-c or approximately $5 \times 10^3 \text{ erg cm}^{-2} \text{ sec}^{-1}$

at the surface of the test tube medium. Stock cultures were initiated by incubation for the first four to seven days under a light intensity of 1000 ft-c at 22°C.

D. (ii). Standard Growth Conditions

Bc medium (50 ml), in cotton-stoppered 200 or 250 ml Erlenmeyer flasks, was inoculated to a final concentration of $3-5 \times 10^2$ colony-forming-units/ml (cfu/ml) from a four-day-old culture (the dilution being 10^2 : i.e. 0.5 ml culture diluted into 50 ml growth medium), and shaken continuously at the rate of approximately 80 oscillations/min on a shaking platform. The light intensity of 1000 ft-c, measured outside the flask, was provided in a light régime of 16 hours light - 8 hours dark. After four days of growth at $32 \pm 1^\circ\text{C}$, the log phase cultures were used for growth studies, experiments on the response to ultra-violet light or drugs and for mutation induction. Unless otherwise stated the wild-type strain 1192 was used in these studies.

D (iii). Contamination Check

Stock cultures and test cultures were tested for contamination by plating a sample of the culture on nutrient broth plates (Table II-4), and incubating overnight at 37°C . Any contaminated cultures were rejected.

Table II-4

Medium for Contamination Check

Preparation of Medium:

| | |
|---------------------------------|---------|
| Difco Bacto Nutrient Broth..... | 8 g/l |
| Difco Bacto Agar..... | 10 g/l |
| NaCl..... | 5 g/l |
| Pyrex-distilled water..... | 1000 ml |

D (iv). Collection of Coenobia

The coenobia were harvested by centrifuging in a Sorvall Desk Centrifuge SP/X head at 2100 ± 100 rpm (approx. $500 \times g$) for 5 minutes. The supernatant was decanted and the culture was then resuspended in Bm or potassium phosphate buffer (KH_2PO_4 : K_2HPO_4 ; pH 6.8; 6.7×10^{-3} M). (Hereafter potassium phosphate buffer will be designated phosphate buffer). This process was repeated and, after a second resuspension, the culture was said to be washed twice.

E. Growth Characterisation

Growth was defined as the increase in colony-forming-units/ml in liquid culture, or the ability to form visible colonies on solid medium.

E (i). Growth in Liquid Media

To determine the ability of a culture to grow in liquid media, a five ml aliquot of a log phase culture was harvested, washed twice and resuspended at one-half the original concentration in Bm. After incubation under standard growth conditions for 24 hours, the test medium, inoculated to a concentration of approximately $3-5 \times 10^2$ cfu/ml, was incubated under standard growth conditions. The volume of medium in the flask was maintained at the original level by topping-up the culture with sterile

distilled water. The original level was indicated by a line on the optical density tube. This addition reduced or eliminated effects caused by concentration of the medium.

Growth was monitored by absorbance at 560 nm (A_{560}) in a Coleman spectrophotometer 6C. The culture was grown in 125 ml Erlenmeyer flasks, modified so that the optical tube (14 mm light path; 18 mm outside diameter) was affixed where the mouth of the flask had originally been. The mouth was relocated further down the side of the flask.

Growth was also followed by plating on Bc solid medium. A sample (0.1 to 0.3 ml) of the culture previously diluted in Bm to yield a final coenobial concentration of 5×10^2 - 2×10^3 cfu/ml, was mixed with 2.5 ml of top agar (kept liquid at 45°C) and poured onto a plate of Bc solid medium. The plates were scored for visible colony formation after 5 - 7 days at 32°C.

Pour-plating, instead of spread-plating, was used, unless otherwise stated, as the method of determining the concentration of coenobia. All concentrations up to approximately 400 cfu/ml pour-plated efficiently whereas spread-plating was only efficient at low coenobial concentrations (Table II-5).

The growth in the various liquid media was defined by

Table II-5

Comparison of the Spread-Plating and Pour-Plating
Techniques

| | dilution | colonies/plate | cfu/ml |
|----------------|--------------------|----------------|-----------------------|
| Expt. 1: | | | |
| Pour-plating | .1/10 ² | 37, 40, 48 | 4.2 x 10 ⁴ |
| Spread-plating | .1/10 ² | 39, 44, 45 | 4.3 x 10 ⁴ |
| Expt. 2: | | | |
| Pour-plating | .2/10 ² | 86, 88, 88 | 4.4 x 10 ⁴ |
| Spread-plating | .2/10 ² | 90, 81, 73 | 4.0 x 10 ⁴ |
| Expt. 3: | | | |
| Pour-plating | .1/10 ¹ | 392, 440, 448 | 4.3 x 10 ⁴ |
| Spread-plating | .1/10 ¹ | 319, 540, 391 | 4.2 x 10 ⁴ |
| Expt. 4: | | | |
| Pour-plating | .1/10 ¹ | 427, 442, 440 | 4.4 x 10 ⁴ |
| Spread-plating | .1/10 ¹ | 365, 397, 476 | 4.1 x 10 ⁴ |

the duration of lag phase, specific growth rate in the exponential growth phase and final cfu/ml after entering senescent phase. The growth rate was expressed as the number of coenobial doublings each day.

E (ii). Growth on Solid Media

Further growth characterisation was carried out by determining the efficiency of plating on solidified media, comparing the results obtained by spread-plating at low concentrations of coenobia/ml or by pour-plating, on Bc, to those on Bm (plus supplement). A culture grown under standard growth conditions was washed, resuspended and incubated for 24 hours in Bm before plating on the test media.

F. Ultra-Violet Irradiation Apparatus

The source of ultra-violet light (UVL) was the central part of two 15-watt germicidal lamps (Sylvania 15GT8) suspended over a shaking platform. A sliding door between the suspension and the light, controlled the dose of UVL supplied to the coenobial suspension. The incident dose rate, 95% emitted at 254 nm (manufacturer's specifications), was supplied to the samples at 5, 10, or 20 $\text{ergs mm}^{-2} \text{sec}^{-1}$, determined by the distance between the suspension and the light. The lamp was calibrated by use of a Blak-Ray UV

Meter (J-226) and by a biological cross-calibration with bacteriophage T4. The inactivation of T4, to the 10% level of survival, occurred at approximately 175 ergs mm^{-2} with the UV-lamp used in these studies, compared to approximately $160 - 170 \text{ ergs mm}^{-2}$ published by Harm (1968).

G. Standard Ultra-Violet Irradiation Procedure

Cultures to be irradiated for ultra-violet (UV) dose-response characteristics and for mutation induction were grown under standard growth conditions for four days in Bc. After four hours illumination on the fourth day, the culture was harvested and washed twice, either in Bm or in phosphate buffer, as in Chapter II, D. Growth Conditions. The coenobia were then resuspended in the washing medium at $5-10 \times 10^4$ cfu/ml. After an agitation period of 10-15 minutes on the shaking platform of the UV-apparatus, the suspension was exposed to UVL with agitation continuing throughout the irradiation period.

The ultra-violet lamp was turned on at least 15 minutes before use.

Irradiation and plating were done at room temperature, in very low light intensity. Incubation of the plates or cultures for the first 24 hours at 32°C was carried out in the dark to prevent photoreactivation (See Chapter V). Following this period in the dark, the plates were incubated

under standard conditions of light and temperature for 5 - 7 days prior to scoring. Irradiated cultures for liquid growth analysis and mutant isolation were incubated with agitation under standard growth conditions.

H. Treatment with Chemicals (Mutagens and Antimetabolites)

To study the effect of chemical mutagens and anti-metabolites on Eudorina elegans 1192 cultures grown under standard conditions were treated as follows:

a) NTG or EMS was added to a coenobial suspension ($5-10 \times 10^4$ cfu/ml), which had been washed twice and resuspended in phosphate buffer. This suspension was agitated continuously during treatment.

b) cultures to be treated with sulfanilamide, streptomycin, penicillin or 8-azaguanine were washed twice and concentrated two fold in Bm. After incubation for 24 hours in Bm under standard conditions, the suspension was inoculated into the media containing the antimetabolite. After various times of treatment the cultures were diluted ten-or one hundred-fold into phosphate buffer, and plated on Bc plates.

CHAPTER III

GROWTH AND QUANTITATION STUDIES OF EUDORINA ELEGANS

INTRODUCTION

Rayns and Godward (1965) described the growth characteristics of an individual coenobium from its liberation during one generation to the formation of sixteen, or more, coenobia, and their subsequent release in the next generation. They also analysed the mitotic characteristics of the population during the growth cycle. However no intensive study of the growth and plating characteristics of a population of coenobia has been reported in the literature. This section describes such experiments and provides the basis for quantitative investigations into the responses to ultra-violet radiation and drugs, mutagenesis and biochemical and physiological genetic studies.

MATERIALS AND METHODS

Techniques used in studies of growth of Eudorina elegans in liquid and on solid minimal, supplemented minimal and complete media are described in Chapter II: D. Growth Conditions and E. Growth Characterisation.

RESULTS AND DISCUSSION

A. Efficiency of Dilution

Table III-1 shows two results in which the ability of this organism to suspend readily to give a uniform suspension was tested. Three samples were removed from each of the well-agitated cultures, and diluted into Bm. These suspensions were then plated on Bc. The results indicate that the cultures were homogeneous suspensions, and repeated sampling and dilution gave reproducible results. This homogeneous suspension property of the cultures could be used in the determination of growth parameters by the absorbance of the culture, and by plating of a well-agitated culture at various times.

B. Use of the Absorbance of the Culture for Growth

Characterisation

Because the organism suspended uniformly in turbulent culture, the possibility of using absorbance measurements of a growing culture as a method of growth determination was investigated.

The relationship of the absorbance of the culture in Bm to wavelength was followed to determine the wavelength

Table III-1

Efficiency of Dilution

Three samples were taken from two, four-day-old cultures of Eudorina elegans 1192, diluted into Bm and plated on Bc plates. Four plates were made from each sample.

| | dilution | colonies/plate | cfu/ml |
|-----------|--------------------|-----------------------|------------------------------|
| Sample 1a | .2/10 ² | 68, 76, 72, 83 | 3.8 x 10 ⁴ |
| Sample 1b | .2/10 ² | 65, 75, 82, 58 | 3.5 x 10 ⁴ |
| Sample 1c | .2/10 ² | 64, 77, 65, 63 | 3.4 x 10 ⁴ |
| | | Mean ± Standard Error | 3.6 ± 0.12 x 10 ⁴ |
| Sample 2a | .2/10 ² | 51, 56, 41, 69 | 2.7 x 10 ⁴ |
| Sample 2b | .2/10 ² | 46, 56, 49, 50 | 2.5 x 10 ⁴ |
| Sample 2c | .2/10 ² | 40, 46, 50, 37 | 2.2 x 10 ⁴ |
| | | Mean ± Standard Error | 2.5 ± 0.15 x 10 ⁴ |

at which monitoring of turbidometric values of a culture could be made. The wavelength chosen, 560 nm, where pigment absorption is minimal, is between the two absorption peaks of chlorophyll (See Figure VI-4).

The relationship between absorbance at 560 nm and cfu/ml was determined as follows: a log phase culture, concentrated four-fold, was serially diluted. The absorbance of each of these dilutions was determined, and a sample of the dilution plated on Bc plates. The absorbance in the range 0.03 to 0.8 was proportional to the cfu/ml (Figure III-1). Below the absorbance of 0.03, the change was very small for a relatively large change in the number of coenobia/ml, and above the absorbance of 0.8, light scattering and pigment absorption interfered to distort the linear relationship.

C. Growth Measurements of the Wild-Type Organism

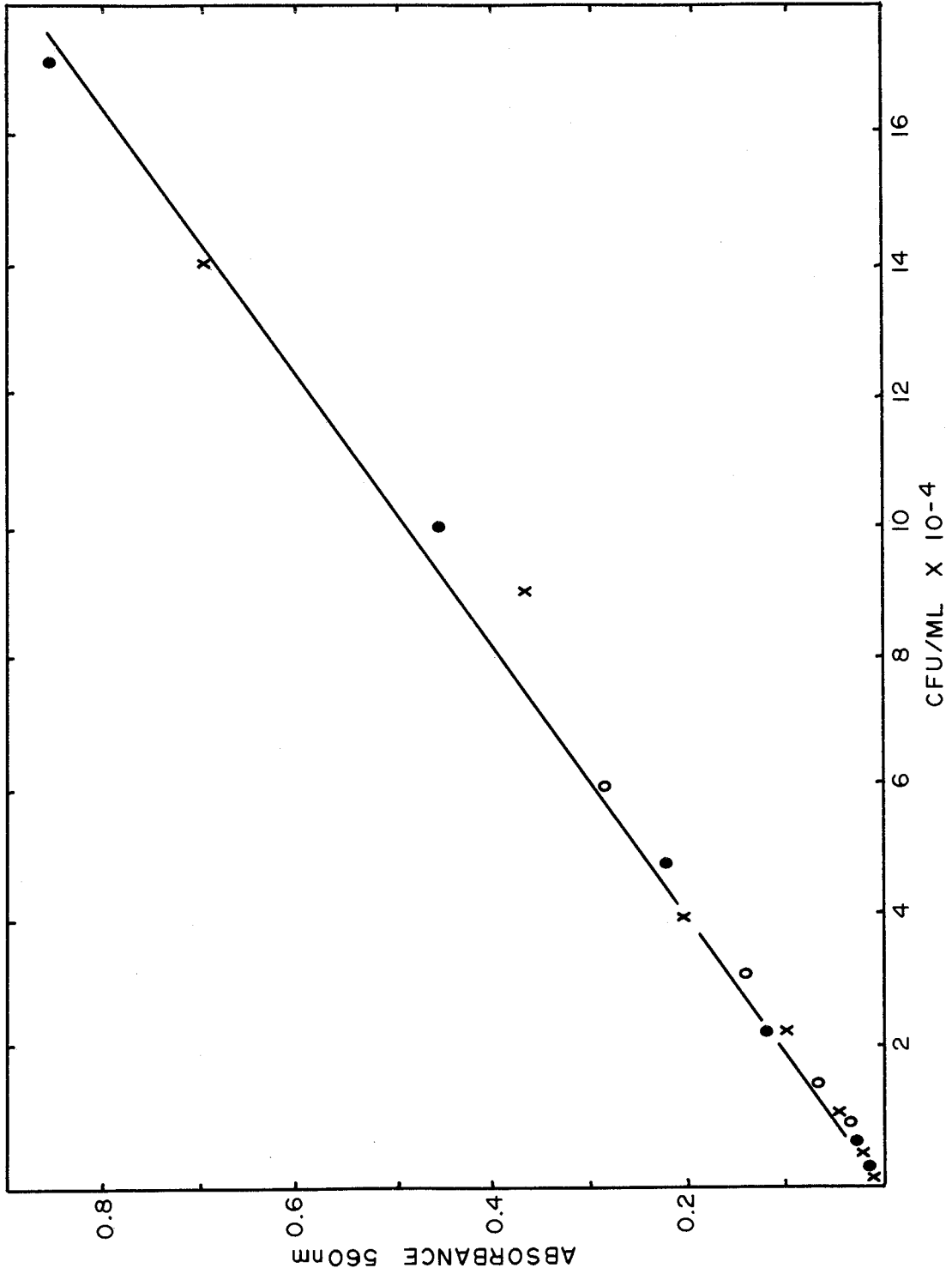
C (i). Growth on Solidified Media

Growth was studied using the two methods as outlined in Chapter II: E. Growth Characterisation. These methods were used, extending the usual growth/no growth ability in liquid or on plates, as a preparation for mutant studies. Many mutants are "leaky"; that is, they

Figure III-1. The relationship between absorbance at 560 nm and colony-forming-units/ml of a log phase culture of Eudorina elegans 1192.

- Experiment 1; ○ Experiment 2;
- × Experiment 3.

This line was fitted by observation.



grow on minimal and minimal-supplemented media, but to a reduced degree from that of the wild-type organism. Only a comparison of growth characteristics of the mutants on the various media with those characteristics of the parent organism indicate that such are indeed mutant.

Efficiency of plating determinations were made by plating samples of log phase cultures on Bm, Bm-supplemented, Bc and Vm media (Table III-2; Appendix I). The number of colonies on Bc was considered as 100% and the colony numbers obtained on other media were related to this standard for each experiment. This table includes results obtained by plating Eudorina elegans 1192 on Bm with complex supplements at the concentrations given in Table II-3, and the results of plating both 1192 and 1193 on Bm supplemented with the compounds added at the same levels as are present in Bc. It is significant that the plating efficiencies of the organism on the latter plates are similar but those on the former are extremely variable.

C (ii). Growth in Liquid Media

A comparison of the growth of Eudorina elegans 1192 in the two complete liquid media is given in Figure III-2.

TABLE III-2

Efficiencies of Plating of Eudorina elegans 1192 and 1193.

(c.f. Appendix I)

A. Efficiencies of plating of Eudorina elegans 1192 on Bm, Bm + supplemented (Table II-3), Bc and Vm media.

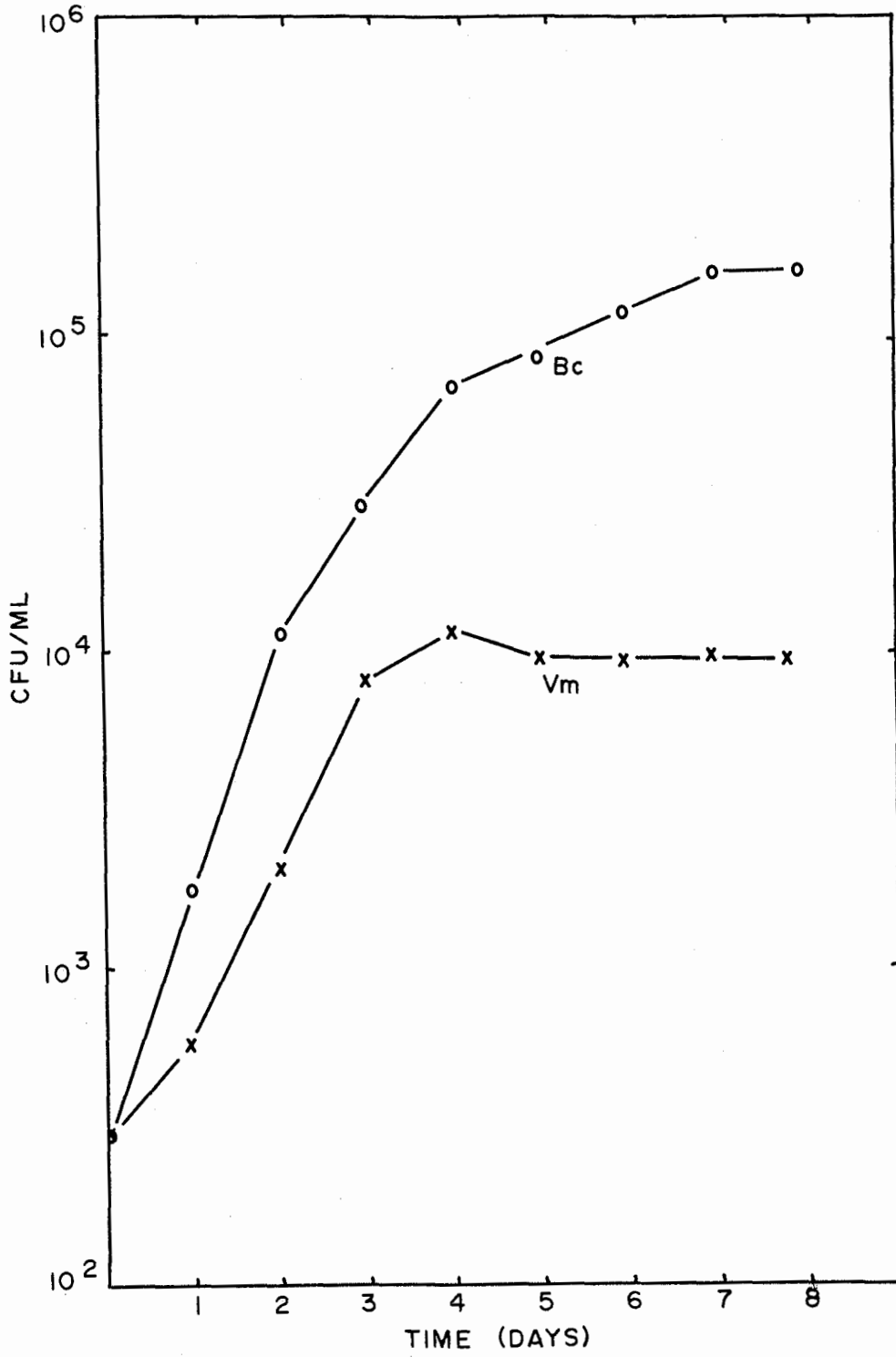
| <u>Media</u> | <u>Efficiencies of Plating (%)</u> |
|--------------------------------------|------------------------------------|
| <u>Bc</u> | 100 |
| <u>Bm</u> | 70 |
| <u>Vm</u> | 140 |
| <u>Bm</u> + Yeast extract (2.0 g/l) | 220 |
| <u>Bm</u> + Casamino acid (2.5 g/l) | 60 |
| <u>Bm</u> + Sodium acetate (1.0 g/l) | 50 |
| <u>Bm</u> + Nucleic acids | 90 |

B. Efficiencies of plating of Eudorina elegans 1192 (1) and 1193 (2) on Bm, Bm + supplemented, and Bc media, where the concentrations of the additions are the same as those to Bc (Table II-2).

| <u>Media</u> | <u>Efficiencies of Plating (%)</u> | |
|--------------------------------------|------------------------------------|-----|
| | (1) | (2) |
| <u>Bc</u> | 100 | 100 |
| <u>Bm</u> | 80 | 100 |
| <u>Bm</u> + Yeast extract (0.4 g/l) | 80 | 120 |
| <u>Bm</u> + Casamino acids (0.4 g/l) | 80 | 120 |
| <u>Bm</u> + Sodium acetate (0.2 g/l) | 80 | 100 |
| <u>Bm</u> + Beef extract (0.2 g/l) | 100 | 160 |

Figure III-2. Growth of E. elegans 1192 in Bc and in Vm.

