# RECOVERY FROM CHEMICAL- AND ULTRAVIOLET LIGHT-INDUCED POTENTIALLY LETHAL DAMAGE IN *EUDORINA ELEGANS*

by

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ABSTRACT

The colonial green alga *Eudorina elegans* was used in a study of cellular repair processes for damage due to various inactivating agents. Reactivation was estimated by survival and growth of the organisms following the inactivating treatment with ultraviolet light (UVL) and/or drugs. Quantitative and qualitative measurements of repair mechanisms were made by post-UVL treatment with certain drugs whose effects in other systems had been shown to include interference with particular DNA repair processes. Those most extensively used here in this capacity were acridine orange and caffeine. Acridine orange had no effect on dark (non-photoreactivated, or NPR) survival, but interfered markedly with photoreactivation (PR); however, this latter effect was found to be at least partially due to absorption of the PR light wavelengths by acridine orange. Caffeine reduced both PR and NPR survival; NPR survival was reduced slightly at all UVL dose levels, while PR survival was affected only at higher UVL doses.

For an estimation of both the specificity of drugs interfering with repair processes, and the versatility of repair capability in *E. elegans*, other inactivating agents were used in place of UVL. Acridine orange interfered with survival following treatments both with ethyl methanesulfonate and with 4-nitroquinoline-1-oxide; caffeine caused only a slight reduction in survival following either drug.

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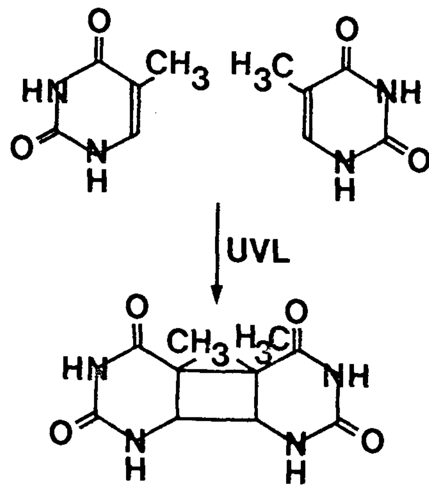
## CHAPTER I

### INTRODUCTION - GENERAL

Ultraviolet light (UVL) has long been used as both a lethal and a mutagenic agent for a great variety of organisms. Its range of effects is fairly wide (Jagger, 1958), causing aberrations in many cellular processes, both nuclear and cytoplasmic. There are many possible targets in the cell for UVL irradiation; protein, RNA and DNA all absorb strongly in the UV range, and thus may be expected to be affected by such radiation. However, the most significant damage (in terms of survival of the organism) is that done to the DNA, as it is the site of the controlling mechanism of cellular processes.

Within the DNA molecule there are several types of aberrations caused by the high-energy photons of UVL (Setlow, 1966 b); these include DNA chain breaks, denaturation, nucleic acid-protein cross-links, hydrates of cytosine and uracil, thymine-uracil heteroadducts (Ikenage *et al.*, 1970) and pyrimidine dimers ($\widehat{TT}$, $\widehat{CT}$ and $\widehat{CC}$). So far, biological importance has been attached almost entirely to pyrimidine dimers, particularly of the thymine-thymine type (Setlow *et al.*, 1965; Setlow, 1966 b). These are formed by the hydration of the 5,6 double bond in the pyrimidine rings of two adjacent thymine molecules, with subsequent formation of new covalent bonds between them (Fig. I-1). This new structure in the DNA will cause disruption and perhpas prevention of DNA synthesis during the next *S* phase in the cell cycle - hence its observed effects of mutation and lethality (Setlow, 1966 b). Thymine dimers have been shown to be produced by UVL

Figure I-1. Thymine dimerization between two adjacent thymines. Dimerization produces two new covalent bonds, with consequent loss of the 5,6 double bond in the pyrimidine ring (after Jagger, 1964).


O
HN
CH3
O
N
H
H3C
O
NH
N
H
O
UVL
O
HN
CH3
H3C
O
NH
O
N
H
N
H
O

irradiation in *Eudorina elegans* (Kemp *et al.*, in prep.), but their biological importance has not yet been shown in this organism. However, for the purposes of this study it will be assumed that the most important UVL-induced DNA damage in *E. elegans* is, in common with other organisms studied, the thymine dimer.

A cell suffering the effects of UVL damage is not neccessarily doomed, as most cells appear to possess some mechanism for the repair of this damage before synthesis begins. Probably many modes of repair have evolved, but so far only two main classes of repair have been found. Both involve the elimination of thymine dimers from DNA, and both are enzymatic; one requires visible light as a physical co-factor, and the other does not. The latter is generally called, simply, dark repair, and the former, photoreactivation (PR). Dark repair is the longer of the two processes, which in bacteria appears to involve four steps (Setlow, 1967; Witkin, 1969; Grigg, 1970):

(a) recognition of the dimer and "nicking" (i.e. producing a single strand break in) the DNA strand on either side of it, releasing a single-stranded oligonucleotide;

(b) depolymerization of the damaged DNA strand 40 to 50 nucleotides to either side of the lesion;

(c) synthesis of a new segment to replace the missing one, using the intact strand as a template; and

(d) joining the ends of the new segment to the original DNA strand.