A culture, resuspended in Bm for 24 hours was inoculated into Bc and Vm, and growth was followed by plating on Bc. Each growth curve is the average of two experiments.



Since the period of exponential growth in Bc was maintained for two days longer than that in Vm, and the yield was larger in Bc than in Vm, Bc was chosen as the complete medium for much of this study, and unless otherwise noted, is the medium referred to as complete medium.

The growth of strain 1192, following A_{560} and cfu/ml with time, in Bm and in Bc is shown in Figures III-3 and III-4 respectively. The growth kinetics of strain 1193 were similar to those shown here. In these studies the coenobial concentration did not exceed the range over which the A_{560} -cfu/ml correspondence was linear (Figure III-1). In Bm, the two growth curves (A_{560} -time, and cfu/ml-time) increased with approximately the same slope over the exponential growth region but this correspondence broke down at the time the culture entered the senescent phase. A similar relationship was seen when the growth in Bc was considered. The constant relationship over the log phase growth, and the breakdown on entry of senescent growth is explained in the following way. Over the log phase of growth, the coenobial size was relatively constant. In senescent phase the curves diverged since the coenobial size decreased with time, approaching stabilization of A_{560} -

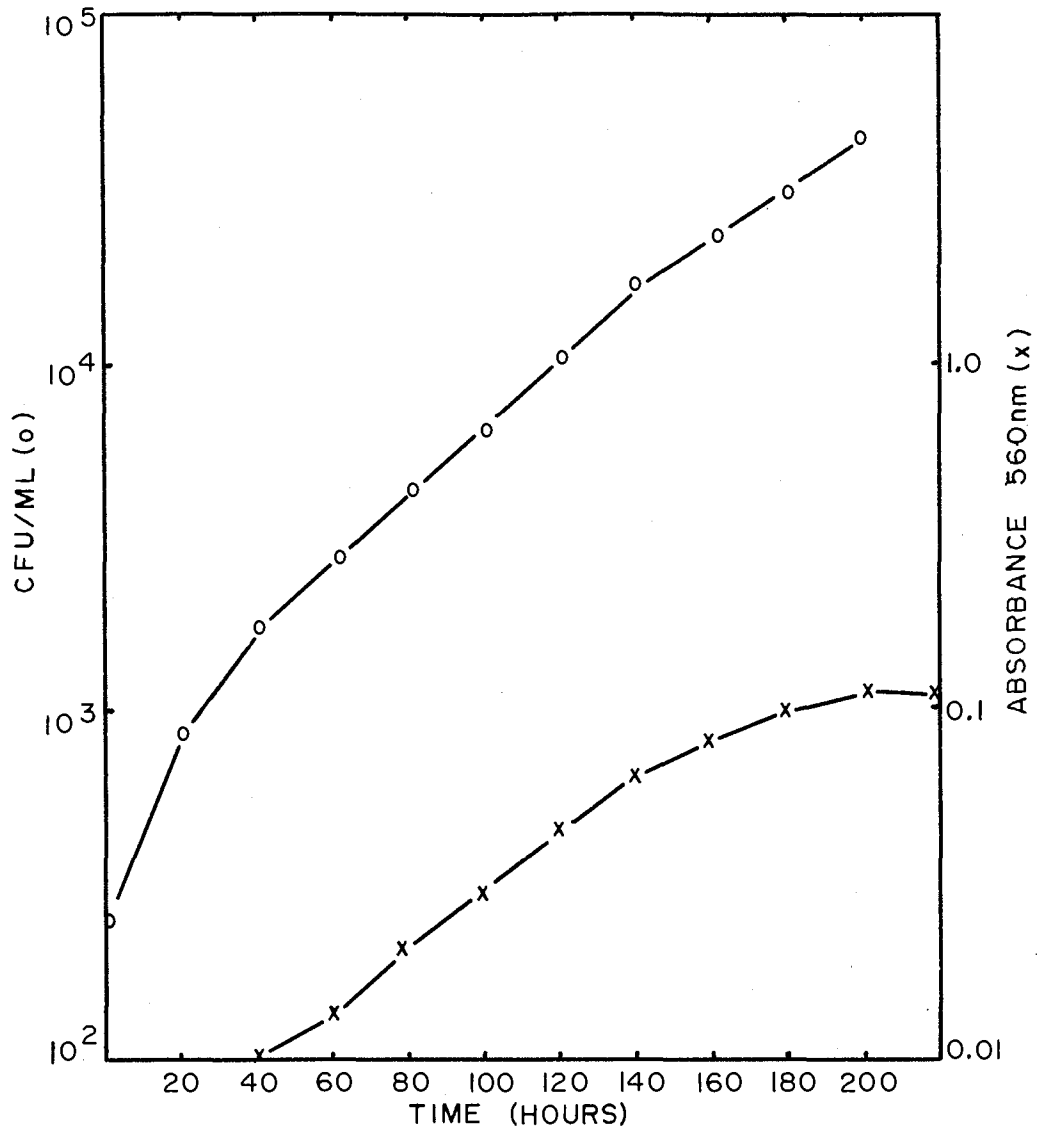
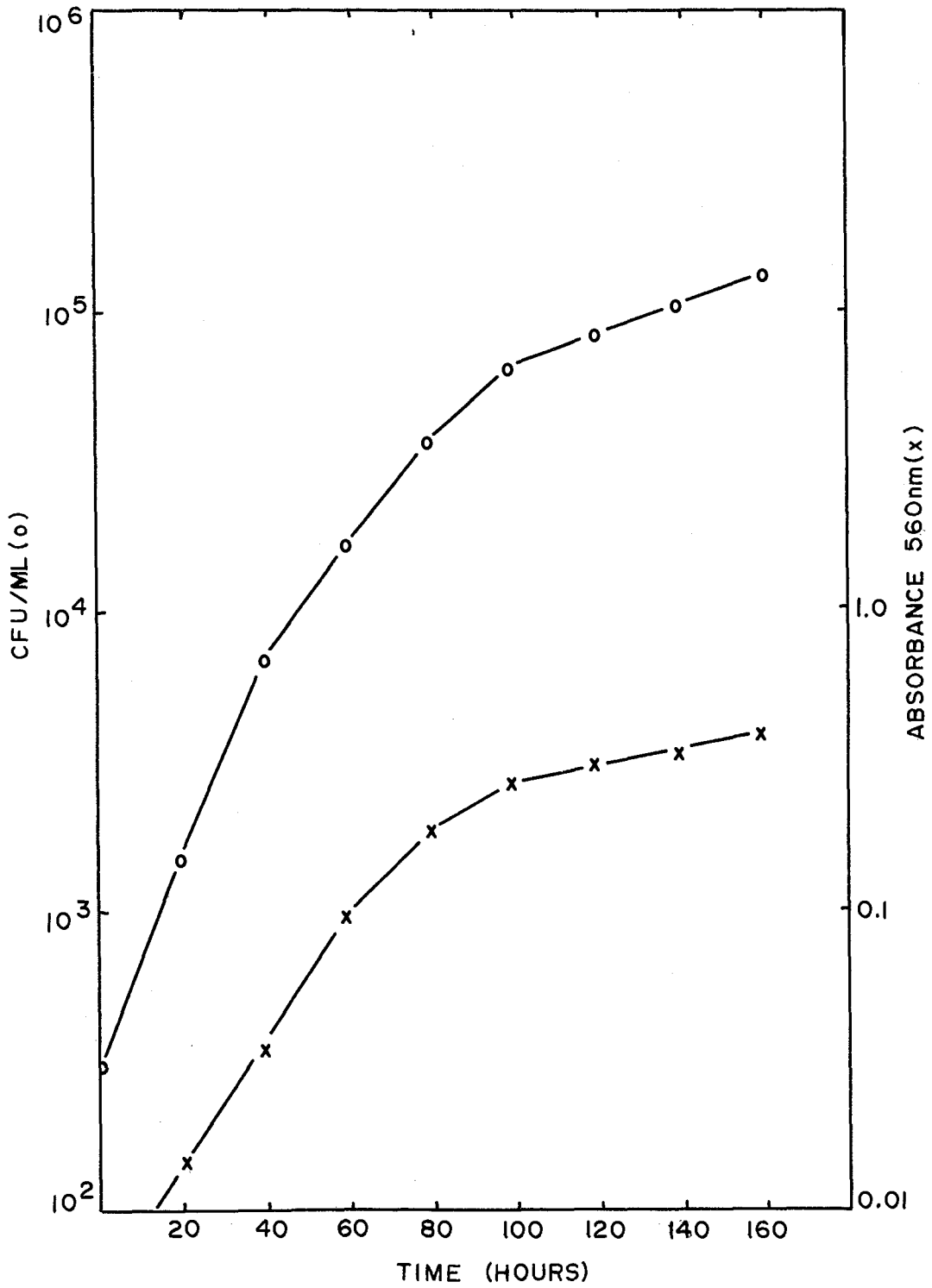


Figure III-3. Growth of E. elegans 1192 in minimal medium. A culture resuspended in Bm for 24 hours was inoculated into Bm and growth followed by absorbance at 560 nm and by plating on Bc. Each curve is the average of 2 experiments.

Figure III-4. Growth of E. elegans 1192 in complete medium.

A culture resuspended in Bm for 24 hours
was inoculated in Bc and growth followed by
absorbance at 560 nm and by plating on Bc.
Each curve is the average of 2 experiments.



time growth (slope = 0) but a continued increase in the cfu/ml-time growth.

The growth in Bm-supplemented media, as stated previously was defined by the lag before entry into log growth, growth rate in exponential phase and maximum number of cfu/ml reached where the growth curve (cfu/ml against time) reaches a slope of zero. Growth rate is expressed as the number of coenobial doublings in one day.

Table III-3 shows these characteristics for growth of Eudorina elegans strain 1192 in Bm, Bm supplemented with the complex supplements (Table II-3) and Bc.

The growth-supporting-and growth-sustaining-abilities of Bm-supplemented with sodium acetate at both 0.2 g/l and 1.0 g/l were very low. Similarly when Bm supplemented with casamino acids was tested, the growth-sustaining-ability was less when the supplement was added at 4.0 g/l, than when added at 2.5 g/l. The large change in pH, with the two concentrations of sodium acetate and with the higher concentration of casamino acids, although in opposite directions, possibly indicates a loss of the buffering capacity of the buffer of Bm, and therefore a depression of the growth-sustaining-ability of the various media.

TABLE III-3

Growth Characteristics of Eudorina elegans 1192 in Liquid Media

| Media | Time in Lag (days) | Growth Rate | Maximum Number of cfu/ml x 10 ⁻⁴ | Initial | pH After 11 Days |
|----------------------------|-----------------------|-------------|---|---------|------------------------|
| <u>Bm</u> | 0 | 0.7 | 4.0 | 6.9 | 7.1 |
| <u>Bc</u> | 0 | 2.0 | 11 | 6.8 | 8.2 |
| <u>Bm</u> + Yeast extract | 0 | 2.0 | 11 | 6.8 | 7.8 |
| <u>Bm</u> + Sodium acetate | | | | | |
| 0.2 g/l | 3 | 0.3 | 2.8 | 6.8 | 8.1 |
| 1.0 g/l | 3 | 0.4 | 1.0 | 6.4 | 8.4 |
| <u>Bm</u> + Casamino acids | | | | | |
| 2.5 g/l | 0 | 0.6 | 4.0 | 6.8 | 6.6 |
| 4.0 g/l | 0 | 0.7 | 2.1 | 6.8 | 5.2 |
| <u>Bm</u> + Nucleic acids | 0 | 1.0 | 4.5 | 6.8 | 7.8 |

However, if the increased efficiencies of plating of coenobia on Bm supplemented with sodium acetate at 0.2 g/l, and with casamino acids at 0.4 g/l, over those plated on 1.0 g/l sodium acetate and 2.5 g/l casamino acids are considered with the results obtained in liquid media, it is possible that the loss of the buffering capacity is not totally responsible for the depression of the growth-sustaining-ability. The concentrations of some of the additions to the minimal medium (Table II-3) may be too great and the suppression of growth arises from concentration inhibition.

A concentration inhibition caused the failure of the complete vitamin mix (Eversole, 1956) given in Table II-3 to support growth. Kemp (personal communication) has been able to obtain good growth on a vitamin mixture in which the concentration of many of the vitamins has been reduced.

D. Continuous Subculture of Eudorina elegans in Bc

After Bc was shown to support growth of Eudorina elegans, the response to constant culture in Bc was determined. The ability to maintain the organism in continuous culture was desirable since cultures would always be available.

The constant nature of growth in continued subculture is shown in Figure III-5, in which the absorbance at 560 nm was followed for approximately eight days with subculturing into Bc with an inoculum of $3-5 \times 10^2$ cfu/ml every fourth day. Under normal conditions the four-day-old culture was used as the source of the inoculum for continued culture and of experimental material.

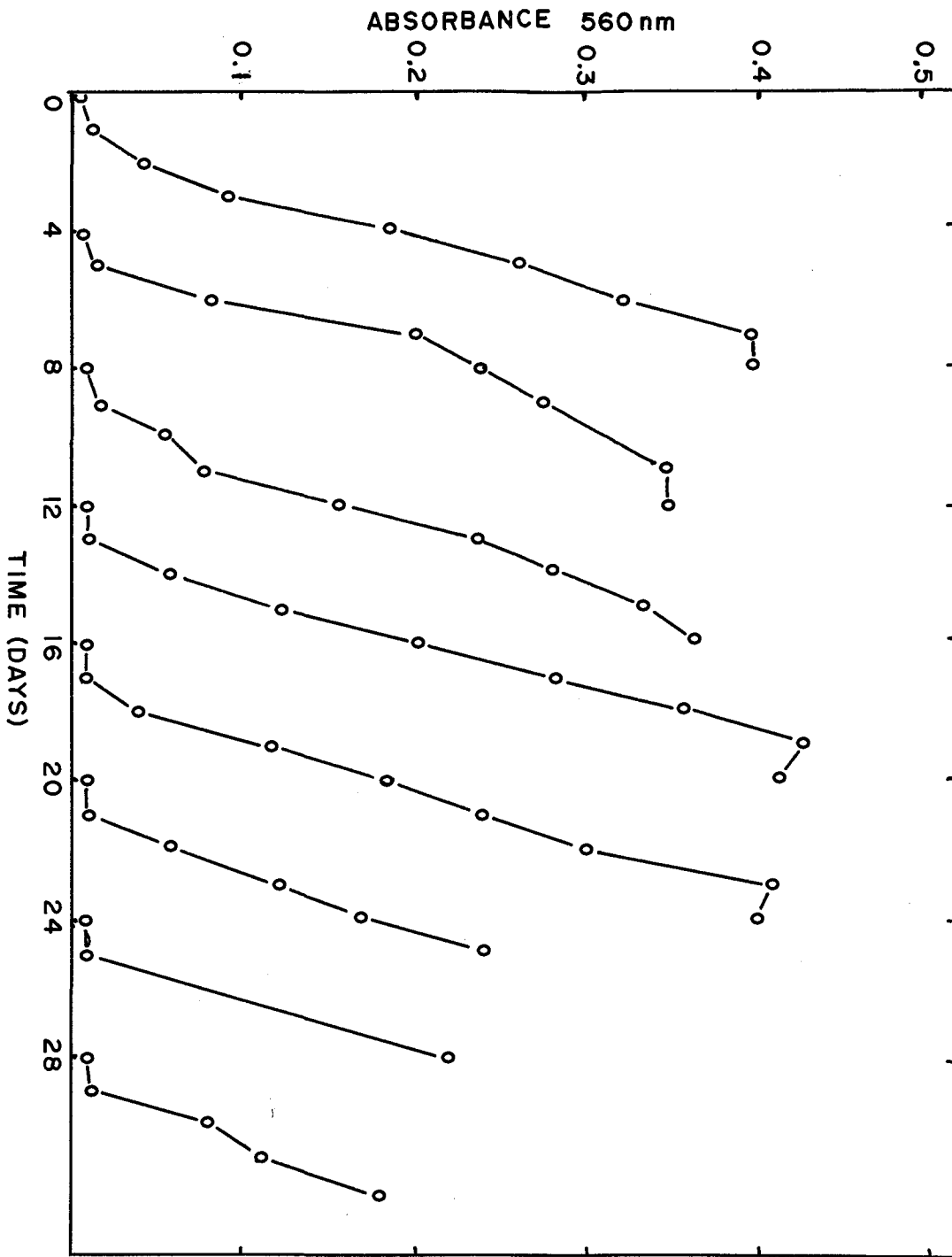


Figure III-5: Effect of repeated subculture into Bc on growth of E. elegans 1192.

CHAPTER IV

EFFECTS OF DRUGS ON EUDORINA ELEGANS

INTRODUCTION

Genetic studies of an organism require a large number of mutants. To facilitate induction and isolation of mutants, agents which are highly mutagenic, and agents which selectively inactivate wild-type coenobia following mutagen-treatment, while leaving mutants with a selective advantage, must be used.

As the initial step in finding such agents, the inactivation abilities of several compounds on Eudorina elegans were studied. Compounds tested with N-methyl-N' nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS), which are potent mutagens in other test organisms, and streptomycin, 8-azaguanine (8-AG), sulfanilamide, and penicillin, which might be useful in mutant enrichment studies.

Many ways to enrich for mutants have been presented in the literature. Most techniques were developed for concentration of auxotrophic mutants by inactivation of wild-type cells. 8-Azaguanine is bactericidal to Bacillus megaterium

under growth conditions (Mangalo and Wachsman, 1962). When mutants and wild-type cells are present in the same medium which allows growth of wild-type cells but will not support the growth of auxotrophic mutants, 8-azaguanine selectively inactivates the wild-type organism (Wachsman and Mangalo, 1962), resulting in an increase in the percentage of auxotrophic mutants in the population. The penicillin method developed initially by Davis (1948) with Escherichia coli, and by Lederberg and Zinder (1948) with E. coli and Salmonella strains relies on the same principle.

Streptomycin and sulfanilamide have not been successfully used in enrichment studies. The results with Eudorina elegans (Mishra, 1967) and with Chlamydomonas reinhardi (Weaver, 1952) show that streptomycin and sulfanilamide respectively inactivate wild-type organisms. Lederberg and Zinder (1948) were unable to demonstrate that streptomycin would act as a selective agent with Salmonella strains.

MATERIALS AND METHODS

A. NTG and EMS Treatment

NTG or EMS was added to a 10 ml coenobial suspension

in phosphate buffer. The concentrations used were

| | |
|------------|-------------------------|
| <u>NTG</u> | 50 μ g/ml |
| <u>EMS</u> | 2×10^{-4} M/ml |

The reaction mixture was agitated, and after various times of treatment at room temperature, samples were removed for plating on Bc medium. The samples were washed twice in phosphate buffer to stop the reaction, plated at the appropriate dilution and incubated for 5 - 7 days under standard conditions of light and temperature prior to scoring. A control culture was similarly treated except that the mutagen was omitted.

Initially the EMS reaction was quenched by dilution of the sample into 6% thiosulfate for 10 minutes. Sodium thiosulfate reacts with free alkyl groups, and thereby inactivates the residual EMS (Loveless and Stock, 1959). However this treatment proved lethal to the organism and was replaced by washing in phosphate buffer.

B. Streptomycin, 8-Azaguanine, Sulfanilamide and Penicillin

Treatment

The general method used for antimetabolite experiments

is given in Chapter II: H. Treatment with Chemicals (Mutagens and Antimetabolites). The concentrations and treatment media are as follows:

| | | |
|---------------|------------------|--------------------------------|
| Streptomycin | phosphate buffer | 0, 500 $\mu\text{g/ml}$ |
| 8-Azaguanine | <u>Bm</u> | 0, 1520 $\mu\text{g/ml}$ |
| | <u>Vm</u> | 0, 15.2, 152, $\mu\text{g/ml}$ |
| Sulfanilamide | <u>Bm</u> | 0, 100, 1000 $\mu\text{g/ml}$ |
| Penicillin | <u>Bm</u> | 0, 300, 600 $\mu\text{g/ml}$ |

RESULTS

Figure IV-1 shows a comparison of the inactivation kinetics of EMS and NTG on Eudorina elegans 1192. The exponential inactivation with NTG was caused by the high concentration of NTG used, since Kemp and Malloy (personal communication) were able to show by decreasing the concentration of NTG, that inactivation occurred with similar kinetics to those of EMS and UV-inactivations.

Growth was slightly stimulated by addition of 15.2 $\mu\text{g/ml}$ 8-azaguanine to Vm, but the organism was rapidly inactivated by growth in Vm containing 152 $\mu\text{g/ml}$. However 8-azaguanine affected growth in Bm only by increasing the lag period and did not cause inactivation at concentrations up to 1520 $\mu\text{g/ml}$ (Figure IV-2).

Sulfanilamide enhanced the growth of Eudorina in Bm (Figure IV-3).

Figure IV-1. Survival of E. elegans 1192 after exposure
in phosphate buffer to NTG and to EMS.

x NTG (50 μ g/ml; o EMS (2×10^{-4} M/ml).

Each curve is the average of two experiments.

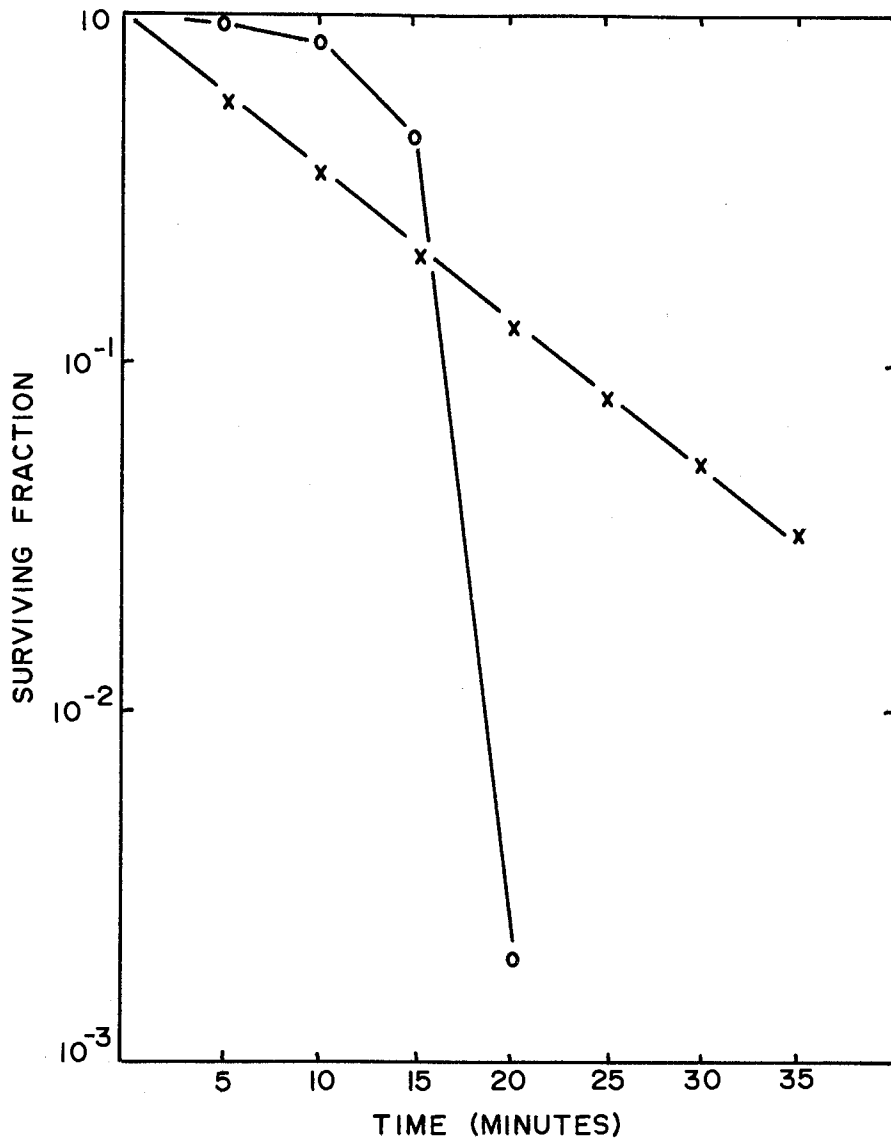


Figure IV-2. Effect of 8-azaguanine on the growth of E.

elegans 1192. A culture resuspended for 24 hours in Bm was inoculated into Bm and Vm containing 8-azaguanine, and growth was followed by plating on Bc. The concentrations of 8-azaguanine are given in $\mu\text{g/ml}$. * Vm;

● Bm.

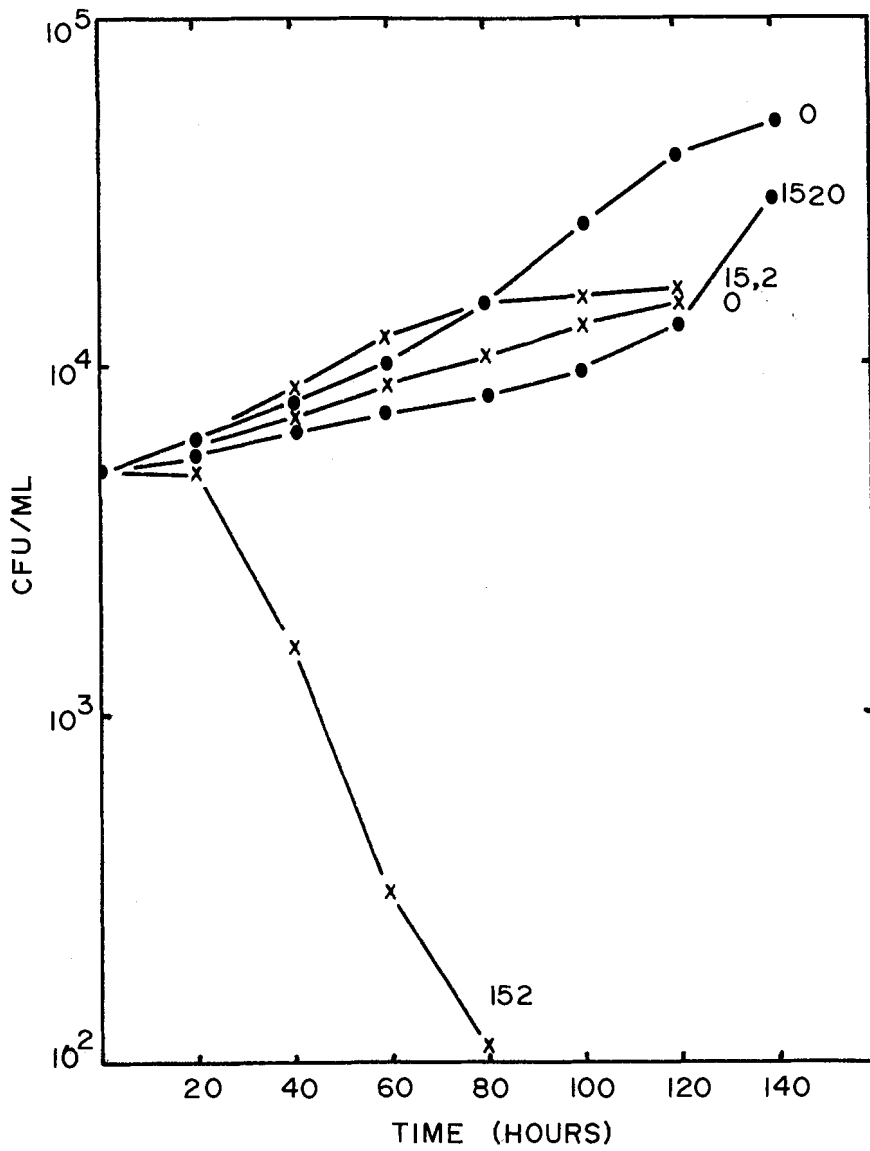
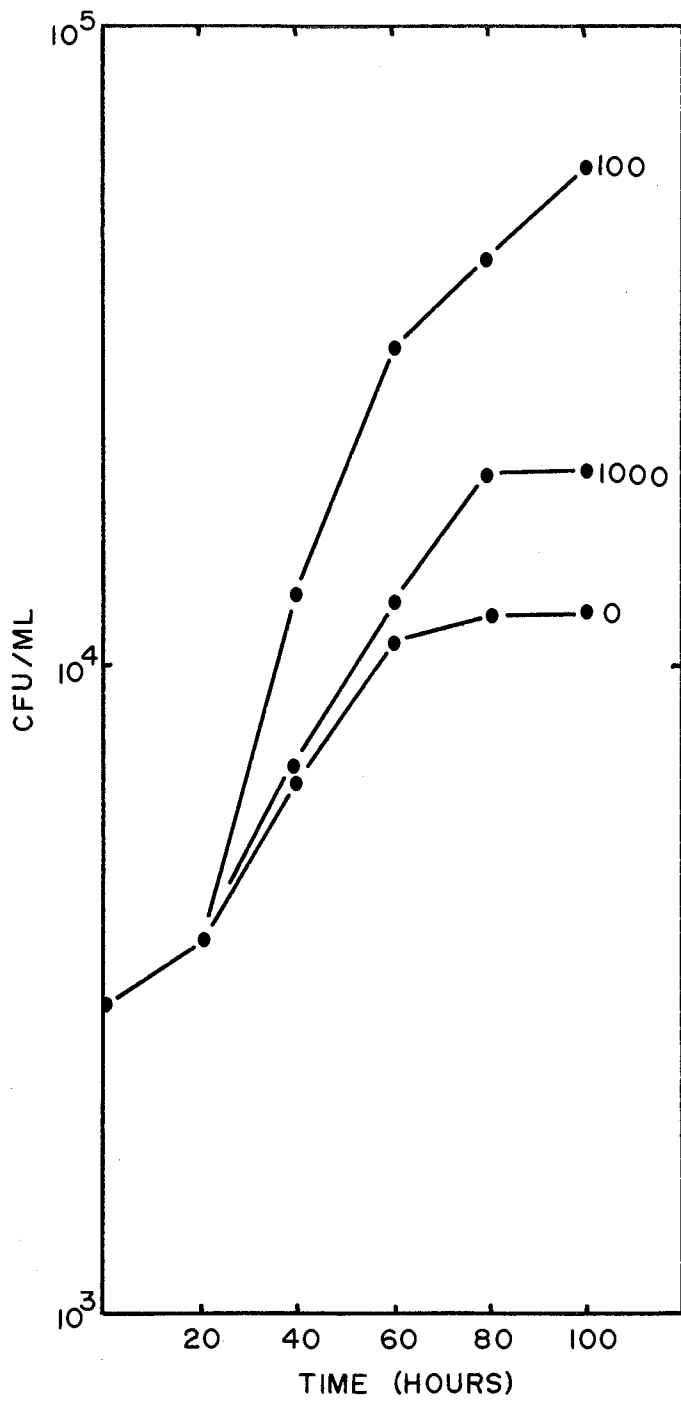


Figure IV-3. Effect of sulfanilamide on the growth of E. elegans 1192. A culture resuspended for 24 hours in Bm was inoculated in Bm containing sulfanilamide, and growth was followed by plating on Bc. The concentrations of sulfanilamide are given in $\mu\text{g/ml}$.



Streptomycin rapidly inactivated the wild-type coenobia when treated in phosphate buffer (Figure IV-4).

In a single experiment penicillin had no effect on the lag or growth rate of Eudorina in Bm.

DISCUSSION

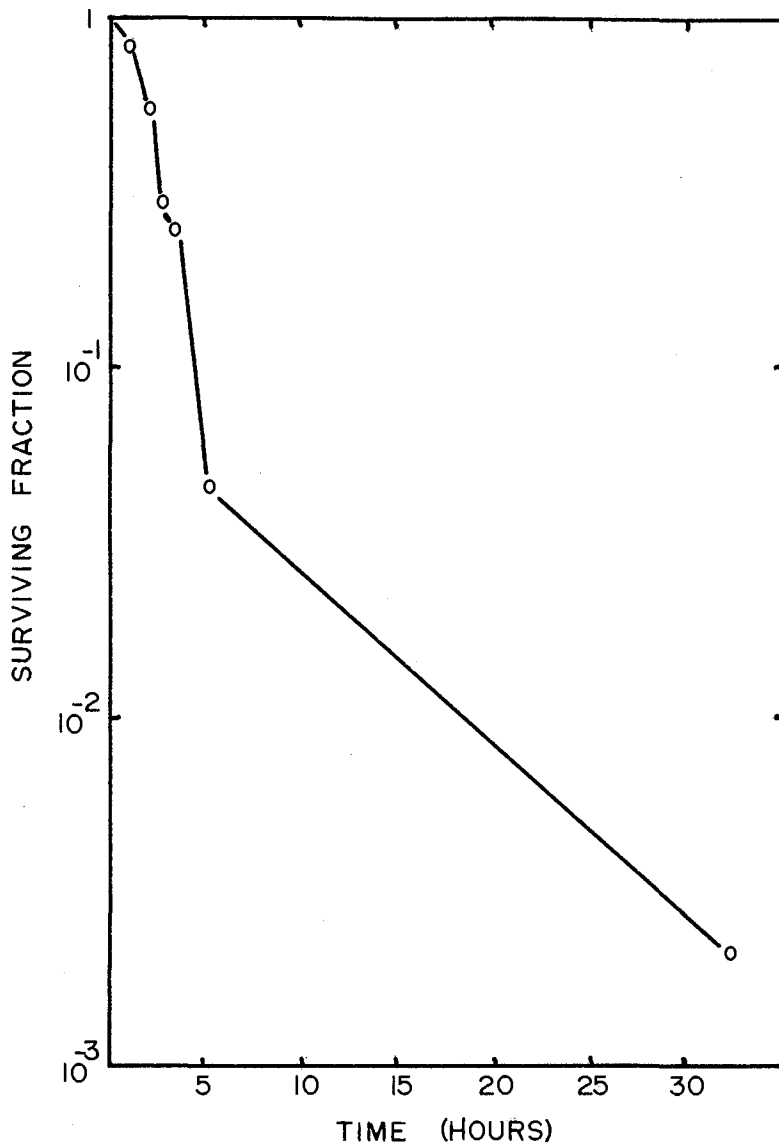
A. Inactivation by NTG and EMS

From the results presented in this thesis and from results elsewhere (Kemp and Malloy, personal communication), NTG is mutagenic to Eudorina elegans.

NTG might possibly be used in its lethal capacity as an enriching agent for mutants by reducing the concentration used, and, with UVL and EMS, to elucidate the nature of the mechanism involved in repair of damaged DNA, although the broad spectrum of action of NTG might have certain drawbacks.

The mode of action of NTG in lethality, and in mutagenesis, is unknown, except that it probably acts through its breakdown product diazomethane (Cerdá-Olmeda and Hanawalt, 1968). In vitro studies with DNA show that NTG alkylates guanine and adenine (Kriek and Emmelot, 1964); in vivo it causes single strand breaks in the DNA (Yoshida and Yuki, 1968; Olsen and Baird, 1969) and gross chromosomal aberrations (Gichner et al., 1963; Kaul, 1969). Malling

Figure IV-4. Survival of E. elegans 1192 after exposure to streptomycin. A culture was starved for 24 hours in phosphate buffer, treated with streptomycin (500 μ g/ml) and plated on Bc.



and De Serres (1970) using Neurospora crassa found that the genetic alterations caused by NTG are point mutations and chromosomal deletions.

Repair of damage leading to inactivation and/or mutation by NTG has been shown in several systems. NTG-induced single strand breaks are reduced in number with time prior to isolation of DNA (Olsen and Baird, 1969). The mutation frequency is maximum if Paramecium aurelia is exposed to NTG at or just before the onset of DNA replication and is minimum when exposed just after replication (Kimball, 1970). These results indicate that NTG causes damage which can be fixed as mutation, which may or may not lead to lethality, or can be repaired.

No studies on the induction of mutations by EMS in Eudorina have been carried out to present in this laboratory. However EMS, an alkylating agent, has been shown to be a very effective mutagen in a number of test organisms. Some of these are bacteriophages T_2 (Loveless, 1958), and T_4 (Kreig, 1963), E. coli and Salmonella typhimurium (Loveless and Howarth, 1959), Saccharomyces cerevisiae (Lindegren et al., 1965), Chlamydomonas reinhardi (Loppes, 1969a, 1969b) and Drosophila (Fahmy and Fahmy, 1957).

Eudorina appears to be much more sensitive to EMS than does Chlamydomonas reinhardi (Loppes, 1968). It is possible

that the EMS stock is the same as that designated by Loppes as HT-EMS (high toxicity EMS). Loppes (1968) found that this solution contained small amounts of toxic impurities. On the other hand Loppes (1966a, 1969b) has found the methylating agent methyl methanesulfonate was more effective in mutant induction in Chlamydomonas than EMS. This differential effect has not been studied in Eudorina but it might be a profitable approach.

B. Enrichment

Agents which will increase the fraction of mutants in a mutagen-treated population must be able to inactivate wild-type coenobia in minimal growth medium. The abilities of sulfanilamide, penicillin, and 8-azaguanine to meet this requirement are reported here. Streptomycin is able to inactivate growing coenobia in Eudorina (Mishra, 1967).

Sulfanilamide and penicillin appeared to be unable to inactivate Eudorina on minimal medium. They are therefore unsatisfactory as an enriching agent under the conditions used.

8-Azaguanine, although unable to inactivate E. elegans under minimal medium conditions, did kill the organism in complete medium, a condition in which the growth rate was rapid compared to that in minimal medium. It may be possible

to enrich for mutants by supplementing Bm containing 8-azaguanine with a complex supplement, say casamino acids, on which all wild-type coenobia and amino acid-requiring mutants will grow rapidly, take up the drug, and be inactivated whereas any vitamin or nucleic acid-requiring mutants will not grow, and therefore will not be inactivated. However, at present it is only possible to say that since 8-AG will not inactivate wild-type coenobia in Bm under the conditions used, it is not suitable as an enriching agent.

Streptomycin inactivates wild-type coenobia under growth (Mishra, 1967) and non-growth conditions. The wild-type coenobia under conditions of starvation are essentially under the same conditions that auxotrophic mutants are when in a medium in which they are unable to grow. These results suggest that streptomycin will be unsatisfactory for mutant enrichment. However, a reduction in the concentration of streptomycin might make it more satisfactory.

If streptomycin will differentially inactivate wild-type and mutant coenobia then its use as an enriching agent might cause mutations of non-chromosomal genes. It is a mutagen for non-chromosomal genes (Sagar, 1960; 1962; Gillham and Levine, 1962) in Chlamydomonas, and these genes are present and mutable in Eudorina elegans (Mishra, 1967).

CHAPTER V

THE RESPONSE OF EUDORINA ELEGANS TO ULTRA-VIOLET LIGHT

INTRODUCTION

Ultra-violet light (UVL) is known to be lethal and mutagenic to many organisms, including bacteria -- E. coli (Guthrie, 1949; Kelner, 1949a; blue green algae -- Anacystis nidulans (Kumar, 1963; Singh, 1968); fungi -- Saccharomyces cerevisiae (Cox and Parry, 1968) and Coprinus macrorhizus (Wentworth and McClaren, unpublished); and green algae -- Chlamydomonas species (Lewin, 1952; Ebersold, 1954; Gowans, 1956). Although Mishra (1967) found that Eudorina elegans is sensitive to the lethal action of UVL, he was unable to isolate mutants following this treatment.

A study of the response of Eudorina to UVL was undertaken for two reasons. First, since UVL was to be used as a mutagen, it was important to know the inactivation kinetics of colony-formation, and the conditions which influence the inactivation rate and the extent of inactivation. It was difficult to use the methods of Mishra (1967) since the technique was not well outlined, and the results presented by Mishra, were significantly different from preliminary observations on Eudorina elegans obtained in this laboratory.

Therefore dose-response curves, photoreactivation, and growth of irradiated Eudorina were studied. Second, the responses to UVL and accompanying treatments yield information on the ability of the organism to enzymatically repair irradiation-induced genetic lesions (Haynes, 1964; Barnhart and Cox, 1970). The capability of Eudorina elegans to repair irradiation damage, as indicated by the changes in UV-response with pre- and post-irradiation conditions was considered, as preliminary to attempts to isolate repair-deficient mutants.

This intensive study was made with the following quotation of Roberts and Aldous (1949) as a guideline:

"In conducting any radiation experiments the following factors should be considered as parameters and carefully controlled: (a) organism; (b) conditions of growth (media, temperature, aeration); (c) phase of growth cycle; (d) conditions during radiation (media, temperature, surface or liquid); (e) radiation (wavelength, time, intensity); (f) conditions after irradiation (media, temperature, time before plating); and (g) conditions for growth of colonies (media, temperature and time)."

Coenobial inactivation, or killing, as used in this study, means reproductive death, and is determined by the inability of the irradiated organism to multiply to form a visible colony on solid medium.

MATERIALS AND METHODS

The Ultraviolet Irradiation Apparatus and Standard Ultraviolet Irradiation Procedure are described in Chapter II, Sections F and G. For determination of UVL-sensitivity, appropriate dilutions were made into the medium used for irradiation, and samples were pour-plated onto Bc plates.

The parental strain, Eudorina elegans 1192, was used in the experiments discussed here, unless otherwise noted.

A. The Effect of Irradiation Conditions (Media and Dose Rate) on Dose-Response

To determine the effect of the suspension medium on dose-response, the culture was suspended in Bm or phosphate buffer, and treated under standard irradiation conditions. A comparison of the dose rate effect upon UV-inactivation was made at 5, 10, and 20 ergs $\text{mm}^{-2} \text{sec}^{-1}$.

B. The Response of Photoreactivating Light

B (i). Photoreactivation Ability as a Function of UV-Dose

Immediately after an irradiated culture was plated, the plates were placed under light conditions of 1000 ft-c intensity.

B (ii). Decay of Photoreactivation Ability

To determine the kinetics of decay of photoreactivation ability with time of incubation in the dark, several plates from an irradiated culture were incubated for various lengths of time in the dark, prior to incubation in the light.

B (iii). Time Required for Completion of Photoreactivation

Plates of UV-treated coenobia which had been placed in the light immediately after irradiation, were transferred at intervals to dark conditions. After 24 hours, all plates were returned to the light.

C. The Effect of Pre-Irradiation Starvation on Dose-Response

A four-day-old, log phase culture was resuspended in phosphate buffer and incubated under standard growth conditions for 24 hours to starve the organism. After four hours illumination on the fifth day, the culture was washed twice, resuspended in phosphate buffer at $5-10 \times 10^4$ cfu/ml, and irradiated under standard irradiation conditions at $10 \text{ ergs mm}^{-2} \text{ sec}^{-1}$.

D. The Effect of Post-Irradiation Holding on Dose-Response

Coenobia irradiated under standard irradiation conditions, were inoculated into either Bm or phosphate buffer and held in the dark at 32°C. Platings were made at 0, 6, and 24 hours following inoculation. The total time for maintaining cultures

under dark conditions was 48 hours following irradiation. The plates were then incubated in the light under standard conditions of light and temperature.

E. The Effect of Combined Pre-UV-Starvation and Post-UV-Holding on Dose-Response.

A starved culture of Eudorina was irradiated and inoculated into phosphate buffer for holding in the dark, prior to plating on Bc plates.

F. The Effect of UV-Treatment on Growth

An irradiated culture was resuspended into Bc in the dark, held in the dark under standard conditions of temperature and agitation for 24 hours, and then incubated in the light under standard conditions. Samples were plated at 24-hour intervals on Bc plates.