This mode of repair, as can be seen, involves enough DNA synthesis so as to be detectable by the use of radioactive tracers, and is often called

"unscheduledDNA synthesis", or "repair replication" to distinguish it from the synthesis observed during _S_ phase in the normal cell cycle (Cleaver, 1969; Rauth, 1970).

The mechanism for PR appears much simpler, and thus is much more efficient than dark repair. The PR enzyme, being itself a chromophore (cf. Jagger, 1958) or using energy transferred from another chromophore is activated by light in the range of 300 to 450 nm (Jagger, 1958). In its activated form, it recognizes pyrimidine dimers and splits them _in situ_. The variation from organism to organism in the peak wavelength for PR suggests several different types of PR; in fact, even within one organism there may be several types, as in _Streptomyces griseus_ (Jagger _et al._, 1970).

Both of these repair mechanisms depend, for the amount of damage each can repair, on the length of time between UVL irradiation and the onset of the next synthesis phase in the cell cycle. Once DNA synthesis has occurred, any damage still remaining is apparently "fixed", and no longer repairable. Thus the observed UVL-induced lag in growth (Roberts and Aldous, 1949) is to the advantage of the organism, for it allows some time for repair to take place, thus reducing mutation and lethality. Any treatment which increases the length of this lag period, then, will increase the amount of repair possible. This is the principle upon which are based the phenomena known as photoprotection and liquid holding recovery. The former is accomplished by irradiating organisms with light of 300 to 450 nm prior to UVL irradiation; this results, as observed mainly in bacteria (Jagger, 1964; Jagger _et al._, 1964; Setlow, 1966 a) and fungi (Jagger, 1964), in an increased growth lag following UVL irradiation; consequently more dark repair can take place,

and increased survival and reduced mutation rates are observed. (This mechanism seems also to account for the process known as indirect photoreactivation, which has been observed in nonphotoreactivable (phr$^-$) strains of E. coli (Jagger et al., 1970) following post-UVL irradiation with light of 310 to 380 nm wavelength.) Liquid holding recovery (Alper and Gillies, 1958; first reported by Roberts and Aldous, 1949) involves lengthening the growth lag by post-UVL holding in a non-nutrient solution (such as a phosphate buffer), again resulting in greater survival and lower mutation rates than observed with immediate transfer to growth-sustaining media.

Many organisms possess both dark repair and PR; all organisms so far studied, with the exception of certain UV-sensitive bacterial and fungal mutants (Hill, 1958; Elder and Beers, 1965; Chang and Tuveson, 1967; Nishoka and Doudney, 1969, etc.) appear to be able to undergo repair replication, or dark repair. Many mammalian cell systems have been reported deficient in PR ability (Trosko et al., 1965; Cleaver, 1969; Rauth, 1970); only recently was the PR of UVL-induced DNA lesions reported for a mammalian cell system (Shaeffer et al, 1971). Thus, with the exception of those mammalian cells deficient in PR ability, the mechanisms for averting serious consequences to the cell from UVL irradiation seem to be fairly constant from one organism to another, at least among those studied. Most work in this field, however, has been conducted with heterotrophic systems (mainly prokaryotes), while little attention has been paid to the repair capabilities of autotrophs. Also, all quantitative work has been done using unicellular organisms - naturally enough, for good quantitative results

in this field are more easily obtained from such a system. However, to quote Loppes (1969), "it is necessary for organisms of different evolutionary levels to be investigated if results are to be generalized".

Thus, because of this lack of existing knowledge of many of the genetic and physiological aspects of autotrophic, and of multicellular organisms, the colonial green alga Eudorina elegans Ehrenberg promised to be an interesting subject for study. This alga grows well in axenic culture; its size and growth habit make possible the performance of operations with it which have previously been done mainly with bacteria, yeasts, and mammalian cells. It has the capability of dividing vegetatively indefinitely, and so can be maintained in continuous culture; but it is heterothallic, and under certain conditions the sexual cycle may be induced, allowing formal genetic studies. Growth and division characteristics of the organism have been studied by Rayns and Godward (1965); Goldstein (1964), Mishra (1967), and Mishra and Threlkeld (1968) have contributed to characterization of the mating types and genetic system; and Wentworth (1970) has conducted a preliminary study of UVL- and drug-sensitivity, as well as of mutant isolation and characterization in E. elegans.

E. elegans exists mainly as a coenobium of 16 chlamydomonad-like cells; occasionally coenobia of 8 or 32 cells are encountered. Each of these cells has the potential of dividing 4 times in rapid succession (Rayns and Godward, 1965), remaining in the original coenobial envelope, so that each of the 16 cells is now a cluster of 16 small cells. This we call a "mulberry" form; it is fairly stable, but as maturity of the

stage increases, it breaks down into 16 new coenobia (premature breakdown may sometimes be caused by centrifuging or by UVL irradiation). Under culture conditions presented here (see Materials and Methods - General), the time required for one "generation" (i.e. from 1 to 16 coenobia) is about 1 day.

It was suggested by Wentworth (1970), and by Kemp and Wentworth (1971), that E. elegans possessed little or no dark repair capability, though its PR system was very efficient. With this observation as a starting point, it was decided that the UVL sensitivity and repair capabilities of E. elegans should be more carefully studied. This study, then, was initiated with the purpose of discovering just what DNA repair capabilities are present in E. elegans, and what some of their characteristics are; and, hopefully, of determining what significance these might have to the survival of the organism. This involves a study of both UVL- and drug-sensitivity, using mainly the effects of various physical parameters to characterize these sensitivites. The only measure of sensitivity used is survival, as the genetic system of the organism is not yet sufficiently worked out to allow measurement of mutation rate.