RESULTS

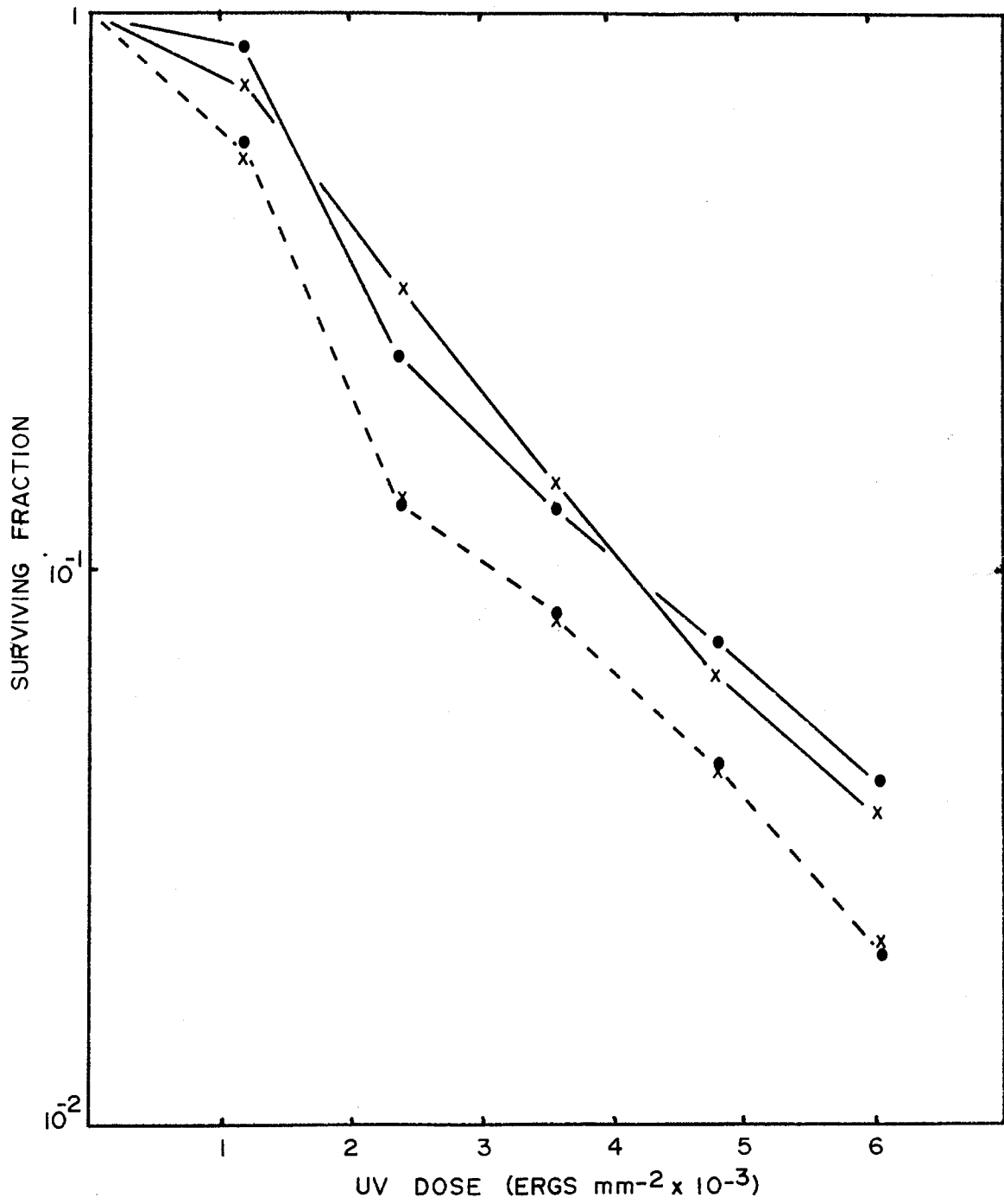
A. Effect of Irradiation Media and Dose Rate on Dose-Response

The UV-inactivation curves of Eudorina elegans in the two suspension media (Bm and phosphate buffer) are shown in Figure V-1. These results indicated that the response to UVL is affected similarly by both media. Therefore, in subsequent experiments, the suspension medium was chosen

Figure V-1. Effect of the irradiation medium on the dose-response of Eudorina. A culture was suspended in Bm and phosphate buffer, irradiated with $10 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ and plated on Bc.

* phosphate buffer; ● Bm.

Experiment 1: solid line. Experiment 2: broken line.



for the convenience of the particular study.

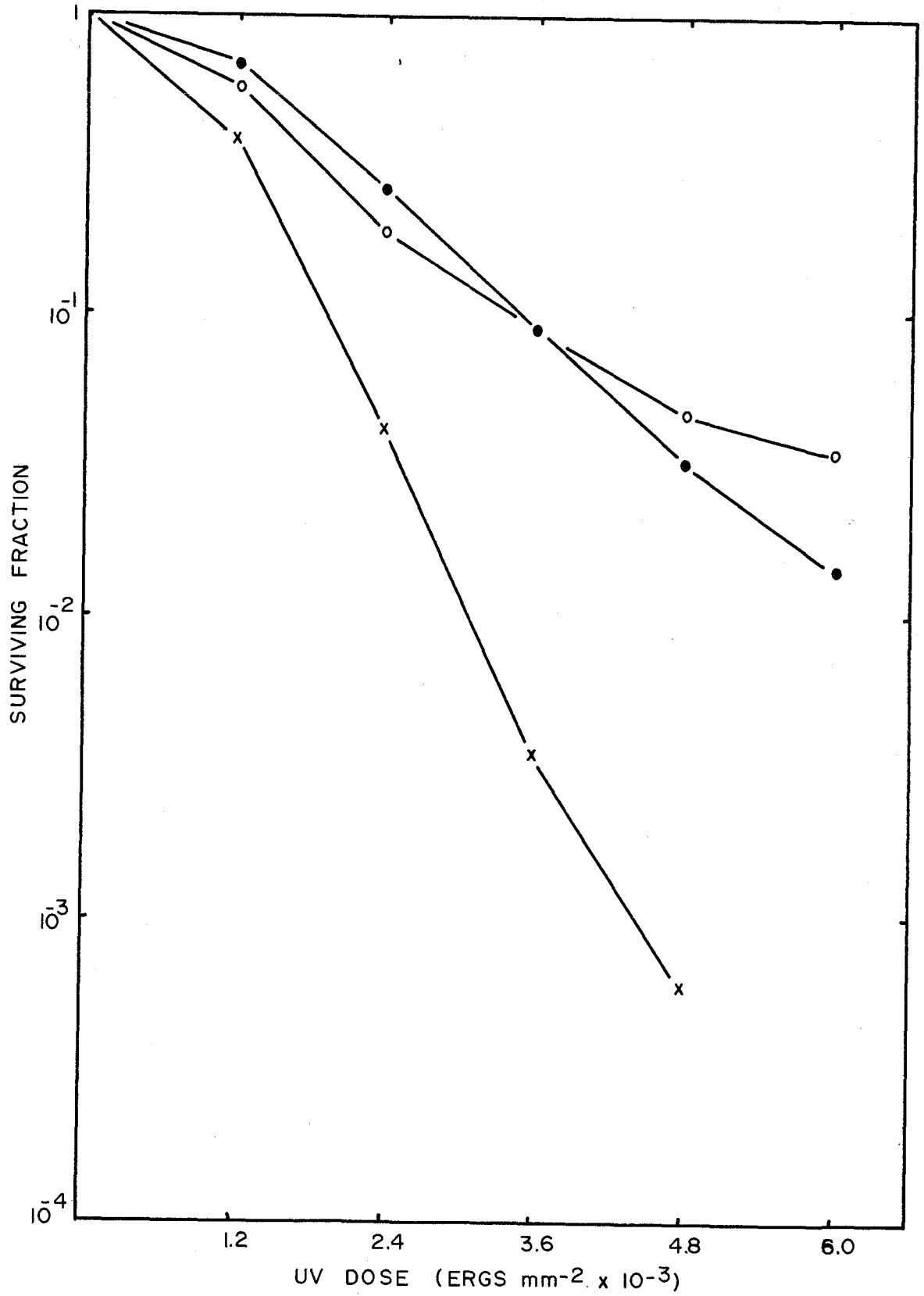
The effect of the dose rates, 5, 10, and 20 ergs $\text{mm}^{-2} \text{sec}^{-1}$, on survival is illustrated in Figure V-2. As the dose rate increases from 5 to 20 ergs $\text{mm}^{-2} \text{sec}^{-1}$, the shoulder in the low-dose region is reduced, and the rate of exponential inactivation is increased. This rate of inactivation is increased two to three times when the irradiation is supplied at 20 ergs $\text{mm}^{-2} \text{sec}^{-1}$ from the inactivation rate when the dose is applied at 5 ergs $\text{mm}^{-2} \text{sec}^{-1}$.

Cultures irradiated at 5 and 10 ergs $\text{mm}^{-2} \text{sec}^{-1}$ have similar exponential inactivation rates; however resistance to UVL develops more rapidly when irradiation is supplied at the lower dose rate, as indicated by the decrease in the inactivation rate at 400 ergs mm^{-2} (Fig. V-2). This resistance is probably the result of a type of repair which occurs during the irradiation period at the low dose rate but which does not have sufficient time to function at 10 ergs $\text{mm}^{-2} \text{sec}^{-1}$.

These kill curves are different from that reported by Mishra (1967). The curves presented here each have a shoulder, resembling the gamma-irradiation inactivation curve of Mishra, whereas the UV-dose-response curve, presented by Mishra, lacks this shoulder.

Figure V-2. Survival of E. elegans 1192 after exposure to UVL at various dose rates.

o 5 ergs mm⁻² sec⁻¹; ● 10 ergs mm⁻² sec⁻¹;
x 20 ergs mm⁻² sec⁻¹. Each curve is the
average of 4 experiments.



B. Photoreactivation, Decay of Photoreactivation, and Time for Completion of Photoreactivation

Figure V-3 shows the UV-inactivation curves of Eudorina elegans under conditions of visible light or photoreactivation (PR) and of darkness or no photoreactivation (non-PR). Introduction to the bright light following irradiation prevented any detectable inactivation of the colony-forming-ability for total doses up to $7200 \text{ ergs mm}^{-2}$ (dose rate = $10 \text{ ergs mm}^{-2} \text{ sec}^{-1}$). At higher doses PR could not overcome the increased irradiation damage.

Since the PR-inactivation and non-PR-inactivation curves, have similar slopes, visible light is said to be dose-modifying (Dulbecco, 1955; Kilbey, 1969). The illustrated experiment gives a dose-modifying-factor of 5.3 at 10% survival. (That is, the inactivation of Eudorina elegans to a level of 10% survival requires 5.3 times as much irradiation if photoreactivation occurs than under non-PR conditions).

The decay of the ability to reverse the lethal effects of a dose of $3600 \text{ ergs mm}^{-2}$ UVL by photoreactivation was followed by plating on Bc and incubating the plates in dark conditions for various periods before transfer to the light. The results are presented in Figure V-4. Irreversible inactivation of the coenobia began almost immediately and continued for approximately 24 hours, after which there was no further decay. This

Figure V-3: Survival of E. elegans 1192 after various doses of UVL at $10 \text{ ergs mm}^{-2} \text{ sec}^{-1}$, with and without photoreactivating light post-treatment.

◦ photoreactivated; ● non-photoreactivated
(Figure V-2). The photoreactivation response is the average of 2 experiments.

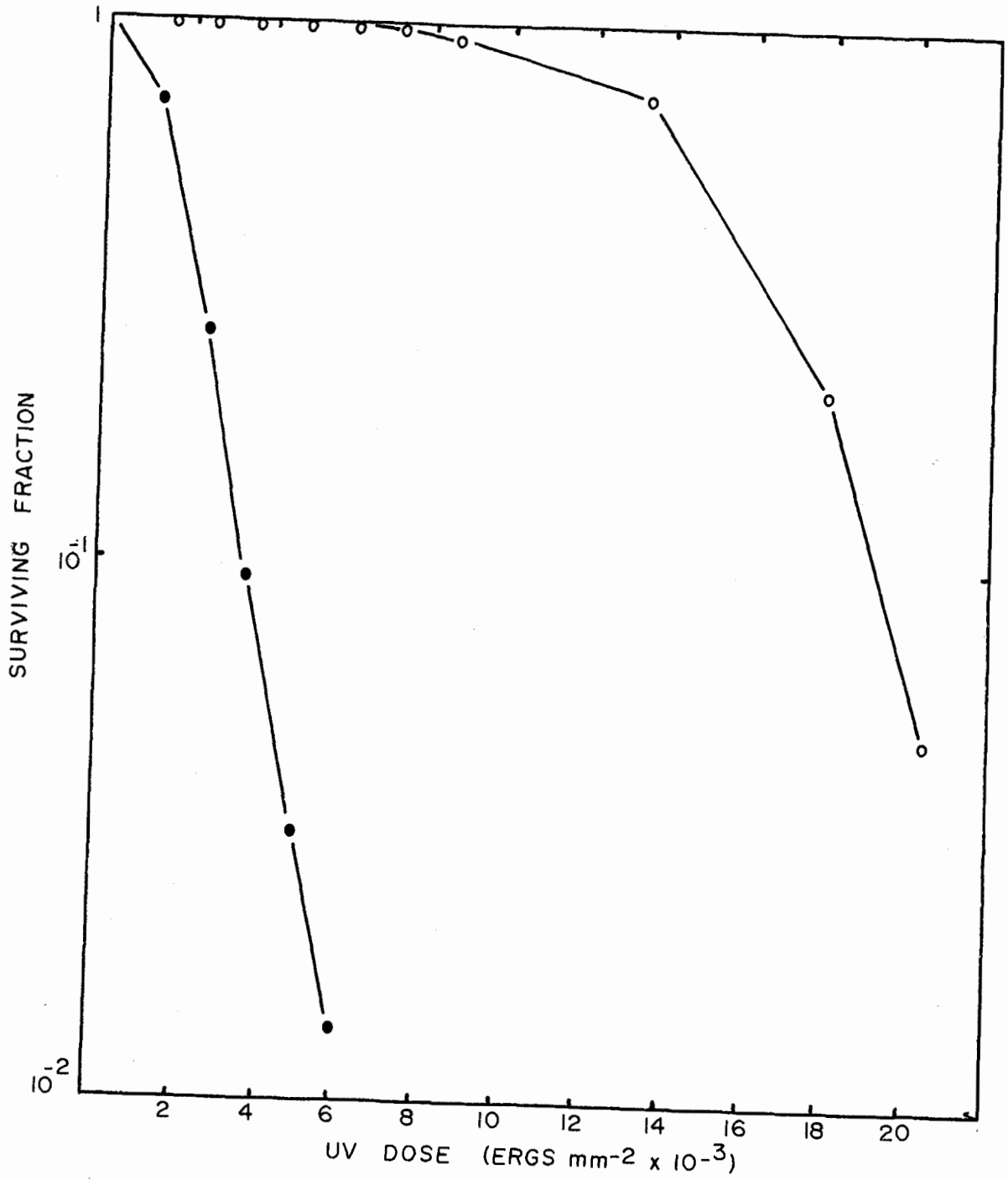
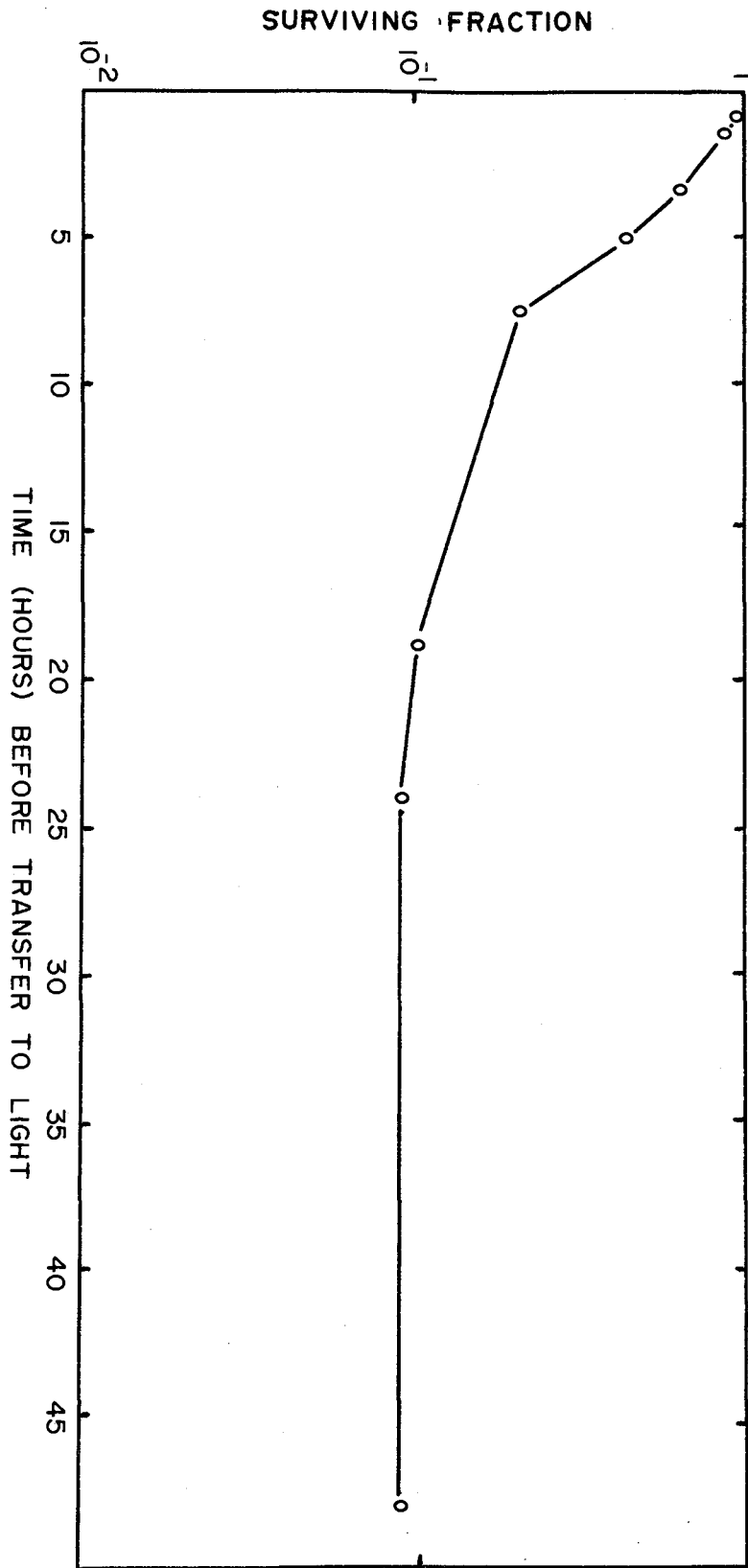


Figure V-4. Decay of photoreactivation ability of E. elegans 1192. Plates prepared from a culture irradiated with $3600 \text{ ergs mm}^{-2}$ were transferred to intense light after various times under dark conditions.



result indicated that cultures, irradiated by mutation induction or dose-response in which no photoreactivation was to occur, had to be kept in the dark for at least 24 hours. Therefore irradiated cultures were routinely held in the dark for 24 hours prior to incubation in the light. Mishra (1967) held irradiated cultures in the dark for 6 hours to prevent PR before placing them in the light. This dark incubation period appears to be significant with respect to mutation induction and UV-inactivation, and will be considered more thoroughly in the discussion of this section.

When plates with UV-irradiated coenobia were incubated in the light for various periods of time prior to removal to the dark conditions, the photoreactivation kinetics were obtained. Figure V-5 shows the results of this treatment, following irradiation of Eudorina elegans 1192 and 1193 with $4800 \text{ ergs mm}^{-2}$. It is important to note that the rate and extent of PR is dependent on the intensity of the PR-light, and therefore at lower intensities PR to the same extent would only occur at much longer incubation times (Kelner, 1949b).

C. Pretreatment

The UV-dose-response of Eudorina, after a 24-hour starvation period yields the result shown in Figure V-6.

Figure V-5. Time required for the completion of photoreactivation of Eudorina. Plates prepared from a culture irradiated with $4800 \text{ ergs mm}^{-2}$ were transferred to dark conditions after various times in intense light.

o E. elegans 1192; x E. elegans 1193.

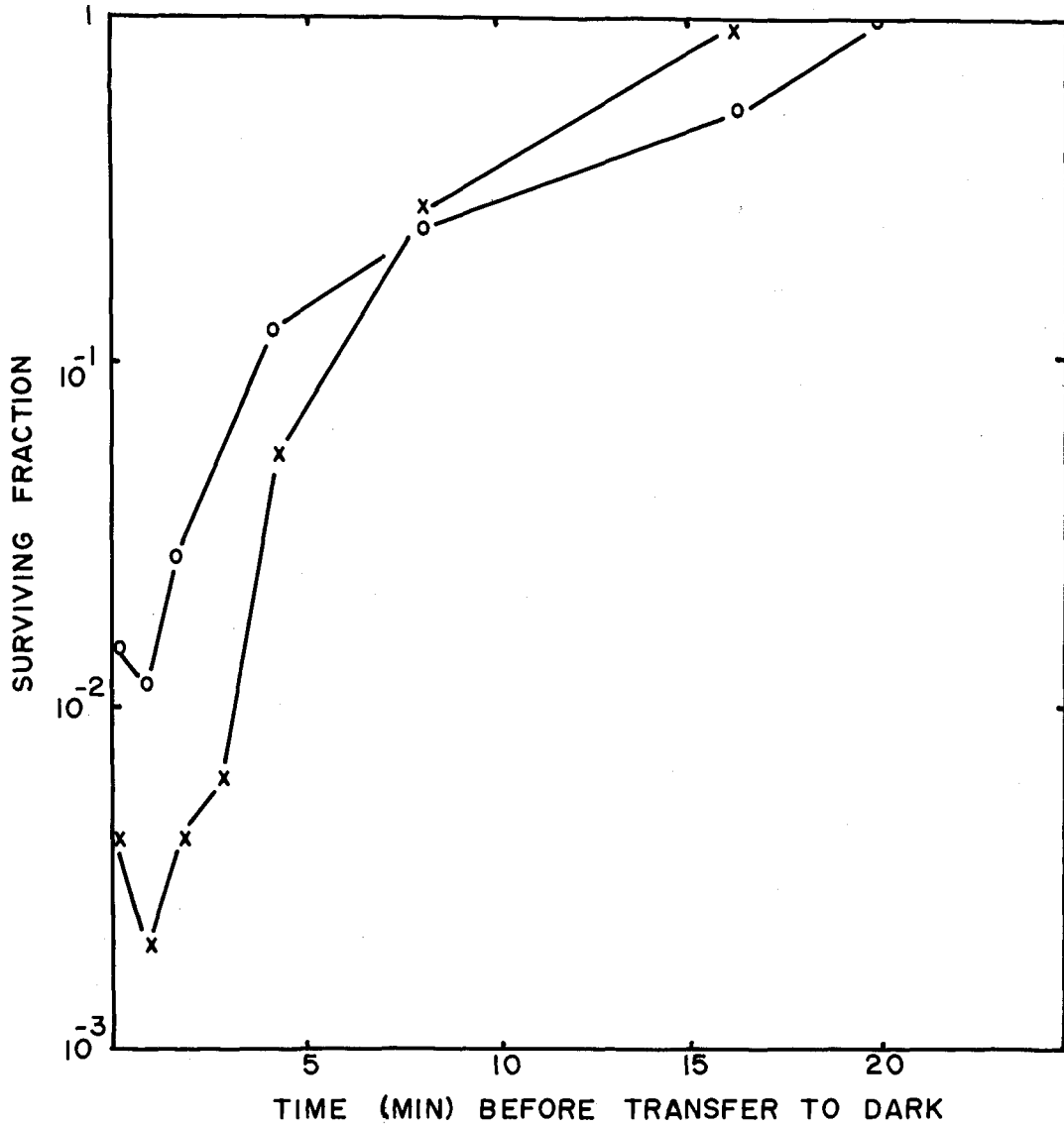
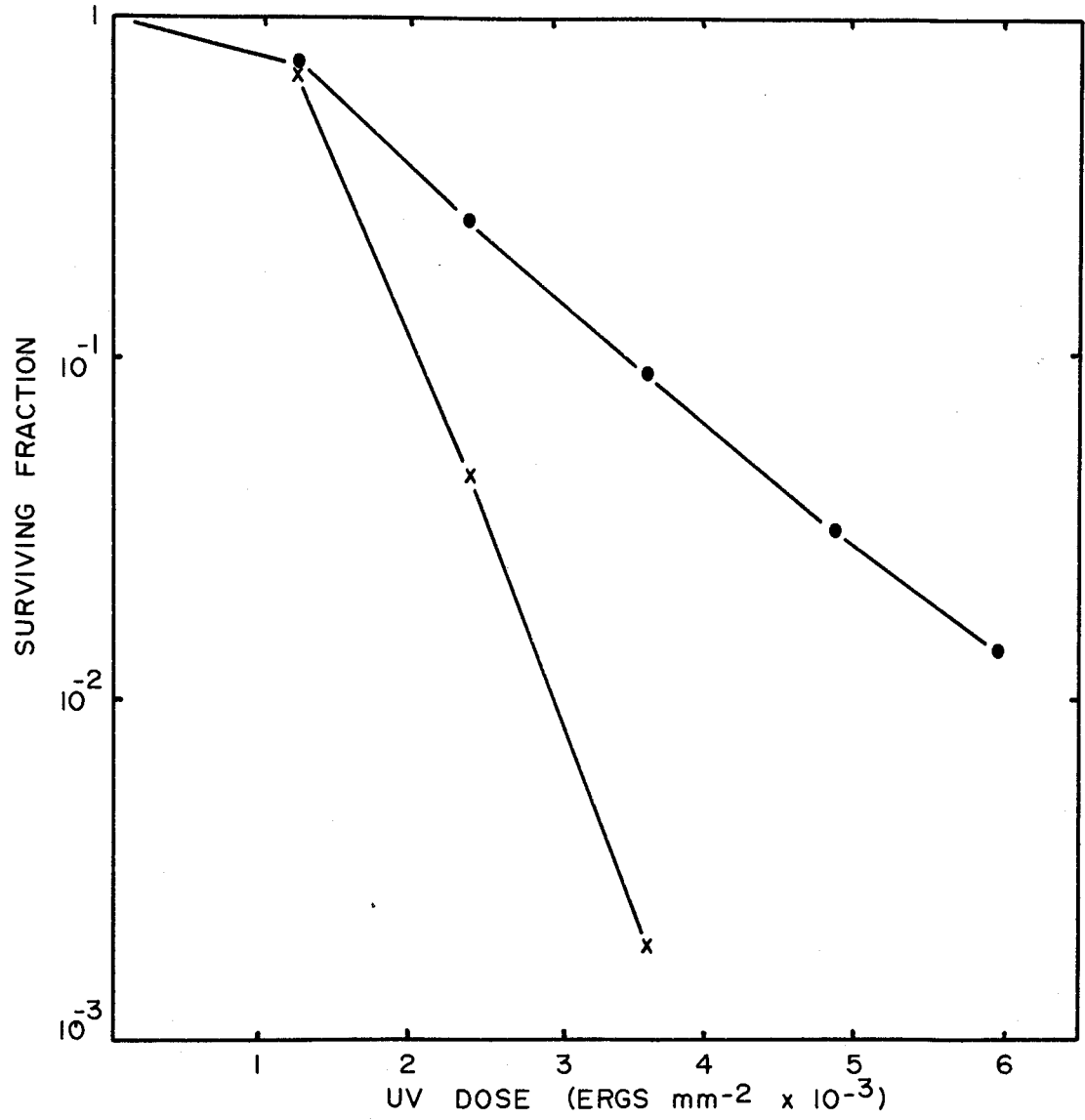


Figure V-6. Effect of pre-irradiation starvation on the survival of Eudorina to UVL.

x with pre-UV-starvation; ● without pre-UV-starvation (Figure V-2). The starved-irradiated response is the average of 2 experiments.



There is an increase in the exponential inactivation rate compared to that of the non-starved culture, indicating that non-growing coenobia are more sensitive to UVL than are the growing coenobia.

D. Post-treatment

Dark storage of a culture not previously exposed to UVL shows no decrease in colony-forming-ability.

The effect of holding in irradiation media on the survival of UV-treated coenobia is shown in Figures V-7 a, b and c. In this set of experiments all samples were held in the dark for a total of 48 hours after irradiation. This extended dark treatment, while preventing any PR of the coenobia held in the liquid media for 24 hours before plating on BC, had no effect on dark survival in the form of further inactivation.

These figures show that dark-holding for 24 hours in non-growth medium (phosphate buffer) following irradiation at the dose rates of 5, 10, and 20 ergs $\text{mm}^{-2} \text{sec}^{-1}$ results in a slight but consistent increase in survival of treated coenobia. Holding in growth medium (Bm) yields results which show no enhancement or only slight enhancement of survival when the surviving fraction is compared to the control, plated immediately after irradiation, and subsequently handled

Figure V-7a. Survival of E. elegans 1192 after exposure to UVL at $5 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ followed by incubation in the irradiation media prior to plating on Bc.

- plated immediately after irradiation;
- x held in phosphate buffer for 6 hours prior to plating;
- v held in Bm for 6 or 24 hours prior to plating;
- held in phosphate buffer for 24 hours prior to plating.

Each symbol is the average of 2 experiments.

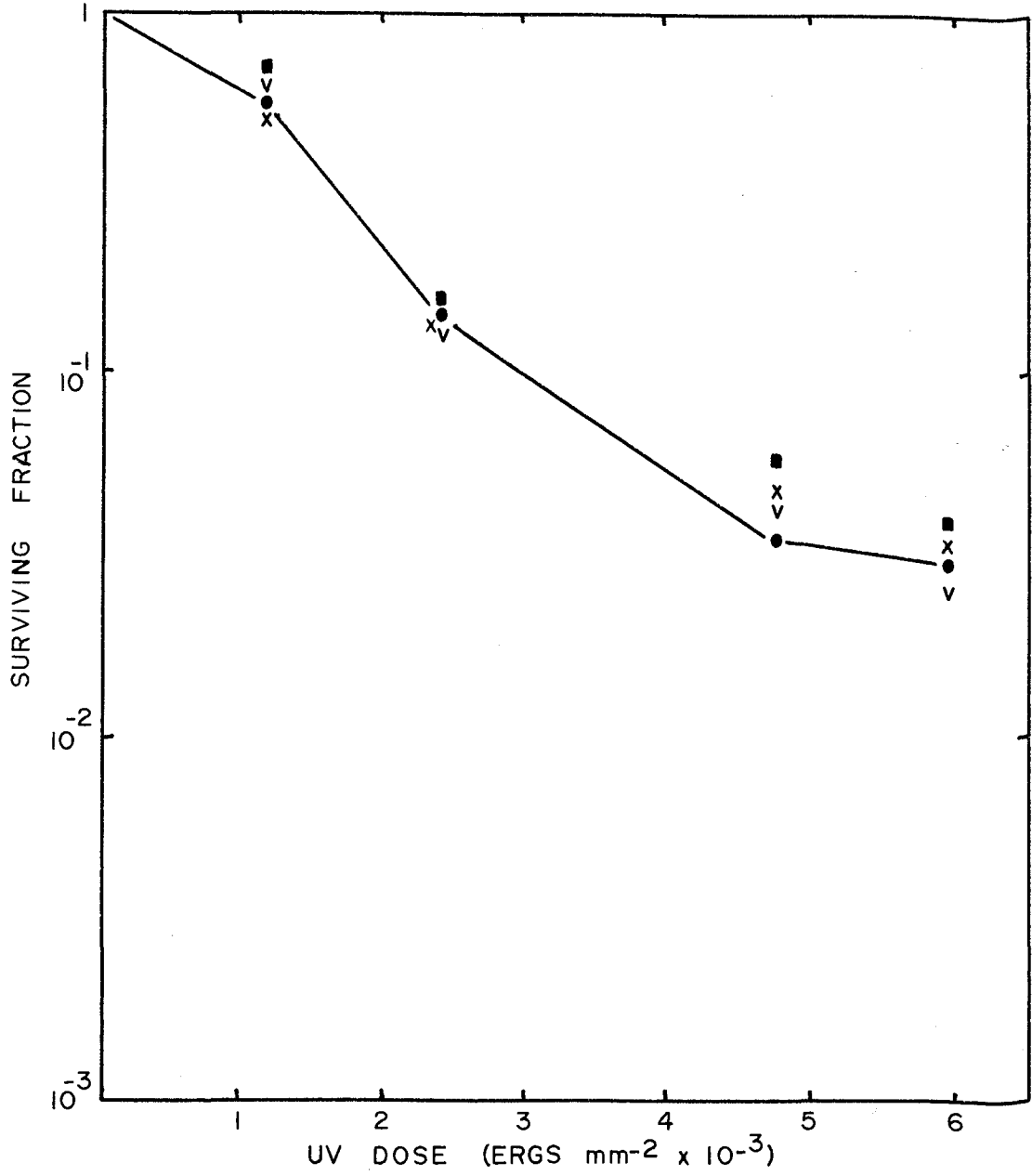
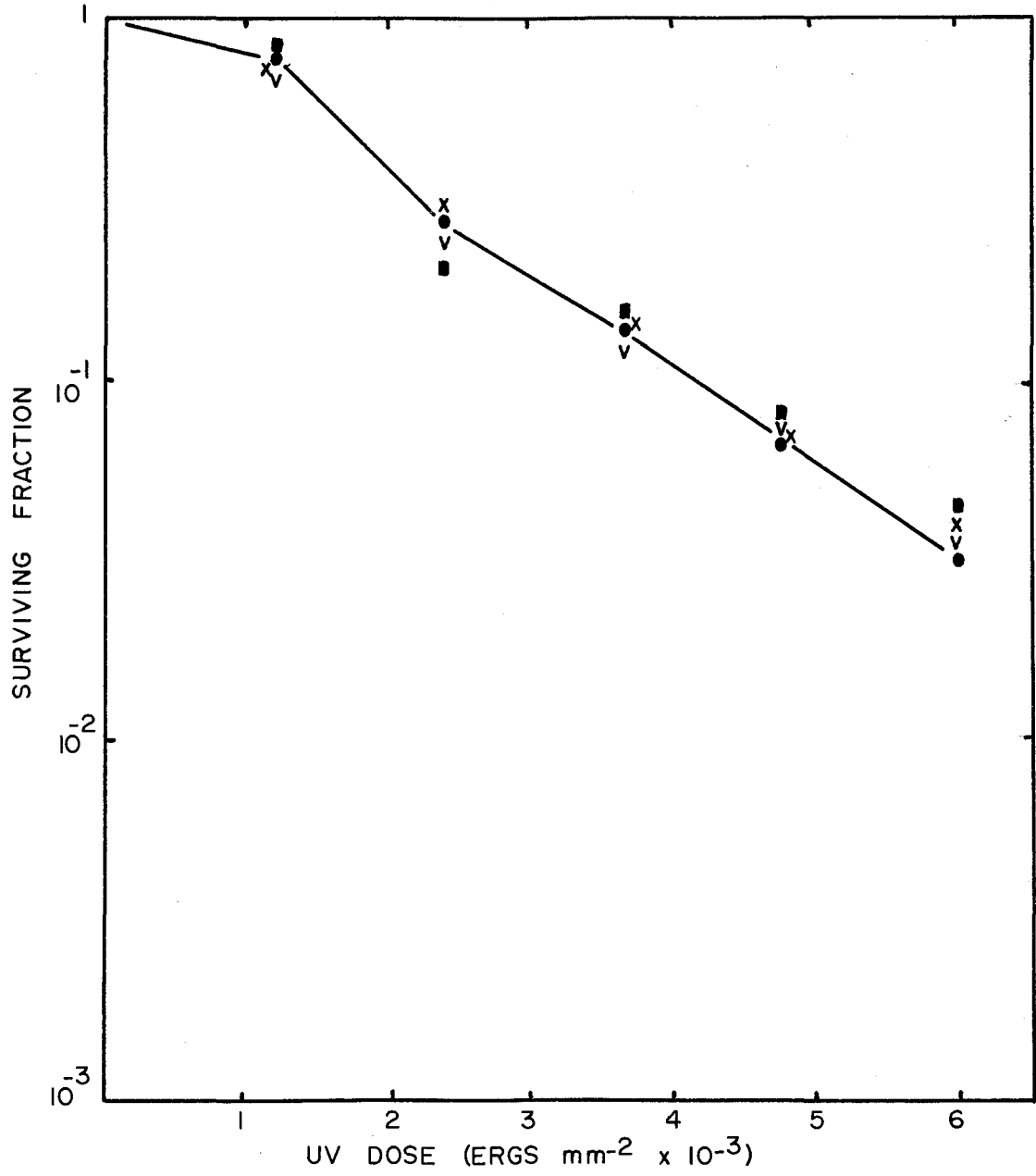


Figure V-7b. Survival of E. elegans 1192 after exposure to UVL at $10 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ followed by incubation in the irradiation media prior to plating on Bc.

- plated immediately after irradiation;
- × held in phosphate buffer for 6 hours prior to plating;
- v held in Bm for 6 or 24 hours prior to plating;
- held in phosphate buffer for 24 hours prior to plating.

Each symbol is the average of 3 experiments.



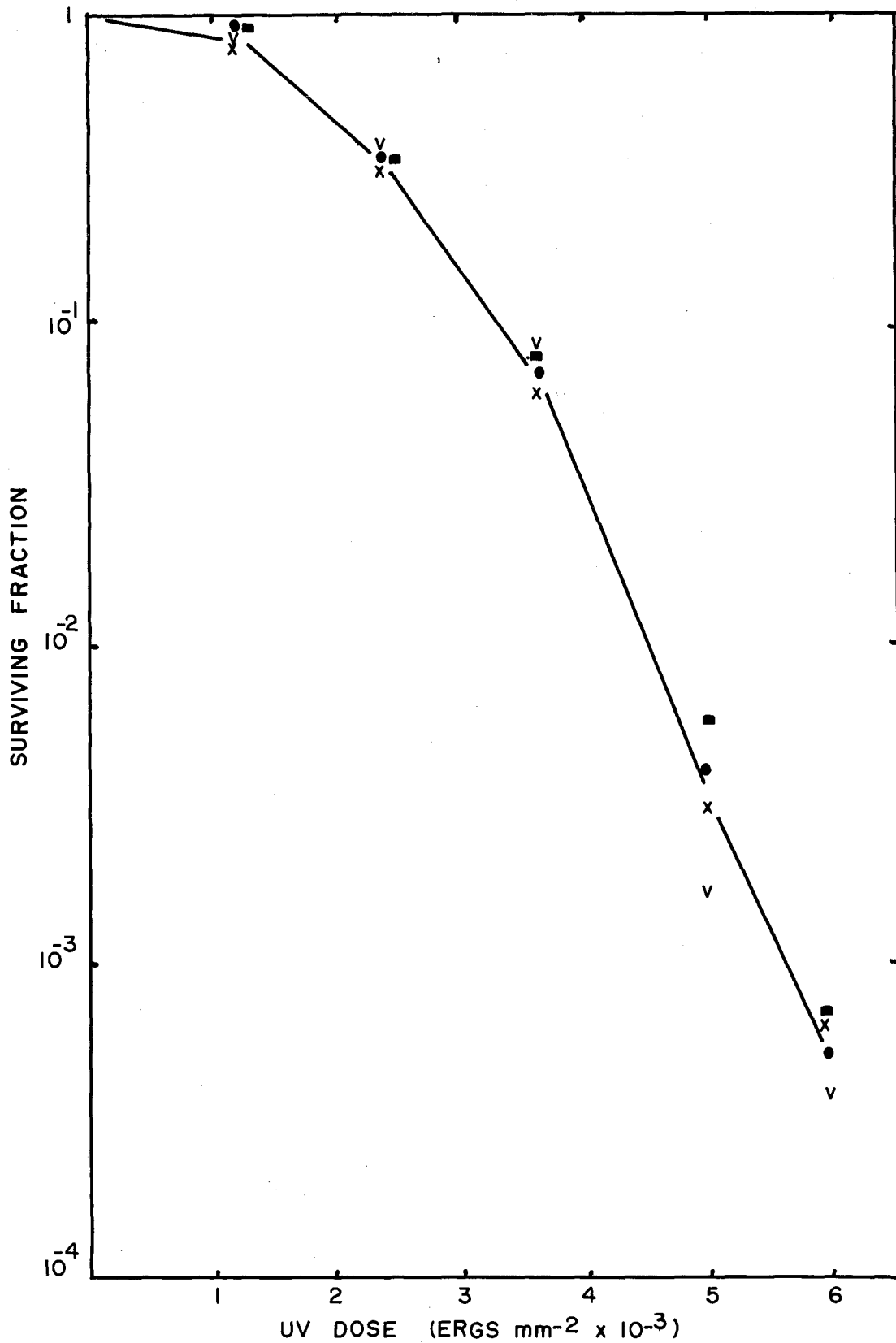


Figure V-7c. Survival of E. elegans 1192 after exposure to UVL at $20 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ followed by incubation in irradiation media prior to plating on Bc.

- plated immediately following irradiation;
- x held in phosphate buffer for 6 hours before plating;
- v held in Bm for 6 or 24 hours before plating;
- held in phosphate buffer for 24 hours before plating.

Each symbol is the average of two experiments.

in the normal manner. It is difficult to determine at this time whether the differences derived from holding are significant or not.

E. Effect of Combined Pre-UV-Starvation and Post-UV-Holding on Dose-Response

The effect of combined pre-UV-starvation and post-UV-holding in phosphate buffer on dose-response is shown in Figure V-8. The rate of inactivation is much greater than the rate with no starvation and no holding, and with holding following UV-treatment. However there is an increased survival with holding following irradiation of the starved culture.

F. Growth

Coenobia taken from cultures in the log phase of growth and irradiated with a total dose of 4800 ergs mm⁻², upon reinoculating into complete liquid medium show a lag prior to resumption of exponential growth. Control cultures do not show this delay (Fig. V-9). The exponential growth-rate of irradiated cultures was reduced compared to the non-irradiated coenobial growth rate. This UV-induced lag in cell multiplication has been noted for other organisms [e.g. Escherichia coli (Deering, 1958) and Micrococcus lysodeikticus (Elder and Beers, 1965)].

When mutation studies were undertaken this delay and

Figure V-8. Effect of combined pre-UV-starvation and post-UV-holding on the survival of E. elegans 1192.

- non-starved, non-held (Figure V-7b)
- non-starved, held for 24 hours in phosphate buffer (Figure V-7b);
- starved, held in phosphate buffer;
- x starved, non-held (Figure V-6).

The starved-held response is the average of 3 experiments.