# CHAPTER II

## MATERIALS AND METHODS - GENERAL

### A. The Organism:

The strains of *Eudorina elegans* used in this study were obtained from the Algae Culture Collection, Indiana University, Bloomington, Indiana, U.S.A. They are listed as compatible mating types (Starr, 1964), designated 1192 and 1193 (described by Goldstein, 1964, as 56m and 56f, respectively). The two strains gave qualitatively similar results under the experimental conditions employed and were thus used interchangeably.

### B. Media:

The medium used for maintaining cultures for experimental purposes consisted of a modified Bristol's salt solution (Cain, 1965), with trace elements provided by Gaffron's mixture (Hughes *et al.*, 1958), and enriched by the addition of 200 ml Euglena medium per litre of final medium. This medium is denoted Bristol's complete medium, or Bc, throughout this work; the basic medium, not enriched, is denoted Bristol's minimal medium, or Bm. The composition of all media mentioned above is given in Appendix I.

Solid medium for plates was obtained by adding 1.5% w/v Difco agar to Bc medium; top agar for the pour plating technique was made by the addition of 0.6% w/v Difco agar to Bc medium. All media were sterilized by autoclaving at 15 p.s.i. for 15 minutes.

### C. Chemicals:

All inorganic compounds added to growth media were reagent grade;

the source of organic supplements are given in Appendix I.

Stock solutions of acridine orange (J.T. Baker Chemical Co., New Jersey), 15 mg/ml, caffeine (Eastman Organic Chemicals, New York, N.Y.), 0.1 M, and acriflavine (Matheson, Coleman and Bell, Norwood, Ohio), 15 mg/ml, were made up in Bm and kept at 5°C; any solutions more than 3 months old were discarded. Iodoacetic acid (Matheson, Coleman and Bell, Ohio), mitomycin C (Sigma Chemical Co., Missouri), and deoxyadenosine (Calbiochem., Los Angeles, Calif.) were either added directly to the medium to the desired concentration, or made up into solutions in Bm immediately prior to use. Ethyl methanesulfonate (Eastman Organic Chemicals, New York, N.Y.), which was obtained in 8 M liquid form, was added directly to the culture to be treated. 4-nitroquinoline-1-oxide (Daiichi Pure Chemical Co., Tokyo; obtained from H. Stich, University of British Columbia) was kept in a stock solution of 0.01 M in 95% ethanol, stored at 5° C; any solution more than 3 months old was discarded.

D. Growth Conditions:

Stock cultures were maintained on Bc slants and cloned periodically for ability to grow on both Bm and Bc, as described by Wentworth (1970).

Growth conditions for experimental cultures were kept standard in an attempt to keep variability due to life cycle differences to a minimum. A 5-ml aliquot of a late logarithmic phase culture (approximately 2.5 x $10^4$ colony forming units, or cfu, per ml) was inoculated into 50 ml Bc in a 200- or 250-ml Erlenmeyer flask. The culture was grown on a shaking platform (approximately 80 oscillations/min) at $32 \pm 1$°C, under banks of

40-watt Cool White fluorescent lights (light intensity about 1000 ft-c, measured outside the flasks). Light was provided on a cycle of 16 hr light: 8 hr dark. After 48 hr growth under these conditions, the culture (in late logarithmic phase) was harvested for experiment. Cultures were harvested by centrifuging in a Sorvall Desk Centrifuge SP/X head at 2100 ± 100 rpm (approximately 500 x g) for 5 min. The supernatant was decanted and the culture resuspended in sterile phosphate buffer ($K_2HPO_4$ and $KH_2PO_4$, final concentration 1.7 x $10^{-3}$ M. pH = 6.8 to 7.0). This suspension was then re-centrifuged under the same conditions, and resuspended in phosphate buffer to the final coenobial concentration desired. Coenobial concentration was estimated by measuring absorbance of the suspension at 560 nm in a Coleman Spectrophotometer 6C (Wentworth, 1970).

In all experimental work presented here, the original coenobial concentration, or input cfu, was assayed as a control; this was done by taking an aliquot of the resuspended culture before experimental treatment, and diluting it in sterile phosphate buffer for plating.

Considerable quantitative variability was observed between experiments, due apparently to life cycle variations in the organism. However, qualitatively similar results were obtained from duplicate experiments; thus each figure presented in Results sections represents the results of a single typical experiment, unless otherwise indicated. Plating error was found to be within a range of ± 10% about the calculated survival level at each assay point.

# CHAPTER III

## INTRODUCTION - UVL

Most kinetic studies of UVL inactivation and effects of various post-UVL conditions on repair capabilities have been conducted with prokaryotes (Roberts and Aldous, 1949; Witkin, 1961, 1966 a&b; Setlow, 1966 a; Harm, 1967, 1970; Barnhart and Cox, 1970), and almost entirely with heterotrophic systems (Jagger, 1958; Nasim, 1968; Kilbey and Smith, 1969; Kiefer, 1970). Davies (1965) did a preliminary study of UVL inactivation and repair in *Chlamydomonas*, from which he concluded that both dark repair and PR were present; Van Baalen (1968) studied post-UVL survival in *Agmenellum*, considering only PR and the effects of UVL irradiation on photosynthesis. PR enzyme has been extracted from several green plants (Elder and Beers, 1965; Saito and Werbin, 1969, 1970), and action spectra for the enzyme determined *in vitro*. Kinetic studies of UVL inactivation and reactivation in green plants, then, form a large gap in knowledge of DNA repair mechanisms. This study, hopefully, will help to fill the gap.

Previous work, particularly with bacteria (Setlow, 1966; Witkin, 1966 a) and mammalian cells (Cleaver, 1969; Rauth, 1970) have made extensive use of drugs to interfere with particular repair processes, thus aiding in the characterization of the repair mechanisms present. Those most commonly used were caffeine (Cleaver and Thomas, 1970; Rauth, 1967; Harm, 1971) and acriflavine (Witkin, 1961; Alper, 1963; Doudney *et al.*, 1964; Davies, 1965; Setlow, 1966); both have been shown to interfere with dark repair. The mode of action of caffeine has not been determined, but it appears to

have several effects (Cleaver, 1969; Harm, 1971). Acriflavine has been shown fairly clearly to intercalate into the DNA molecule and interfere with the excision of pyrimidine dimers (Lerman, 1961, 1963; Setlow, 1966 b). Other drugs used include crystal violet (Witkin, 1961), iodoacetate and dinitrophenol (Elder and Beers, 1965), and mitomycin C (Cleaver, 1969). Some of these have been used in this study as an aid to characterizing repair processes, and to enable fairly direct comparison to results obtained in other systems.

## MATERIALS AND METHODS - UVL

### A. Standard Irradiation Method:

UVL irradiation of the harvested culture (see Materials and Methods - General) was carried out in liquid; the culture, in a plastic disposable Petri dish, was exposed to light from the central part of two Sylvania G15T8 germicidal lamps (95% emission at 254 nm, by manufacturer's specifications) while agitated on a shaking platform. Dose rates used were 10 and 20 ergs $mm^{-2}$ $sec^{-1}$, as measured by a Blak-Ray UV Meter (J-226), and cross-calibrated by a phage T4 inactivation curve (Harm, 1968; Wentworth, 1970). These two rates produced the same survival levels per total dose. Total doses were timed, and delivered by opening and closing a door beneath the UVL lamps. Aliquots were taken between exposures, and diluted appropriately for survival assay.

Survival was assayed by the pour plating technique, using top agar (kept soft at 45°C) and plate agar of the same composition. All opera-

tions during and after UVL irradiation were carried out under G.E. "Gold" incandescent or fluorescent lamps to prevent unwanted PR; these lamps emit only at wavelengths above 500 nm. All survival assays were plated in duplicate or quadruplicate at each dose level, and the average among the plates taken. All experiments were done with internal controls to ensure that comparisons made between experiments were valid; quantitative variability between experiments made this essential.

B. Post-UVL Conditions:

For PR conditions, plates were transferred immediately to standard growth conditions of 32 ± 1°C, under Cool White fluorescent lights, at an intensity on top of the plates of approximately 1000 ft-c. To test for dark (NPR) repair, plates were incubated for 48 hr in the dark (32 ± 1°C) before transfer to light conditions as above. All plates were incubated until colonies had appeared and were countable (5 to 6 days). Survival was noted, then, in terms of colony forming units, or cfu.

Light of higher intensity than that used for regular PR was obtained from a Sylvania 500T3/Q/CL/U 120 V tungsten-halogen lamp in a Dicrolite Co. housing. This housing was equipped with a rear mirror to transmit near infrared and reflect visible light, and a front mirror to reflect near infrared and transmit visible light. A 5-cm water layer (in a plexiglass tray) was placed between the lamp and the cultures to be photoreactivated in order to further reduce infrared radiation. Light intensity obtained in this way was approximately 4000 ft-c incident on the plates.

C. Chemicals:

To aid in the characterization of repair processes, certain drugs were used to treat UVL-irradiated cultures. Unless otherwise stated, this was done by addition of the drug to both the plate agar and the top agar, to the same concentration. Caffeine, acridine orange, and acriflavine were kept as stock solutions in sterile Bm; further sterilization of these solutions was deemed unnecessary due to the toxicity of the drugs. The concentrations mainly used in the plating medium (caffeine, $2 \times 10^{-3}$ M; acridine orange, 15 μg/ml; acriflavine, 30 μg/ml) did not affect the number of colonies from an unirradiated sample, although a slight reduction in colony size, compared to the control plates (Bc plates) was observed.

D. Light Measurements:

To obtain quantitative measurements of light quality and quantity received by the organisms on plates, an ISCO spectroradiometer was used. Two Cool White fluorescent tubes were suspended above the spectroradiometer sensing head, and the plate or plates desired interposed between the light and the sensor. Intensity readings were taken automatically at 5-nm intervals and the results calculated and plotted by a computer program supplied courtesy of W.S. Duval.

E. Cloning:

For cloning, either pre- or post-UVL, the experimental culture was diluted to approximately 10 cfu/ml. A series of small disposable test

tubes was prepared by placing 0.4 ml of Bc in each; to each was then added 0.1 ml of the diluted culture. These tubes were incubated in the dark at 32 ± 1°C for as long as desired. Generally one set of 50 tubes was plated each day for 4 days following initiation of the experiment.