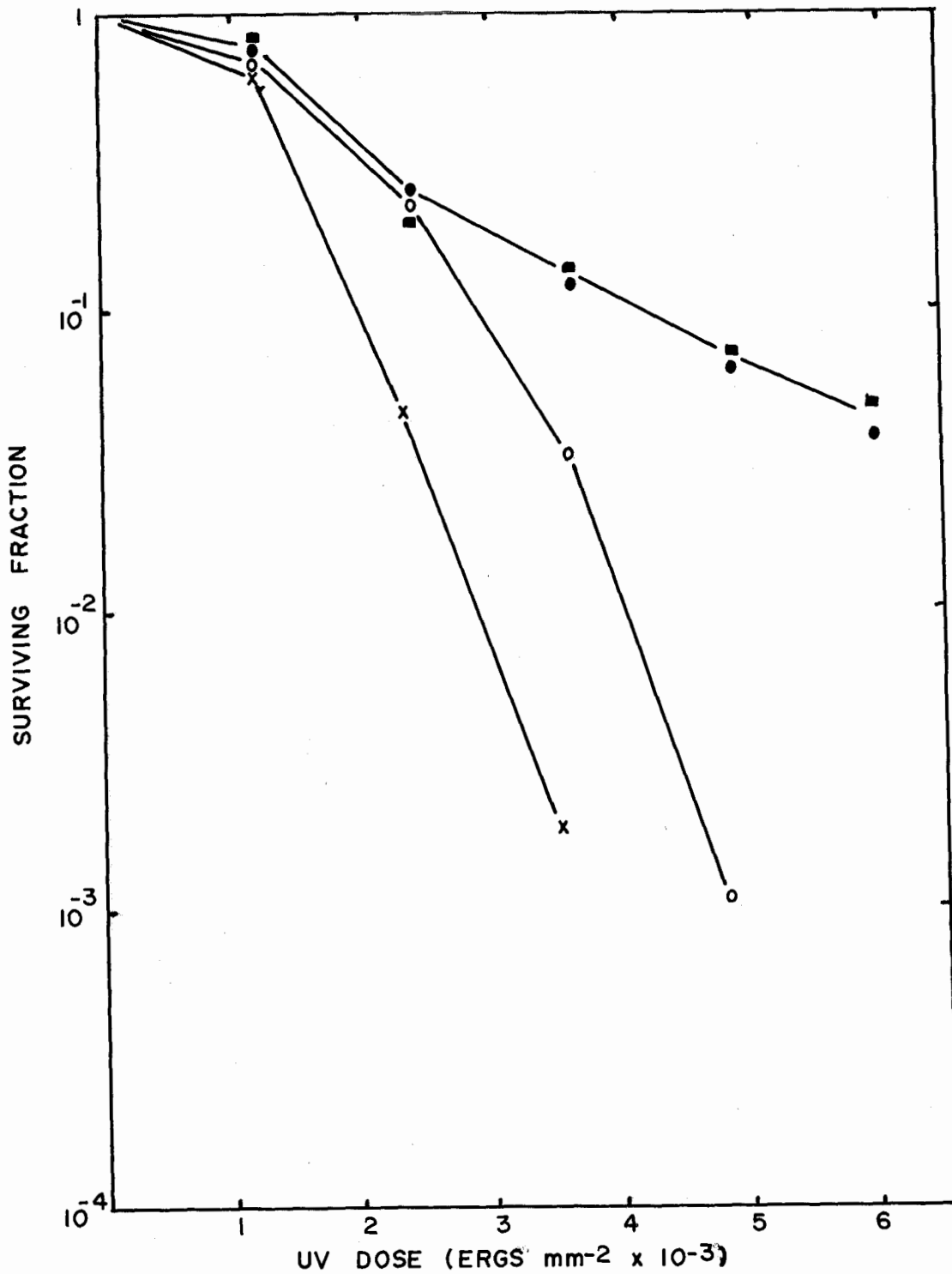
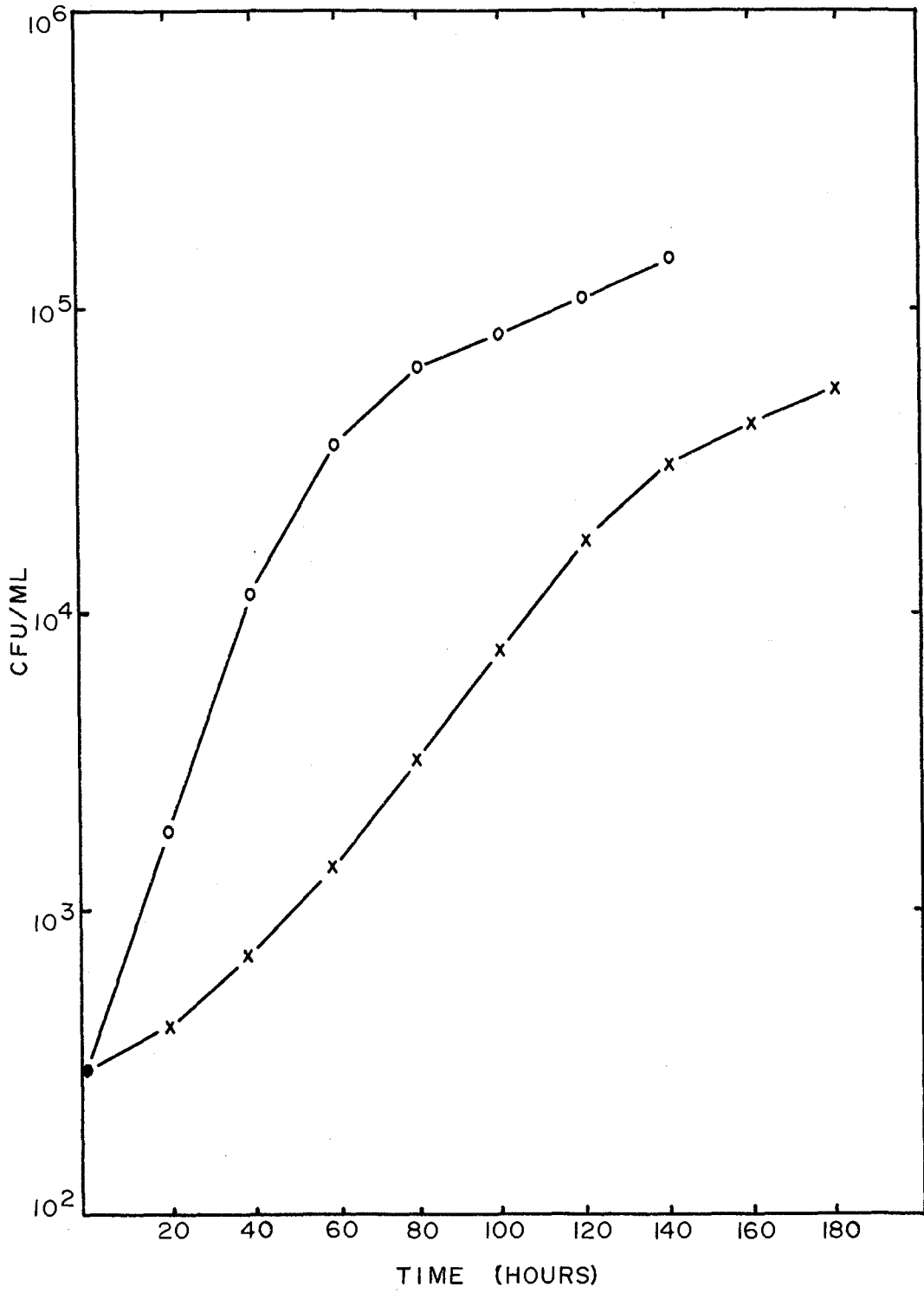


Figure V-9. Effect of irradiation with $4800 \text{ ergs mm}^{-2}$ UVL on the growth of E. elegans 1192.

x with UV-treatment; o without UV treatment. Each curve is the average of 2 experiments.



reduction in exponential growth rate of irradiated cultures were considered in order to ensure separation of mutant and wild-type cells of individual coenobia.

DISCUSSION

Response of Eudorina elegans to UVL-treatment is dependent upon the metabolic state of the coenobia at the time of irradiation, the dose rate of UVL applied to the coenobia, the conditions of storage between irradiation and plating, and the conditions following plating. For example, inactivation of the irradiated organism is less if the plates are incubated in intense light than if the plates are incubated in dark conditions.

A. General Discussion of UV-Inactivation of Eudorina elegans

The dose-response of Eudorina elegans 62m and 62f, reported by Mishra (1967) are different from those presented here. Malloy (personal communication) found that following treatment of the strain of Eudorina elegans used by Mishra, in a similar manner to the method used here, an inactivation response like that of Eudorina elegans 1192 was obtained. However Mishra, found that there was an exponential response with UV-time treatment and therefore with UV-dose. Since the shoulder on the inactivation curve decreases with the

increasing dose rate in the studies reported here, it is possible that the dose rate applied by Mishra was increased over $20 \text{ ergs mm}^{-2} \text{ sec}^{-1}$. But increasing the dose rate would result in 99.9% inactivation occurring at a time much less than 25 minutes of irradiation, in fact, in less than 3.7 minutes, since inactivation at $20 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ results in 99.9% inactivation after 3.7 minutes of irradiation (Fig. V-2).

It is likely that the cultures treated by Mishra for 25 minutes were photoreactivated since, at 32°C there is 30% of the photoreactivable sector, induced by $3600 \text{ ergs mm}^{-2}$ photorepairable at 6 hours in the dark. Mishra grew his organism at 20°C and therefore reduced the rate of growth and probably reduced the rate of PR decay and made more PR possible. Whether the total amount of photoreactivation reached is the same as in experiments presented here is dependent on the time between the end of the 6 hr dark storage and the beginning of the dark period of the light régime, as shown in the following calculations.

The amount of photoreactivation, although proportional to the product of the time of PR and the intensity of the photoreactivating light, is affected directly by the temperature of incubation (Kelner, 1949a, b). Photoreactivation at

32°C at a light intensity of 1000 ft-c is completed in 15-20 min. Therefore at a light intensity of 300-400 ft-c at 32°C, PR would require 60-80 min. If the temperature dependence of photoreactivation in Eudorina elegans is similar to that obtained by Kelner (1949b) with Streptomyces griseus, then a decrease in temperature to 20°C would require five times as long to PR the same sector than at 32°C, or 300-400 min. It must be noted that the effect of temperature is not as proportional as this calculation implies but is only considered as such for simplicity.

Therefore, if the time following dark storage was less than 6 hr. before the regular dark cycle began then the total PR would be less than in the experiment given here; if the time is six hours or more before the regular dark cycle, then the total PR would be equal to that reported here.

This does not explain the lack of a shoulder; it actually means that there must be a shoulder since inactivation followed by photoreactivation would cause a sigmoid curve with a shoulder at low UV-doses.

It is possible to account for Mishra's failure to obtain UV-induced mutants on the basis of this PR, since PR is known to reduce the yield of UV-induced mutations (Kelner, 1949a; Witkin, 1963a, b, 1964).

B. Photoreactivation of Eudorina elegans

Although several thousand UV-treated clones were tested for decreased photoreactivation ability, by storage in the light following irradiation, it has not yet been possible to show that photoreactivation of inactivated colony-forming-units is associated with enzymatic repair of the UV-damage, by isolating mutants lacking the photoreactivating ability (See Chapter VI). It is assumed that such is the mechanism occurring in Eudorina since no other mechanism of PR has been shown to exist in any organism. Mutants, deficient in PR-ability have been obtained in Escherichia coli B (Harm and Hillebrant, 1962), Serratia marcescens (Winkler and Heil, 1969), and in Saccharomyces cerevisiae (Resnick, 1969).

The capacity of photoreactivation, "the restoration of UV-radiation lesions in a biological system with light of a wavelength longer than that of the damaging radiation" is of almost universal occurrence (Jagger, 1958) and is determined by the increased ability of the organism to form a colony on a solid medium under long wavelength conditions, compared to that formed under dark conditions. The results presented for Eudorina do not allow a differentiation between the direct and indirect systems of photorepair. Direct PR is temperature-dependent, photoreactivating light dose-dependent, and

repair results from the splitting of pyrimidine dimers by a light-dependent enzyme (Terry and Setlow, 1962). Indirect PR has only a slight dependence on temperature, no dependence on photoreactivating light dose, is effective only over a narrow wavelength range compared to the direct method and involves a light-independent enzyme (Jagger et al., 1969). Jagger et al. (1969) have shown that indirect PR has no immediate effect on thymine dimers, but encourages a growth delay in which dark repair processes excise dimers.

Significantly the PR system in Eudorina elegans is very efficient, apparently restoring a substantial proportion or all of the damage induced with the UV-dose of $7200 \text{ ergs mm}^{-2}$, which is sufficient to inactivate 99% of the coenobia when treated with UVL at $10 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ under dark conditions. This high efficiency of photorepair suggests the ability to destroy UV-photoproducts which would not be photoreactivable in other organisms, namely Escherichia coli and Saccharomyces cerevisiae (Kilbey and Smith, 1969).

C. Dark Repair of UVL-Induced Damage in Eudorina

It has not been possible to show that the survival of Eudorina elegans from UV-irradiation damage is determined genetically, by isolation of mutants defective in this ability (See Chapter VI). This has been shown for

prokaryotes (e.g. Haemophilus influenza: Setlow et al., 1968; Barnhart and Cox, 1968) and for the eukaryotes, fungi (Holliday, 1965; Chang and Tuveson, 1967; Haefner and Howrey, 1967; Snow, 1967; Cox and Parry, 1968; and green alga Chlamydomonas reinhardi (Davies, 1967).

In single cell systems the shoulder on the UV-inactivation curve has been interpreted as indicative of a dark repair system which becomes saturated in the shoulder region, and only then, after considerable dose has been applied does the slope become exponential, and inactivation occurs (Haynes, 1964; Cox and Parry, 1968). This interpretation has been shown to be correct by the isolation or repair-deficient mutants, which lack the shoulder (Cox and Parry, 1968).

However this interpretation of Eudorina elegans' inactivation shoulder may not be correct since the coenobial character of the organism could be responsible for the shoulder. The coenobium may require the inactivation of several of the cells before coenobial death, and therefore the inactivation curve has multiple hit characteristics. In support of this idea is the fact that plating of UV-irradiated coenobia on acridine orange, in the dark, does not destroy the shoulder of the curve (Wentworth et al.,

1970); acridine dyes are known to inhibit the dark repair of UV-induced lesions by inhibiting pyrimidine dimer excision (Witkin, 1963b; Setlow, 1964), and thereby causes exponential decrease with no shoulder evident in the inactivation curve, or only a very small one, markedly reduced from the original (Singh, 1968).

Also noted as support of a dark-repair mechanism is the marked initial delay in the coenobial multiplication of UV-irradiated coenobia, which is suggested to permit time for the operation of intracellular systems which can lead to the suppression of, or recovery from, some of the UV-damage (Jagger et al., 1964; Haynes, 1964; Elder and Beers, 1965).

Inactivation rates which are lower at low UV-dose rates than those at high dose rates are suggested by Harm (1968) as indicative of the occurrence of dark repair during the irradiation period. The results reported here, comparing 5 and 10 ergs $\text{mm}^{-2} \text{sec}^{-1}$ with 20 ergs $\text{mm}^{-2} \text{sec}^{-1}$ show such differences and are therefore suggestive of dark repair processes in Eudorina.

However, other more concrete evidence for some form of dark repair does exist.

Starvation prior to UV-treatment causes much enhanced inactivation of the non-starved culture. Since the potential

damage is expected to be the same in the starved and non-starved cultures, than the same inactivation kinetics would be expected. However it is possible that there is repair of the potential damage in the actively-metabolising coenobia, since considerably more inactivation occurs in the starved than in the non-starved culture. Alternatively the non-starved coenobia may be able to tolerate a large number of UV-induced lesions.

Holding in liquid starvation or growth medium following UV-irradiation of Eudorina elegans results in very little enhanced survival (Fig. V-7a, b and c). It is possible that the enhancement observed is caused by plating error. In E. coli there is a considerable increase in survival of irradiated wild-type cells are liquid-held, prior to plating (Roberts and Aldous, 1949; Alper and Gillies, 1958; Jagger et al., 1964; Harm, 1968). This reactivation of potentially UV-inactivated cells is not observed when a UV-sensitive strain of Haemophilus influenza is treated (Barnhart and Cox, 1970). It therefore appears that this type of enzymatic dark repair of UV-induced lesions does not occur in Eudorina.

However a combination of starvation and dark-holding in buffer results in enhanced survival of the starved culture, but reduced survival from non-starved cells held in buffer. It appears that the holding allows the repair of some of the

potential damage of the non-metabolising cells, whereas plating on the complete medium following UV-irradiation of this starved culture inhibits the repair. However not all of the potential damage is restored and survival does not reach that of the non-starved, irradiated, held cultures.

Evidence for the existence of a type of dark repair also comes from the differential inactivation observed when irradiated coenobia are grown on plates with and without acridine orange (Wentworth et al., 1970). There is a small reduction in survival on the acridine orange plates compared to the non-acridine orange-containing plates. Acridine dyes are known to interact with DNA (Lerman, 1963), and to block the excision of pyrimidine dimers (Setlow, 1964). This differential inactivation indicates that there is some dark repair.

Thus in Eudorina, there is evidence for a type of dark repair, but this dark repair appears to be different from that in other organisms.

CHAPTER VI

MUTANT INDUCTION, ISOLATION AND CHARACTERISATION

INTRODUCTION

Mutant strains of Eudorina elegans resistant to high concentrations of the drugs dl-methionine-dl-sulfoximine, erythromycin, and streptomycin, have been reported by Mishra (1967). However, his attempts to isolate stable nutrition-requiring, morphological-defective and pigment-deficient mutants were unsuccessful.

The present study was initiated to examine the possibility of obtaining other classes of mutants which were not found and/or not sought after by Mishra [e.g. pigment-deficient, morphological-defective, ultra-violet sensitive, and conditional lethal (temperature-sensitive) and complete auxotrophic mutants]. The mutants which were isolated and characterised include five auxotrophic mutants, two of which are deficient in chlorophylls and one which is unable to undergo normal coenobial breakdown.

MATERIALS AND METHODS

A. Mutant Induction

Mutants were induced either by NTG-treatment or by UVL-treatment.

A (i). NTG-Treatment

The mutants induced by NTG-treatment were isolated from an experiment designed to determine the lethality response of the organism to NTG when grown on plates containing 5.0 $\mu\text{g/ml}$ NTG. The procedure used is described in the following paragraphs.

Coenobia of a log phase culture were spread on the NTG-containing Bc agar and incubated under a light intensity of 1000 ft-c at 22°C for five days. The colonies were then washed from the plates with approximately 5 ml/plate Bc, washed once, and resuspended in Bc. After incubation for seven days under standard growth conditions at 22°C, sufficient growth had occurred to allow any mutant cells of a coenobium to produce daughter coenobia and therefore to be separate from the wild-type cells.

The coenobia were then plated on Bc plates, and grown until colonies were visible; these colonies were tested for ability to grow on Bm and Bc plates at 22°C and 32°C by transferring the colonies to two Bm and two Bc plates and incubating one of each pair at each temperature.

A (ii). UVL-Treatment

A 20 ml culture, grown under standard growth conditions, was suspended in 10 ml phosphate buffer and treated with a

dose of 4800 ergs mm^{-2} UVL.

The culture was washed twice, resuspended in 20 ml Bc medium in a foil-covered flask and incubated under standard growth conditions for 6-8 days. The foil was removed from the flask after 24 hours, the time sufficient to prevent photoreactivation (See Chapter V). After the growth period, the coenobia were plated on Bc, and incubated under standard growth conditions until colonies were visible (5-7 days). The colonies were then tested for a mutant phenotype.

B. Mutant Isolation

The criterion of isolation of auxotrophic mutants was their ability to grow on Bc plates but inability to grow on Bm plates.

B (i). Isolation of NTG-Induced Mutants

Initially colonies on Bc plates, grown from NTG-treated coenobia, were transferred using velveteen, by the replica-plating technique of Lederberg and Lederberg (1952), to two Bm and two Bc plates for identification of temperature-sensitive or complete auxotrophic mutants.

After failing to identify any auxotrophic mutants among approximately 3000 transferred colonies, each of 500 of these colonies was resuspended into 0.2 ml Bm, for 24 hours under

the light intensity of 1000 ft-c at 22°C. It was assumed that the intracellular supply of any requirement would be depleted by this procedure. The suspensions were then spotted, along with a non-treated wild-type control suspension, using a capillary tube, onto two Bm and two Bc plates, and incubated at 22°C and 32°C. From this method two complete auxotrophic mutants were isolated, growing neither at 22°C nor at 32°C on Bm, but at both temperatures on Bc.

B (ii). Isolation of UVL-Induced Mutants

Following growth in Bc liquid medium of a culture treated with 4800 ergs mm⁻² UVL, and growth on Bc plates of the coenobia into colonies, 2000 colonies were picked into 0.2 ml Bm and incubated for 24 hours at 32°C and 1000 ft-c light intensity. These suspensions were then spotted on one Bm plate and 3 Bc plates for treatment as follows. One Bc plate and the minimal plate were incubated under standard light and temperature conditions for five days, for determination of altered nutritional requirements of any of the colonies. The other two Bc plates were used to determine whether any of the colonies showed increased UV-sensitivity from that of the wild-type, by increased inactivation after holding in the dark, or by inability to photoreactivate the UV-inactivated sector. These plates, after drying for 30

minutes, were irradiated with UVL at $3600 \text{ ergs mm}^{-2}$ [giving 10% survival of wild-type coenobia when stored in the dark immediately after treatment (Figure V-2), and 100% survival when placed in the light immediately after treatment (Figure V-3)]. One plate was kept for 24 hours in the dark to prevent PR before incubation under standard growth conditions, and the other plate was placed immediately in the light at 32°C . After seven days of growth, the survival of irradiated colonies, arising from mutagen-treated cultures was compared to the survival of the control colonies.

Those strains, induced by either NTG or UVL, which appeared to have mutant phenotypes, were isolated onto Bc plates for further characterisation.

C. Mutant Characterisation

C (i). Auxotrophic Mutants

The presumptive mutants were then cloned by streaking onto Bc plates and the colonies retested for nutritional requirements on Bm and Bc. After repetition of this cloning step to determine the stability of the mutants, two types of tests were carried out to determine the nutritional requirements.

First a log phase culture was starved for 24 hours and spotted on Bm-supplemented plates. Following determination

of the requirement the mutant was plated onto Bm-supplemented plates to determine the efficiency of plating. Since it was not possible to get the wild-type organism to grow on Bm-vitamin mix (Eversole, 1956), it was assumed that any mutant which grew on yeast extract, but not on any of the other supplements (Table II-3), was defective in its ability to synthesise a vitamin. This was subsequently verified. If a mutant was suspected to have a vitamin-requirement, it was further tested by plating on Bm-supplemented with individual vitamins.

The second method of identification was to grow the mutants in liquid Bm, Bm plus the requirement and Bc, following growth either by A_{560} or cfu/ml, as described in Chapter II E. Growth Characterisation.

C (ii). Pigment-Deficient Mutants: Chlorophyll Determinations

Total chlorophyll and chlorophyll a and b concentrations were determined by the procedure of Arnon (1949) based on the method of MacKinney (1941). The chlorophyll was extracted by shaking the coenobia in 80% acetone and the absorbance of the acetone extract measured at 645, 652, and 663 nm in a 10 mm quartz cell in a Bausch and Lomb Precision Spectrophotometer. The concentrations of chlorophyll a and b were calculated using the equations of Arnon (1949) and the absorption coefficients of chlorophyll a and b given by MacKinney (1941).

The calculations are outlined in Appendix III (from Arnon, 1949).