## RESULTS - UVL

### A. Effect of Lighting Conditions:

The sensitivity of *E. elegans* depends to some extent upon temperature and nutrition (Kemp and Wentworth, 1971), but a much more dramatic effect is seen with a change in lighting conditions (Fig. III-1). The average dose modifying factor (Kelner, 1949) for PR *vs* NPR is 0.2. There is a small "shoulder" region present in the NPR survival curve; when the slope of the exponential inactivation curve is extrapolated it intersects the 100% survival mark at values ranging (among several experiments) from 50 to 800 ergs $mm^{-2}$.

The PR response is dependent upon both PR light intensity and length of exposure time to PR light (Fig. III-2). Lack of adequate equipment prevented the determination of any possible dependence upon PR light wavelength which may be shown by *E. elegans*. Complete loss of PR ability is fairly slow; about 28 to 30 hours dark incubation are required before PR will no longer occur with exposure to white light (Fig. III-3). It was for this reason that dark incubation time for NPR conditions was increased from 24 to 48 hours, thus removing PR as a potential component of "dark" survival.

Figure III-1. Post-UVL survival in E. elegans: photoreactivation (PR) vs non-photoreactivation (NPR). UVL-irradiated cultures were plated and either exposed to PR light immediately (o—o) (PR conditions) or kept in the dark for 48 hr to prevent PR (●—●) (NPR conditions) before moving to the light to complete growth. All incubation was at 32 ± 1°C.