The absorbance-wavelength of the whole coenobia of mutants and wild-type strains were determined using the Coleman Spectrophometer 6C.

RESULTS

Eudorina elegans 1192 and 1193, with which the mutants are compared grew into smooth, raised, dark-green colonies when plated on Bc and Bm. The growth in liquid media of these two strains is shown in Figures III-3 and III-4.

Mutant Types

As presumptive mutants were picked from Bc following preliminary tests for growth on Bc and Bm, they were given a number (M-mutant number), where the numbers were consecutive. Those numbers omitted here in the sequence M-1 to M-56, include mutants which reverted or were lost due to mechanical failure of laboratory equipment, and wild-type organisms which were mistakenly isolated.

There were no temperature-sensitive mutants or mutants with altered ultra-violet light sensitivity found in testing several thousand colonies, treated with NTG and UVL respectively.

A. Nicotinamide-Requiring Mutants

Two mutants, designated M-1 and M-2 were isolated from

the same culture of Eudorina elegans 1193, following growth of this strain on NTG plates. These mutants are very stable, having been maintained in culture for over a year without every having been observed to revert to wild-type.

A comparison of the efficiencies of plating of these mutants are presented in Table VI-1 (Appendix II). These results suggest that the two mutants are of the same origin. However it will not be possible to conclusively determine this until the sexuality system has been developed sufficiently and comparison of growth using nicotinamide precursors [e.g. nicotinic acid, anthranilic acid, indole, tryptophan and kynurine (Gowans, 1956; Brennar, 1955)] have been made.

Figure VI-1 shows the growth response of M-1 to Bm and to supplements of Bm. No other compounds known to be precursors of nicotinamide were tested for their growth-supporting-ability.

It is significant that the growth of M-1 in Bm-supplemented with nicotinamide at 1.0 $\mu\text{g/ml}$ was inhibited to approximately one quarter of the growth in Bm-supplemented with 0.01 and 0.1 $\mu\text{g/ml}$ nicotinamide whereas the parental strain will grow in 1.0 $\mu\text{g/ml}$. Since the concentration of nicotinamide used in the isolation of the mutant was 0.75 $\mu\text{g/ml}$ it is possible that there could be selection against some mutants by this high concentration.

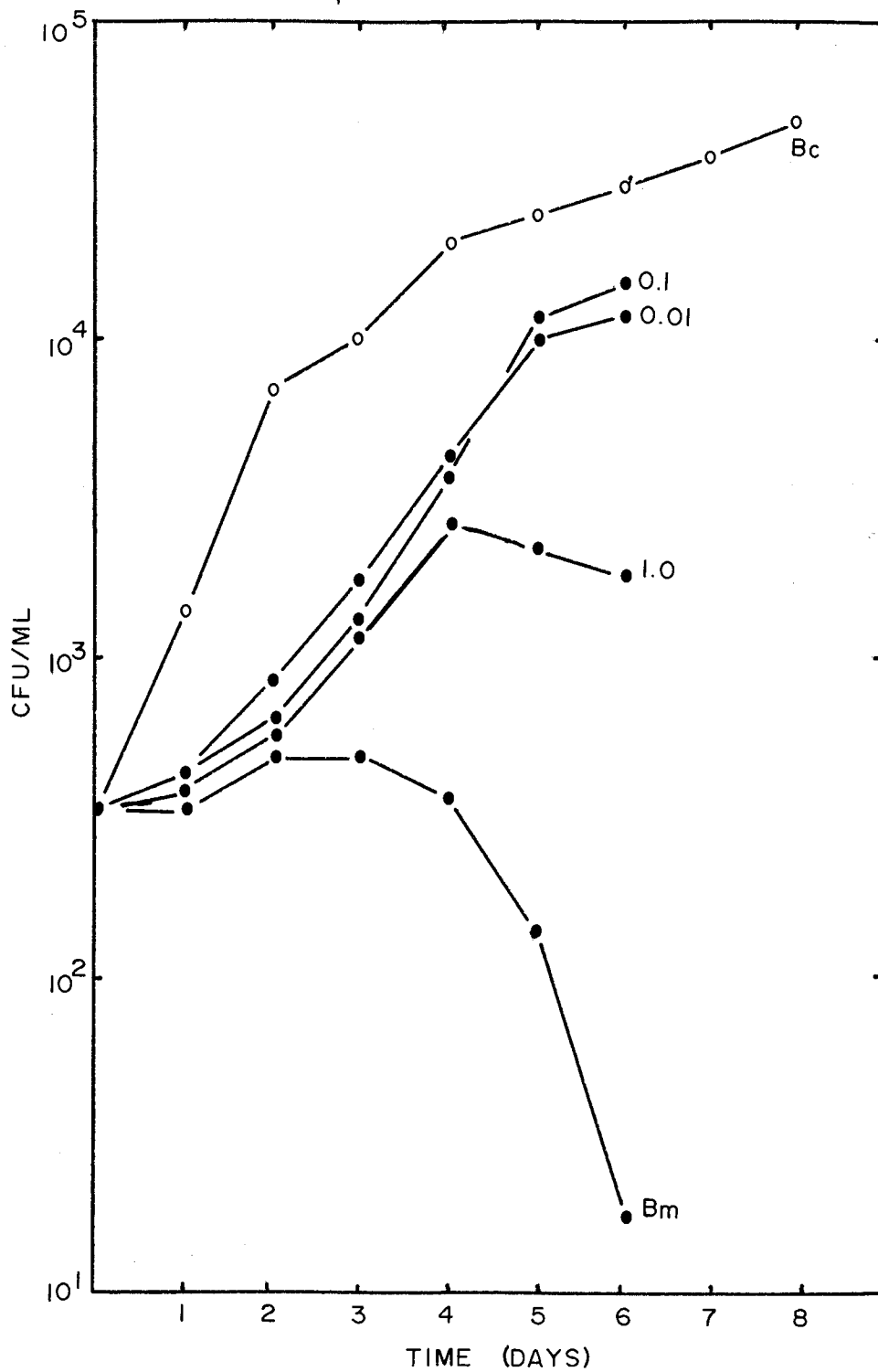
TABLE VI-1

Efficiencies of Plating of Some Auxotrophic Mutants of Eudorina elegans

(c.f. Appendix II)

| Media | Efficiencies of Plating (%) | | | |
|--------------------------------------|-----------------------------|-----|-----|-----|
| | M-1 | M-2 | M-3 | M-6 |
| <u>Bc</u> | 100 | 100 | 100 | 100 |
| <u>Bm</u> | 0 | 0 | 5 | 1 |
| <u>Vm</u> | 200 | 170 | -- | -- |
| <u>Bm</u> + Yeast extract (2 g/l) | 200 | 260 | 0 | -- |
| <u>Bm</u> + Yeast extract (0.4 g/l) | 100 | 130 | 40 | 25 |
| <u>Bm</u> + Beef extract (0.2 g/l) | 100 | 130 | 20 | 0 |
| <u>Bm</u> + Casamino acids (2.5 g/l) | 0 | 0 | 1.5 | 0 |
| <u>Bm</u> + Casamino acids (0.4 g/l) | 0 | 0 | 1.0 | -- |
| <u>Bm</u> + Nucleic acids | 0 | 0 | -- | -- |
| <u>Bm</u> + Nicotinamide (0.75 mg/l) | 170 | 170 | -- | -- |
| <u>Bm</u> + Sodium acetate (1.0 g/l) | 0 | 0 | -- | -- |
| <u>Bm</u> + Sodium acetate (0.2 g/l) | 0 | 0 | 130 | 120 |

Figure VI-1. Effect of nicotinamide on the growth of M-1. A culture resuspended in Bm for 24 hours was inoculated into Bm, Bm supplemented with nicotinamide and Bc. The concentrations of nicotinamide are given in $\mu\text{g/ml}$.



B. Acetate-Requiring, Morphological Defective Mutant

M-6, isolated from UV-treated parental strain 1192, produces large clumps in liquid culture. This clumping is caused by the failure of the daughter coenobia to separate from the parental coenobial envelope when the daughter coenobia reach the 16- or 32-celled stage. The parental coenobial envelope does break down at what appears to be a time when the mass of the clumps becomes too great.

This mutant has an acetate requirement, similar to M-3 (Table VI-1; Appendix II). However it has not been characterized by growth in liquid media, since the growth habit is so irregular.

Spotting or streaking of M-6 on plates, yielded large and small colonies. When either a large or a small colony was streaked, both types of colonies were formed. The ability of the large and small colonies to give rise to both types of colonies indicates that this character is stable and genetic in origin, rather than a temporary phenotype caused by a cytoplasmic alteration.

C. Acetate-Requiring, Pigment Deficient Mutant

M-3 was isolated from ultra-violet light treated wild-type parental strain 1192. A comparison of growth of M-3 in liquid minimal medium supplemented with complex organic

compounds (Figure VI-2) indicates that the requirement is a vitamin. However consideration of the efficiency of plating data presented in Table VI-1 (Appendix II) shows, while there is some growth supported by yeast extract (approximately 40% of that supported by Bc), growth is dependent on the presence of an acetate source. The fact that there is very limited growth in Bm supplemented with sodium acetate (1.0 mg/ml) liquid and solid media is due either to concentration or pH inhibition. Growth in yeast extract indicates either that there is some acetate in the yeast extract or that the organism is capable of using some other component of yeast extract as a carbon source. The former reason satisfies the fact since the amino acids in casamino acids would be expected to be used as carbon sources, and therefore would support growth if many carbon compounds could satisfy the growth requirement. The growth which does occur on casamino acids can be attributed to the fact that the mutation does not totally inactivate the function and sometimes there is some growth (i.e. "leaky" mutant).

It is significant that this mutant is defective in its ability to synthesize chlorophyll (Table VI-2) to the extent that the coenobia contain only one-half the amount of chlorophyll that the wild-type organism does.

Figure VI-2. Effect of organic supplements on the growth of M-3. A culture incubated in Bm for 24 hours was inoculated into the test media and growth was followed by plating on Bc.

- Bm; ● Bm + sodium acetate (1 g/l);
- ▼ Bm + casamino acids (2.5 g/l); ■ Bm
+ nucleic acids; ▼ Bm + casamino acids
(2.5 g/l) + yeast extract (2.0 g/l); × Bm
+ yeast extract (2.0 g/l); ○ Bc.

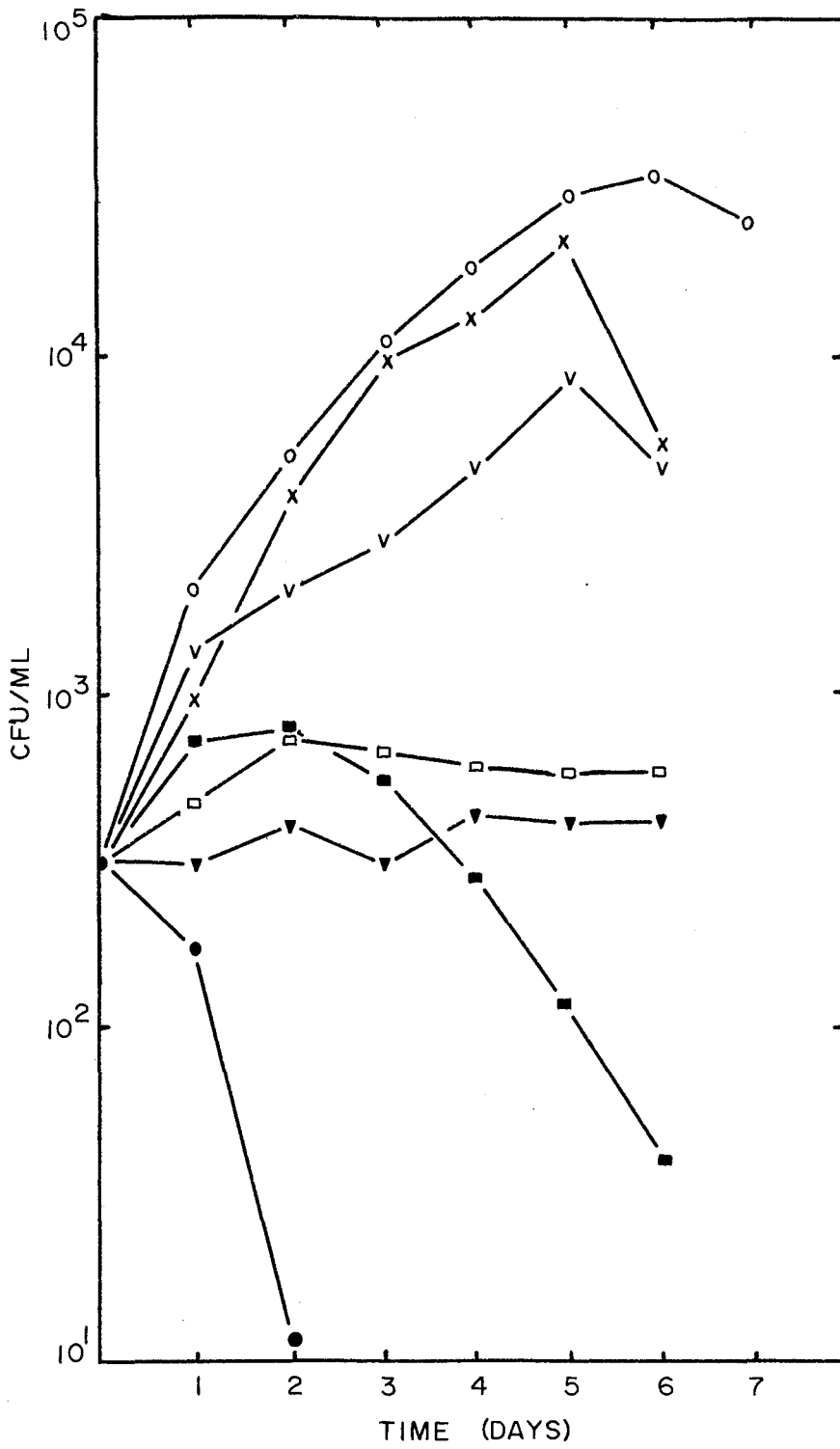


TABLE VI-2
Analysis of the Absorption of Acetone Extracts of Eudorina elegans (c.f. Appendix III)

| Organism | C _a (g/l) | C _b (g/l) | Total Chlorophyll g/l | Ca/Cb | cfu/ml | Chlorophyll g per cfu |
|----------|-----------------------|-----------------------|-----------------------|-------|---------------------|------------------------|
| 1192 | 5.58x10 ⁻³ | 2.24x10 ⁻³ | 7.82x10 ⁻³ | 2.49 | 2.3x10 ⁴ | 3.36x10 ⁻¹⁰ |
| 1193 | 6.95x10 ⁻³ | 2.76x10 ⁻³ | 9.71x10 ⁻³ | 2.52 | 3.9x10 ⁴ | 2.47x10 ⁻¹⁰ |
| M-3 | 3.48x10 ⁻³ | 1.13x10 ⁻³ | 4.61x10 ⁻³ | 3.08 | 2.8x10 ⁴ | 1.63x10 ⁻¹⁰ |
| M-56 | 3.36x10 ⁻⁴ | 1.03x10 ⁻⁴ | 4.39x10 ⁻⁴ | 3.24 | 3.3x10 ⁴ | 1.33x10 ⁻¹¹ |

D. Auxotrophic, Pigment Deficient Mutant

Following an initial lag of 24 hours of the growth of M-56 in Bc growth occurred at a rate equivalent to that of the parental strain 1192 (Figure VI-3). The final coenobial concentration was approximately one-half that of the wild-type. There was little growth of M-56 in Bm; however Kemp (personal communication) has found that this mutant will grow well in Beijerinck's minimal medium (Gowans, 1956) in which ammonium nitrate replaces the sodium nitrate of Bm as the nitrogen source. This suggests that M-56 requires a reduced nitrogen source for growth. No other growth studies have been completed.

Figure VI-4 shows the absorption spectra of suspensions of equal coenobial concentrations (cfu/ml) of log phase cultures of M-56 and of the parental strain. The spectrum of the mutant was depressed at all wavelengths. A comparison of the chlorophylls present (Table VI-2; Appendix III) indicated that both chlorophylls present in the mutant are reduced to about one-twentieth of that in the wild-type organism.

All mutants which have two phenotype changes (see B, C and D above) could have mutations in two loci or a single mutation influencing two pathways. Further studies will be necessary to supply a satisfactory answer to this question.

Figure VI-3. Growth of E. elegans 1192 and M-56 in Bc.

x strain 1192; o M-56.

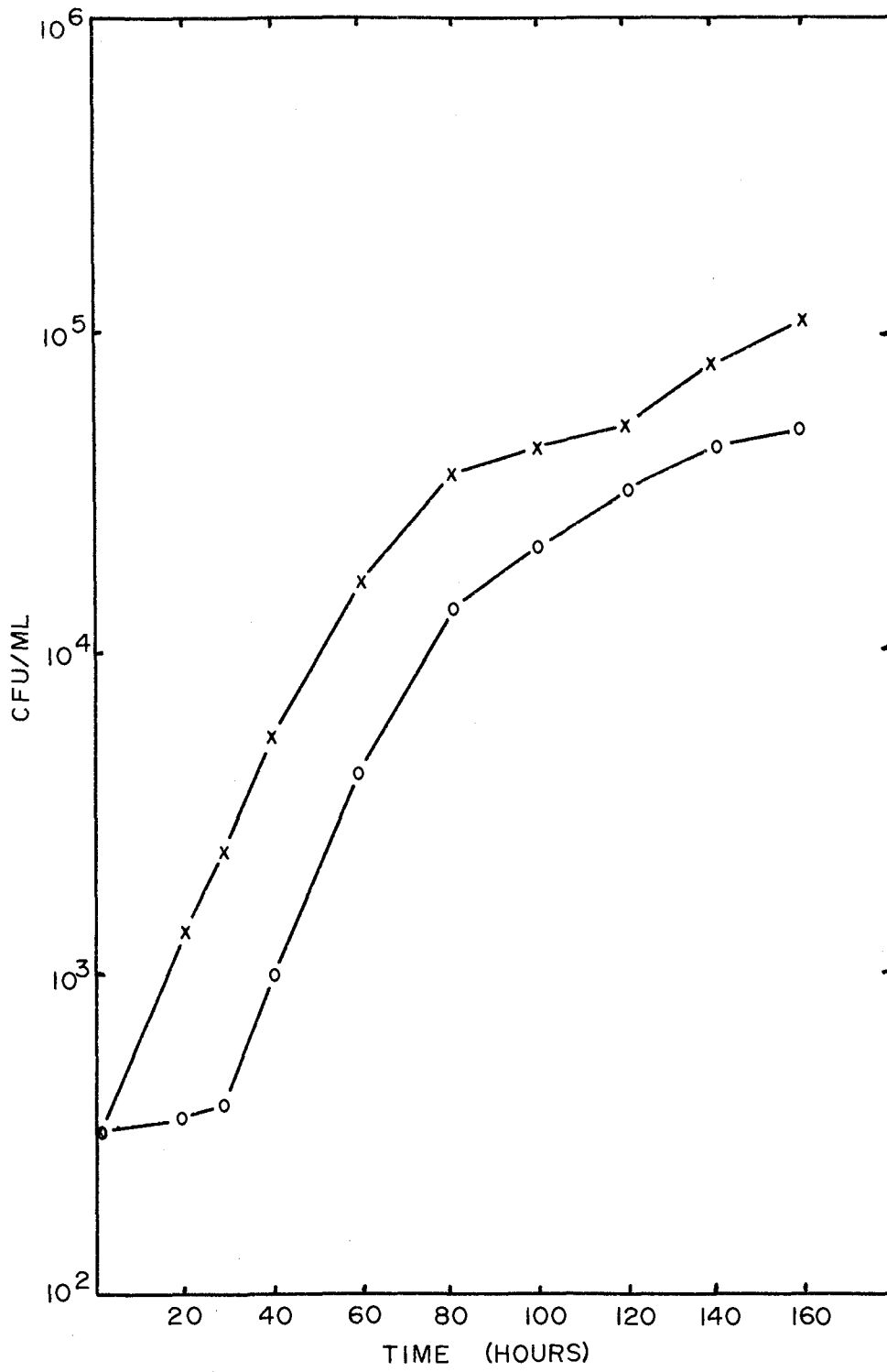
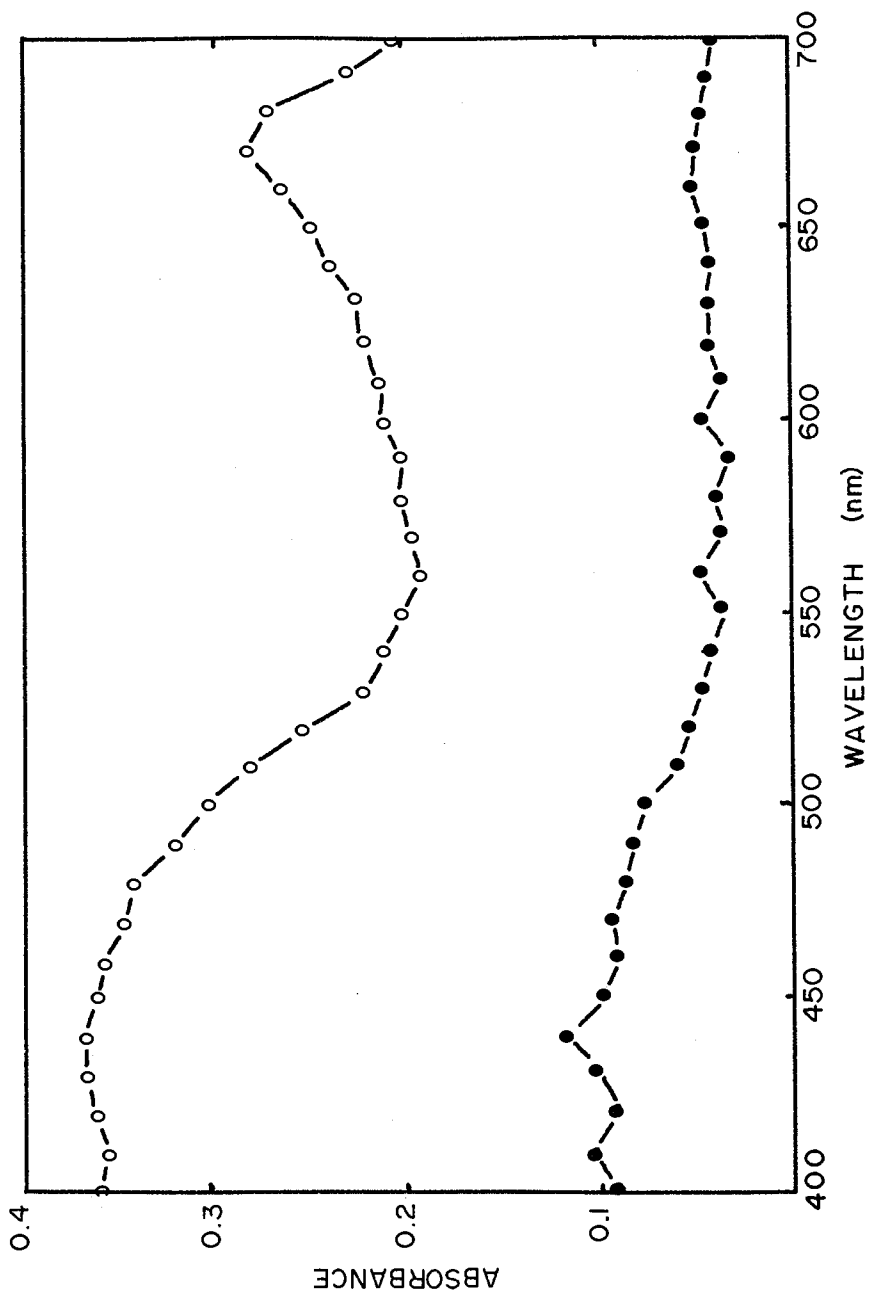


Figure VI-4. Absorption spectra of cultures containing approximately the same number of coenobia of E. elegans 1192 and M-56.