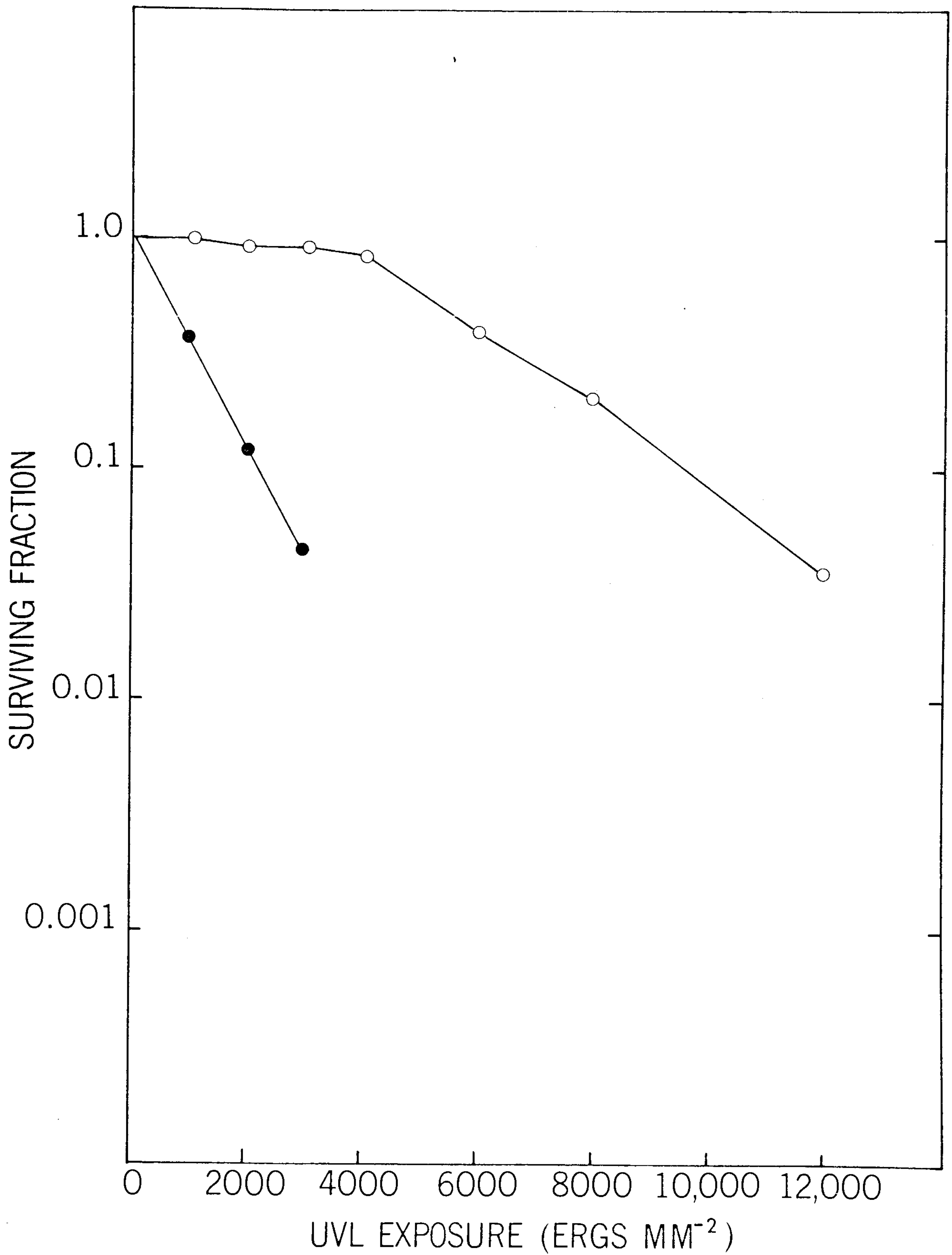
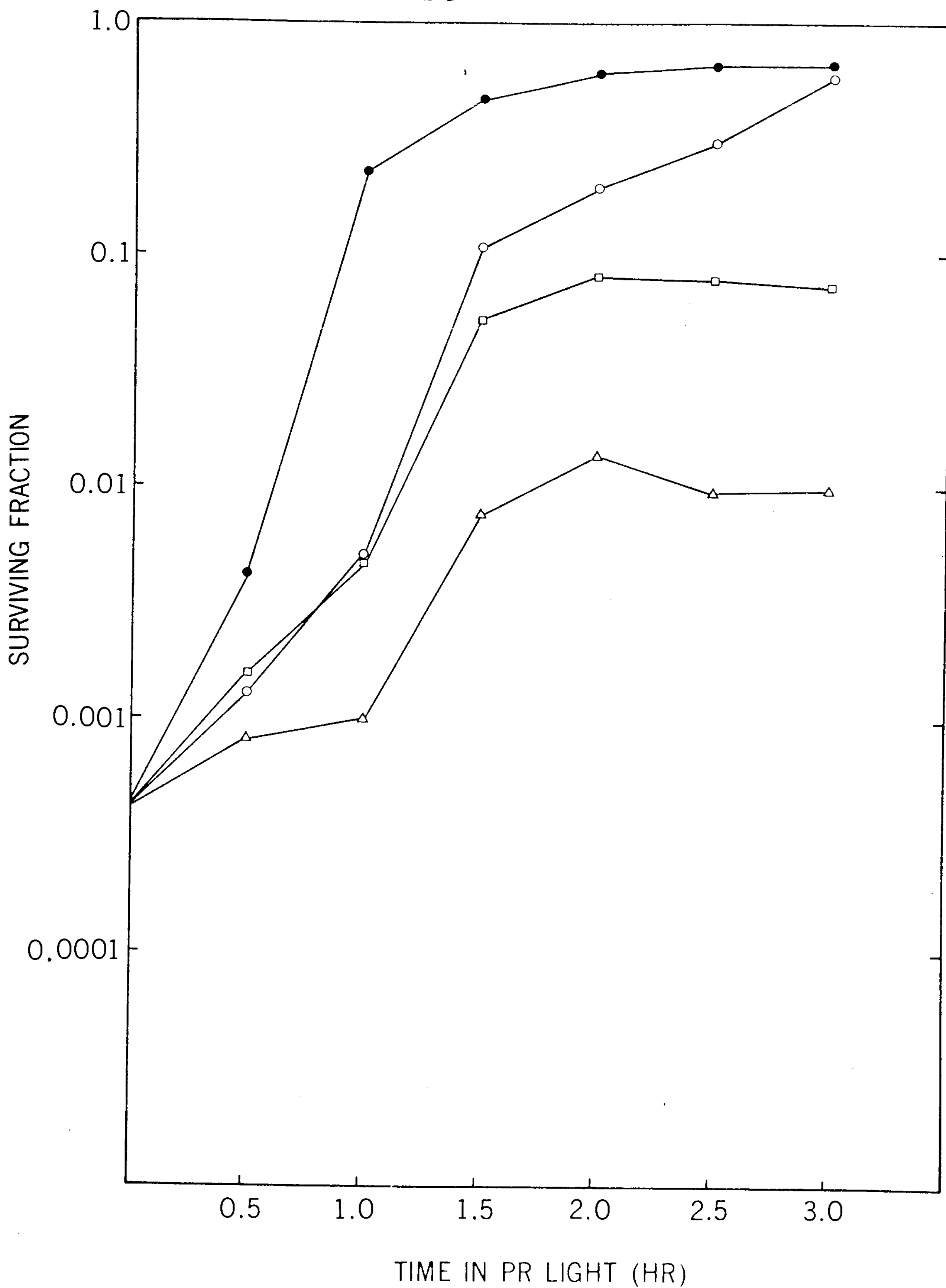
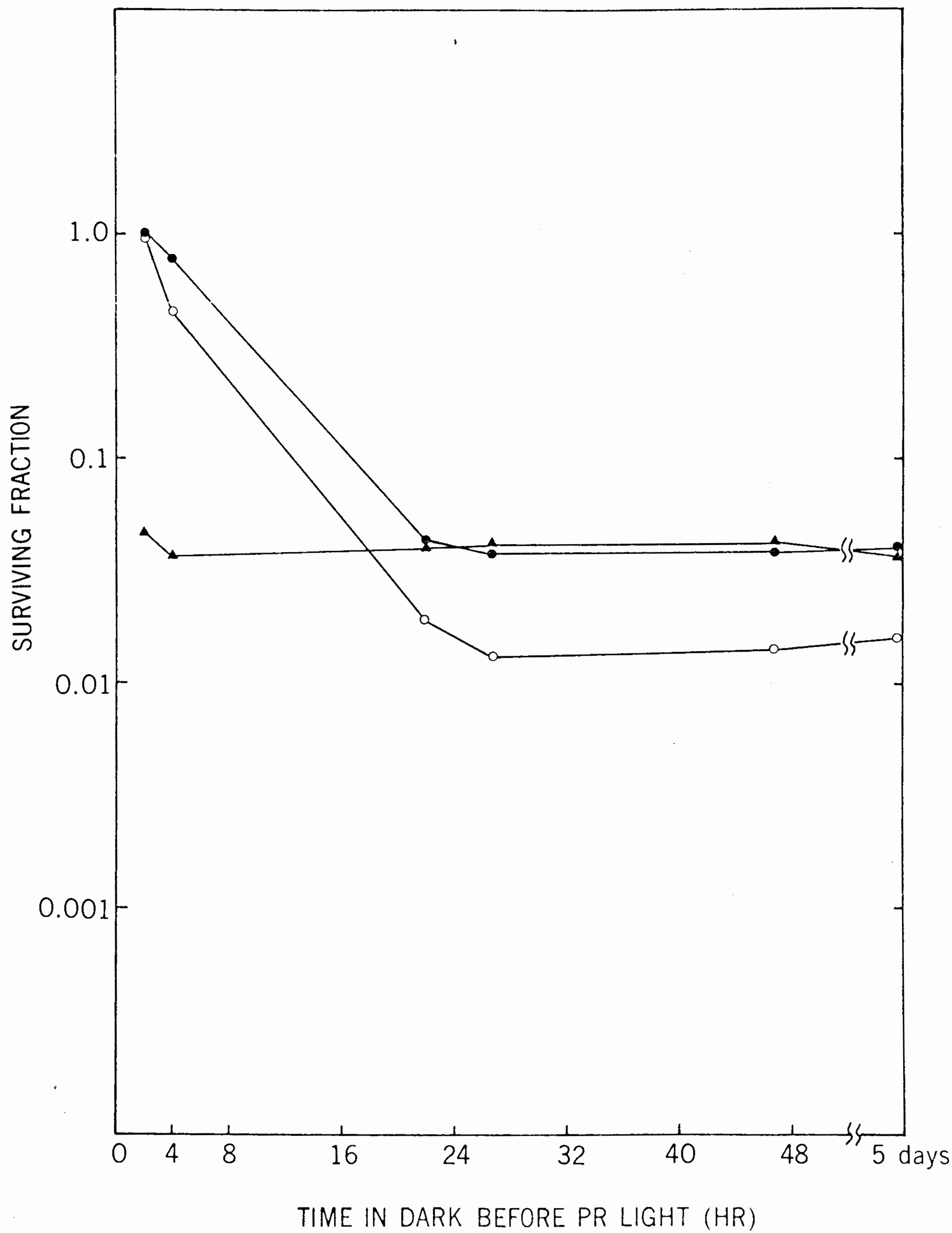

1.0
0.1
0.01
0.001
SURVIVING FRACTION
0
2000
4000
6000
8000
10,000
12,000
UVL EXPOSURE (ERGS MM$^{-2}$)

Figure III-2. Kinetics of PR: the relationship of time to light intensity. Organisms were exposed to 8000 ergs $mm^{-2}$ UVL, plated, and exposed to PR light for given periods of time. Further PR after these time periods was prevented by dark incubation for 48 hr. Highest intensity of light was obtained with a Dicrolite lamp; intensities $\leq$ 1000 ft-c were obtained with the use of Cool White fluorescent lights, with opal plexiglass between the light source and plates for the two lower intensities: 4000 ft-c (●—●), Dicrolite; 1000 ft-c (o—o), Cool White, no plexiglass; 550 ft-c (□—□), Cool White, 1 plexiglass sheet; 300 ft-c (Δ—Δ), Cool White, 2 plexiglass sheets. All points in this figure are normalized against an unirradiated control.



1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
0.5
1.0
1.5
2.0
2.5
3.0
TIME IN PR LIGHT (HR)

Figure III-3. Decay of PR ability: effect of caffeine and of acridine orange (AO) in plating medium. Organisms were exposed to 3000 ergs $mm^{-2}$ UVL, plated, and incubated in the dark for varying periods of time before exposure to PR light. Aliquots were plated on Bc (●—●), on Bc + 2 x $10^{-3}$ M caffeine (○—○), and on Bc + 15 μg/ml AO (▲—▲).


1.0
0.1
0.01
0.001
SURVIVING FRACTION
0
4
8
16
24
32
40
48
5 days
TIME IN DARK BEFORE PR LIGHT (HR)

B. Effects of Acridine Orange:

Acridine orange (AO) was originally used in an attempt to verify the apparent lack of dark repair in *E. elegans* (Kemp and Wentworth, 1971). It is a compound very similar to acriflavine, which has been reported to interfere with NPR excision repair (Witkin, 1961; Davies, 1966; Cleaver, 1969). Acriflavine and AO had similar effects on post-UVL survival of *E. elegans* (Fig. III-4); AO was chosen simply for convenience.

The presence of AO had no effect on NPR survival, but markedly reduced PR survival (Fig. III-4), almost to the NPR level. This effect had not been previously reported (Witkin, 1963), possibly because it may have been dismissed as being due simply to absorption of the PR light wavelengths by AO. Experiments designed to test the light absorption properties of AO in agar plates showed, indeed, that at least a large part of the effect of AO on PR was probably due to selective absorption of the PR wavelengths by the dye. First, a measurement of the spectrum of light emitted by the Cool White fluorescent tubes, and transmitted by the agar plates, with and without AO, was taken by means of an ISCO spectroradiometer (Fig. III-5; see also Appendix II). It can be clearly seen from figure III-5 that the AO substantially reduces the transmitted light in the range of 440 to 540 nm.