○ 1192; ● M-56.



E. Other Mutants

Several mutants had been characterised to the point of determination that they were vitamin-requiring auxotrophs when lost by failure of laboratory equipment or by reversion to wild-type.

DISCUSSION

This investigation revealed that auxotrophic, morphological-defective and pigment-deficient mutants of Eudorina elegans can be induced and isolated. However it is possible that techniques different from those used here will be required before mutational and genetic studies will be advanced to a meaningful stage.

First, the use of a technique for transfer of a large number of colonies will have to be developed. The replica-plating technique of Lederberg and Lederberg (1952) for colony transfer is unsatisfactory for use with Eudorina elegans since the quantity of nutrient carried over by the velveteen from complete to minimal plates is sufficient to allow growth of any auxotrophs into visible colonies. Also the technique of picking presumptive mutants into minimal medium, and depriving them of any exogenous compound before testing is limited by the time and patience of the researcher. It is possible that the technique of Roberts (1959), developed for filamentous

fungi, using a pin-board in place of velveteen, can be used so that there is only a transfer of colonies and only that exogenous material on the envelope of the coenobia. It also seems possible that the method used by Lewin (1952) with Chlamydomonas moewusii to identify the few mutant colonies among a large population of non-mutant colonies, can be adapted for use with Eudorina elegans. Chlamydomonas moewusii will grow in the presence of low concentrations of bromothymol blue or neutral red, remaining its dark green color. However dying cells, in the presence of either of these compounds accumulate the dye, allowing visual determination of dying colonies. If wild-type colonies of Eudorina elegans will grow on Bm containing the dye, but mutants die on the same medium, a technique using this observation for isolation of auxotrophs could be developed.

The growth characteristics of M-3 on Bm and Bm-supplemented media suggest that UVL-induced mutants are not as easily characterised as are those induced by NTG. This could mean that NTG is much more effective in mutation induction than is the UVL, or is harsher causing chromosomal damage, and the use of NTG or other chemicals will be required in place of UVL.

The third alteration in this study might be in the direction of nutrition for growth of mutagen-treated coenobia and

for mutant characterisation. It is highly probable that the failure to find other auxotrophic mutants similar to those obtained in Chlamydomonas studies (Eversole, 1956; Gowans, 1956) is caused by the selection against mutants during the initial growth of the mutagen-treated coenobia, by components of the growth medium. Such a situation has been found in studies of arginine-requiring mutants of Chlamydomonas reinhardi (Loppes, 1966b, 1968, 1969a) and for histidine-requiring mutants of Neurospora crassa (Haas et al., 1952).

The concentration of the individual supplements to Bm (e.g. nicotinamide), even though supporting growth of wild-type, may be too high to support growth of some mutants. During characterisation of a mutant this concentration inhibition of growth could be misinterpreted as a lack of the necessary requirement for growth and therefore yield confusing results.

CHAPTER VII

GENERAL DISCUSSION

At the outset of this investigation, the question was asked concerning the use of Eudorina elegans as a tool to study problems of physiology, morphogenesis and cellular differentiation, and the genetic control of these processes. The question has a very broad scope because such a study is actually attempting to determine how an organism becomes an organism, or, at least, how some essential structure or process of an organism is genetically determined.

The preparation for such a study requires a great deal of basic research before significant advancement can be made in the desired field, or even before an evaluation of the suitability of the organism as a tool to study it can be made.

The processes of cytokinesis and nuclear division of Eudorina elegans have been described in great detail by Goldstein (1964) and Mishra (1967). Also the ultrastructure has been studied by Lang (1963). These investigations, along with that of Rayns and Godward (1965) who present the growth and mitotic characteristics, describe the characteristics of the wild-type organism which are essential to the organism. However to study the processes giving rise to these characteristics stable genetic variants must be obtained.

This study shows that Eudorina can be handled quantitatively, that mutants of Eudorina can be obtained and characterised and that the processes involved in repair of damage induced by ultra-violet light may be different from repair processes under study in other organisms. More specifically the following results were obtained which with the literature results make further studies in the directions indicated possible. The growth characteristics of Eudorina on solid and in liquid minimal, minimal-supplemented and complete media were determined. Inactivation of the wild-type organism by NTG, a mutagen, and EMS, a potential mutagen, of Eudorina occurs very rapidly. From the inactivation responses of Eudorina to the drugs streptomycin, 8-azaguanine, penicillin and sulfanilamide it appears that these agents have little potential for mutant enrichment. It was found, by considering the response of Eudorina to UVL that the response of this strain of Eudorina is different from results published on the UV-sensitivity of another strain, that whereas there is little dark repair of irradiation-induced-lesions, photo-repair restores most UV-damage to a high total dose. Following treatment of coenobia with UVL or NTG auxotrophic, auxotrophic-pigment deficient and auxotrophic-morphological defective mutants were obtained. These mutants were characterised to the level of individual

requirements.

In order to facilitate further work, problems arising in handling this organism have been outlined and possible solutions to them are included in the various sections. These are repeated here.

First, the growth habit of the wild-type organism must be studied more fully at the level of supplements which will be added to the minimal medium to support growth of most auxotrophic mutants. This will probably require a drastic reduction in the concentration of complex supplements (e.g. casamino acids and yeast extract) and of the individual supplements (e.g. nicotinamide).

The UV-induced mutant which has been characterised to the level of a single requirement is leaky, and as such was difficult to characterise. At the other end of the spectrum are those mutants induced by NTG; these mutants have absolute requirements. It is possible that the UV-induced mutants are the result of a single base-pair alterations, whereas the non-reverting stable character of the NTG-induced mutants suggests greater genetic damage. Mutants required for further study must have an easily characterised phenotype, resulting from a mutation which is relatively stable and not of gross deficiencies. While some mutants induced by NTG may result from gross deficiencies, it is unlikely that many will be

caused by such a mechanism. Therefore it is suggested that NTG replace UVL as the mutagen.

Since the proportion of mutants to wild-type coenobia in a treated culture is very small, studies must be made on finding drugs which will inactivate the wild-type coenobia and thus increase the mutant percentage. Also a technique must be found whereby the colonies arising from mutagen-treated coenobia are rapidly and efficiently tested for auxotrophic or other deficiencies.

A study of the genetics of this organism will require studies on the requirements of the sexuality system. To the present, zygotes have not been obtained in several attempts; however the literature contains reports in which inheritance has been studied (Iyengar, 1937; Goldstein, 1964; Mishra, 1967). So it is apparent that this failure is caused by the laboratory conditions and not by the lack of a sexuality system in the organism.

The question might now be asked regarding the direction studies should take after the problems outlined above have been overcome or circumvented.

If a study of the genetics of the developmental sequence (e.g. cell division) is to be undertaken, it will be necessary to use mutants of a type known as conditional lethal. This requirement arises from the fact that such a process is essen-

tial for the viability and growth of the organism, and cannot be restored in a mutant by an addition to the medium. One type of conditional lethal mutant of interest for the study of Eudorina elegans is the temperature-sensitive mutant which will grow only over a restricted region of the temperature range over which the wild-type organism will grow. Temperature-sensitive mutants have been studied to study the essential functions of many organisms. Some of these are E. coli (e.g. DNA synthesis and cell division -- Fangman and Novick, 1968; Hirota et al., 1968), bacteriophage T₄ (e.g. DNA synthesis, head and tail protein synthesis -- Epstein et al., 1963) and mouse L-cells (nucleic acid synthesis or nucleotide metabolism -- Thompson et al., 1970).

There are other types of conditional lethal mutants which are available. Some of these are pH-sensitive (Weaver, 1952) and genome-sensitive (Gorini and Beckwith, 1966).

Therefore the first step in further study of essential functions must be the isolation of conditional-lethal mutants.

Many conditional lethal mutants are likely to require low molecular weight compounds which can be added as supplements to the medium. The added usefulness of these types of mutants will be for studying the formal genetics of Eudorina elegans.

Mutants defective in the essential functions can then be

studied more thoroughly, namely in the elucidation of the characteristic affected and the inheritance pattern. This type of study would require knowledge of the time of gene action within the growth cycle, as studied with Drosophila (Suzuki and Procunier, 1969). This would require chemical (when one macromolecular function is inhibited) and morphological (the time of expression of the defect) investigations. In the study of the growth cycle of mutants, although not totally necessary but very helpful, would be the use of synchronous cultures. This will perhaps be difficult to obtain, but Rayns and Godward (1965) were able to show a partial mitotic synchrony two hours prior to the dark period initiation. Synchrony of cultures of other algae have been obtained by altering the light-dark cycle (e.g. Chlamydomonas, Bernstein, 1960), by additions to the media (Trainor and Rowland, 1968), and by a temperature shift in continuous light (Lorenzen and Venkataraman, 1969). Following from this, the sequence and regulation of gene action in development and differentiation could be determined.

While this statement is very superficial it must necessarily be so since the whole study will be determined by the type of mutants isolated.

A second direction the study of Eudorina elegans might take is that into the repair mechanism of radiation damage.

While much work is at present being done in other organisms on repair of radiation damage, these studies indicate that repair occurs by essentially the same mechanism. However, the results presented here suggest that there is very little, if any, dark repair, and that there is a very efficient photo-repair. The question concerning the type of repair occurring in Eudorina elegans can be approached from the response of the organism to UVL and drugs, and the isolation of mutants, but it will ultimately require chemical studies similar to those done by Setlow and Carrier (1964).

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Appendix I

Data for Efficiencies of Plating of Eudorina elegans 1192 and 1193

A. Efficiencies of plating of Eudorina elegans 1192 on Bc, Bm⁺ supplemented media (Table II-3), and Vm.

| Media | Colonies/plate | Dilution | cfu/ml x 10 ⁻³ | % |
|--|----------------|--------------------|------------------------------|-----|
| <u>Bc</u> | 36, 40 | .2/10 ² | 19 | 100 |
| <u>Bm</u> | 27, 26 | .2/10 ² | 13 | 70 |
| <u>Bc</u> | 63, 65 | .1/10 ¹ | 6.4 | 100 |
| <u>Bm⁺</u> Yeast extract (2.0 g/l) | 135, 148 | .1/10 ¹ | 14 | 220 |
| <u>Bm⁺</u> Casamino acids (2.5 g/l) | 40, 39 | .1/10 ¹ | 4.0 | 60 |
| <u>Bm⁺</u> Sodium acetate (1.0 g/l) | 38, 30 | .1/10 ¹ | 3.4 | 50 |
| <u>Bc</u> | 86, 108 | .2/10 ¹ | 5.2 | 100 |
| | 106, 109 | .2/10 ¹ | | |
| <u>Vm</u> | 135, 170 | .2/10 ¹ | 7.4 | 140 |
| | 119, 165 | .2/10 ¹ | | |
| <u>Bc</u> | 180, 196 | .2/10 ¹ | 9.4 | 100 |
| <u>Bm⁺</u> Nucleic acids | 184, 154 | .2/10 ¹ | 8.5 | 90 |

Appendix I (cont'd)

B. Efficiency of plating on Eudorina elegans 1192 (1) and 1193 (2) on Bc, Bm supplements where supplements are at the concentration of additions to Bc (Table II-2).

| Media | Colonies/plate | Dilution | cfu/ml x 10 ⁻³ | % |
|---|----------------|--------------------|------------------------------|-----|
| 1. <u>Bc</u> | 203, 175 | .2/10 ² | 95 | 100 |
| <u>Bm</u> | 165, 146 | .2/10 ² | 78 | 80 |
| <u>Bm</u> ⁺ Yeast extract (0.4 g/l) | 173, 146 | .2/10 ² | 80 | 80 |
| <u>Bm</u> ⁺ Beef extract (0.2 g/l) | 218, 142 | .2/10 ² | 90 | 100 |
| <u>Bm</u> ⁺ Casamino acids (0.4 g/l) | 132, 175 | .2/10 ² | 77 | 80 |
| <u>Bm</u> ⁺ Sodium acetate (0.2 g/l) | 163, 143 | .2/10 ² | 77 | 80 |
| <hr/> | | | | |
| 2. <u>Bc</u> | 20, 22 | .2/10 ² | 11 | 100 |
| <u>Bm</u> | 20, 24 | .2/10 ² | 11 | 100 |
| <u>Bm</u> ⁺ Yeast extract (0.4 g/l) | 19, 36 | .2/10 ² | 13 | 120 |
| <u>Bm</u> ⁺ Beef extract (0.2 g/l) | 37, 35 | .2/10 ² | 18 | 160 |
| <u>Bm</u> ⁺ Casamino acids (0.4 g/l) | 23, 30 | .2/10 ² | 13 | 120 |
| <u>Bm</u> ⁺ Sodium acetate (0.2 g/l) | 19, 24 | .2/10 ² | 11 | 100 |

Appendix II

Data for Efficiencies of Plating of Some Auxotrophic Mutants of

Eudorina elegans.

| Strain | Media | Dilution | cfu/plate | cfu/ml x 10 ⁻³ | Efficiency of plating |
|--------|--|--------------------|-----------|------------------------------|--------------------------|
| M -1 | <u>Bc</u> | .2/10 ¹ | 119, 119 | 6.0 | 100% |
| | <u>Vm</u> | .2/10 ¹ | 249, 261 | 13 | 200% |
| | <u>Bm</u> + Yeast extract (2.0 g/l) | .1/10 ¹ | 200, 195 | 20 | 200% |
| | <u>Bm</u> + Nicotinamide (0.75 mg/l) | .1/10 ¹ | 164, 169 | 17 | 170% |
| | <u>Vm</u> | .1/10 ¹ | 199, 191 | 20 | 200% |
| | <u>Bc</u> | .2/10 ² | 84, 95 | 45 | 100% |
| | <u>Bm</u> + Yeast extract (0.4 g/l) | .2/10 ² | 104, 83 | 47 | 100% |
| | <u>Bm</u> + Beef extract (0.2 g/l) | .2/10 ² | 95, 92 | 47 | 100% |

In none of the above experiments did M-1 plate on Bm or on Bm supplemented with sodium acetate (0.2 g/l and 1.0 g/l), casamino acids (0.4 g/l and 2.5 g/l) or nucleic acids.

Appendix II (cont'd)

| Strain | Media | Dilution | cfu/plate | cfu/ml x 10 ⁻³ | Efficiency of plating |
|--------|---|--------------------|-----------|------------------------------|--------------------------|
| M-2 | <u>Bc</u> | .2/10 ¹ | 102, 110 | 5.3 | 100% |
| | <u>Vm</u> | .2/10 ¹ | 132, 122 | 7.4 | 170% |
| | <u>Bm</u> + Yeast extract (2.0 g/l) | .3/10 ² | 203, 230 | 72 | 260% |
| | <u>Bm</u> + Nicotinamide 0.75 mg/l) | .3/10 ² | 145, 117 | 44 | 170% |
| | <u>Vm</u> | .3/10 ² | 159, 113 | 45 | 170% |
| | <u>Bc</u> | .2/10 ² | 29, 24 | 13 | 100% |
| | <u>Bm</u> + Yeast extract (0.4 g/l) | .2/10 ² | 38, 29 | 17 | 130% |
| | <u>Bm</u> + Beef extract (0.2 g/l) | .2/10 ² | 32, 36 | 17 | 130% |

In none of the above experiments did M-2 plate on Bm or on Bm supplemented with sodium acetate (0.2 g/l and 1.0 g/l), casamino acids (0.4 g/l and 2.5 g/l) or nucleic acids.

Appendix II (cont'd)

| Strain | Media | Dilution | cfu/plate | cfu/ml x 10 ⁻³ | Efficiency of plating |
|--------|--|--------------------|-----------|------------------------------|--------------------------|
| M-3 | <u>Bc</u> | .2/10 ² | 67, 65 | 33 | 100% |
| | <u>Bm</u> | .2/10 ² | 5, 1 | 1.5 | 5% |
| | <u>Bm</u> + Yeast extract (.4 g/1) | .2/10 ² | 32, 25 | 14 | 40% |
| | <u>Bm</u> + Beef extract (.2 g/1) | .2/10 ² | 20, 12 | 8 | 20% |
| | <u>Bm</u> + Sodium acetate (.2 g/1) | .2/10 ² | 76, 89 | 41 | 125% |
| | <u>Bm</u> + Casamino acids (.4 g/1) | .2/10 ² | 1, 1 | 0.5 | 1% |
| | <u>Bc</u> | .3/10 ² | 65, 54 | 20 | 100% |
| | <u>Bm</u> | .3/10 ² | 3, 2 | 0.8 | 4% |
| | <u>Bm</u> + Yeast extract (2.0 g/1) | .3/10 ² | 0, 0 | 0 | 0% |
| | <u>Bm</u> + Casamino acids (2.5 g/1) | .3/10 ² | 1, 1 | 0.3 | 1.5% |
| M-6 | <u>Bc</u> | .2/10 ² | 146, 129 | 69 | 100% |
| | <u>Bm</u> | .2/10 ² | 0, 1 | 0.3 | 0.4% |
| | <u>Bm</u> + Yeast extract (.4 g/1) | .2/10 ² | 15, 55 | 18 | 25% |
| | <u>Bm</u> + Beef extract (.2 g/1) | .2/10 ² | 0, 0 | 0 | 0% |
| | <u>Bm</u> + Sodium acetate (.2 g/1) | .2/10 ² | 186, 154 | 83 | 120% |
| | <u>Bm</u> + Casamino acids (.4 g/1) | .2/10 ² | 0, 0 | 0 | 0% |

APPENDIX III

Determination of Chlorophyll Concentrations

(From Arnon, 1949):

"The procedure for chlorophyll determination was based on the work of MacKinney (1941) on the absorption of light by aqueous acetone (80%) extracts of chlorophyll. The concentrations of chlorophyll a and b were determined by measuring in a 10 mm cell, the density of 80% acetone chlorophyll extracts with a Beckman spectrophotometer at 663 and 645 $m\mu$ and setting up simultaneous equations using the specific absorption coefficients for chlorophyll a and b as given by MacKinney (1941).

$$(i) \quad D_{663} = 82.04 C_a + 9.27 C_b$$

$$(ii) \quad D_{645} = 16.75 C_a + 45.6 C_b$$

where C_a , C_b are grams per liter of chlorophyll a and b respectively, and D the density values at the respective wavelengths as obtained on the Beckman spectrophotometer.

From equation (ii):

$$(iii) \quad C_a = \frac{D_{645} - 45.6 C_b}{16.75}, \text{ substituting in (i) and}$$

solving for C_b we obtain

$$(iv) \quad C_b = 0.0229 D_{645} - 0.00468 D_{663}; \text{ substituting this expression for } C_b \text{ in (ii) and solving for } C_a:$$

$$(v) \quad C_a = 0.0127 D_{663} - 0.00269 D_{645}$$

Hence total chlorophyll, C, in grams per liter:

$$(iv) \quad C = C_a + C_b = 0.0202 D_{645} + 0.00802 D_{663} \text{ or}$$

expressing total chlorophyll, C, in terms of milligrams per liter:

$$(vii) \quad C = 20.2 D_{645} + 8.02 D_{663}$$

To provide a rough check for the determination of total chlorophyll the light absorption data for chlorophyll a and b (MacKinney, 1949) were plotted and the curves were found to intersect at $\lambda = 652 \text{ m}\mu$. By extrapolation, the value of the specific absorption coefficient for this wavelength was found to be 34.5 . Another equation was therefore set up for total chlorophyll:

$$(viii) \quad D_{652} = 34.5 C_a + 34.5 C_b = 34.5 (C_a + C_b)$$

$$\text{hence (ix) } C = (C_a + C_b) = \frac{D_{652}}{34.5}$$

or expressed as mg per liter:

$$(x) \quad C = \frac{D_{652} \times 1000}{34.5} \dots \dots \dots "$$

The following data were obtained on

Analysis of the Absorption of Acetone Extract of

Eudorina elegans

(c.f. Table VI-2)

| Strain | D ₆₄₅ | D ₆₅₂ | D ₆₆₃ |
|--------|------------------|------------------|------------------|
| 1192 | 0.196 | 0.285 | 0.481 |
| 1193 | 0.243 | 0.355 | 0.599 |
| M-3 | 0.108 | 0.165 | 0.288 |
| M-56 | 0.031 | 0.049 | 0.090 |

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