Second, a comparison was made between post-UVL survival when plated on AO, and survival when plated on Bc and exposed to PR light with an AO plate interposed as a filter. This, as shown in figure III-6, indicated that the effect of AO on PR survival may not be entirely due to filtration of PR light; contact of the cells with AO produces approximately a

Figure III-4. Post-UVL survival of E. elegans: effect of plating on AO or on acriflavine. UVL-irradiated and plated organisms were incubated either under PR conditions (open symbols) or NPR conditions (closed symbols). Aliquots were plated on Bc (○—○, ●—●), on Bc + 30 μg/ml acriflavine (△—△, ▲—▲), and on Bc + 15 μg/ml AO (□—□, ■—■).

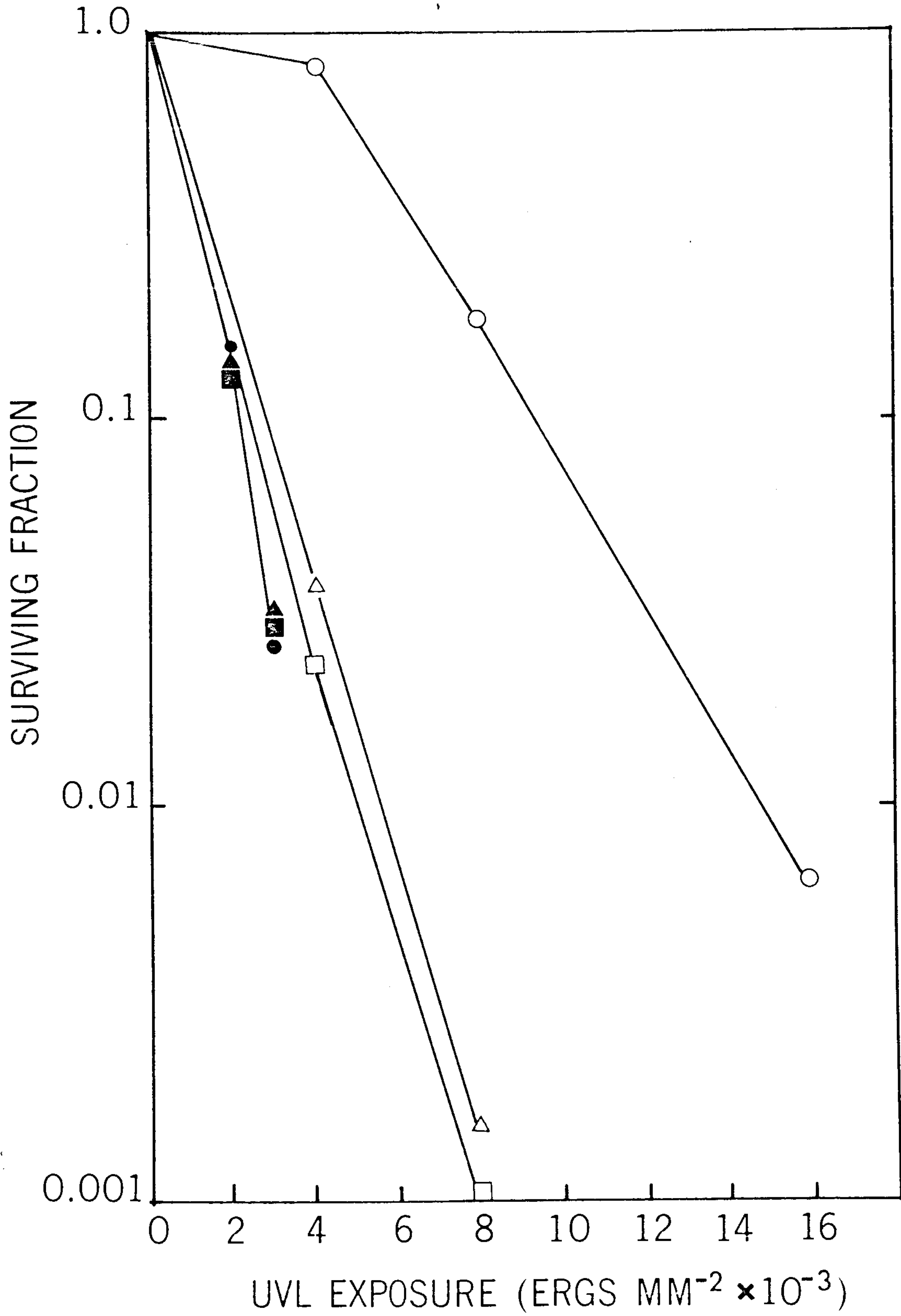

1.0
0.1
0.01
0.001
SURVIVING FRACTION
0
2
4
6
8
10
12
14
16
UVL EXPOSURE (ERGS MM-2 ×10-3)

Figure III-5. Spectroradiometer measurements of emission spectrum of Cool White fluorescent lights, and transmittance through plating media.

Δ—Δ Cool White fluorescent lights, emission spectrum

o—o Cool White fluorescent lights, transmitted through Bc + 15 μg/ml AO agar in plate

●—● Cool White fluorescent lights, transmitted through Bc agar in plate

For comparison of curve shapes, intensities are normalized to 1.0 at wavelength 600 nm. Absolute intensities are given in Appendix II.

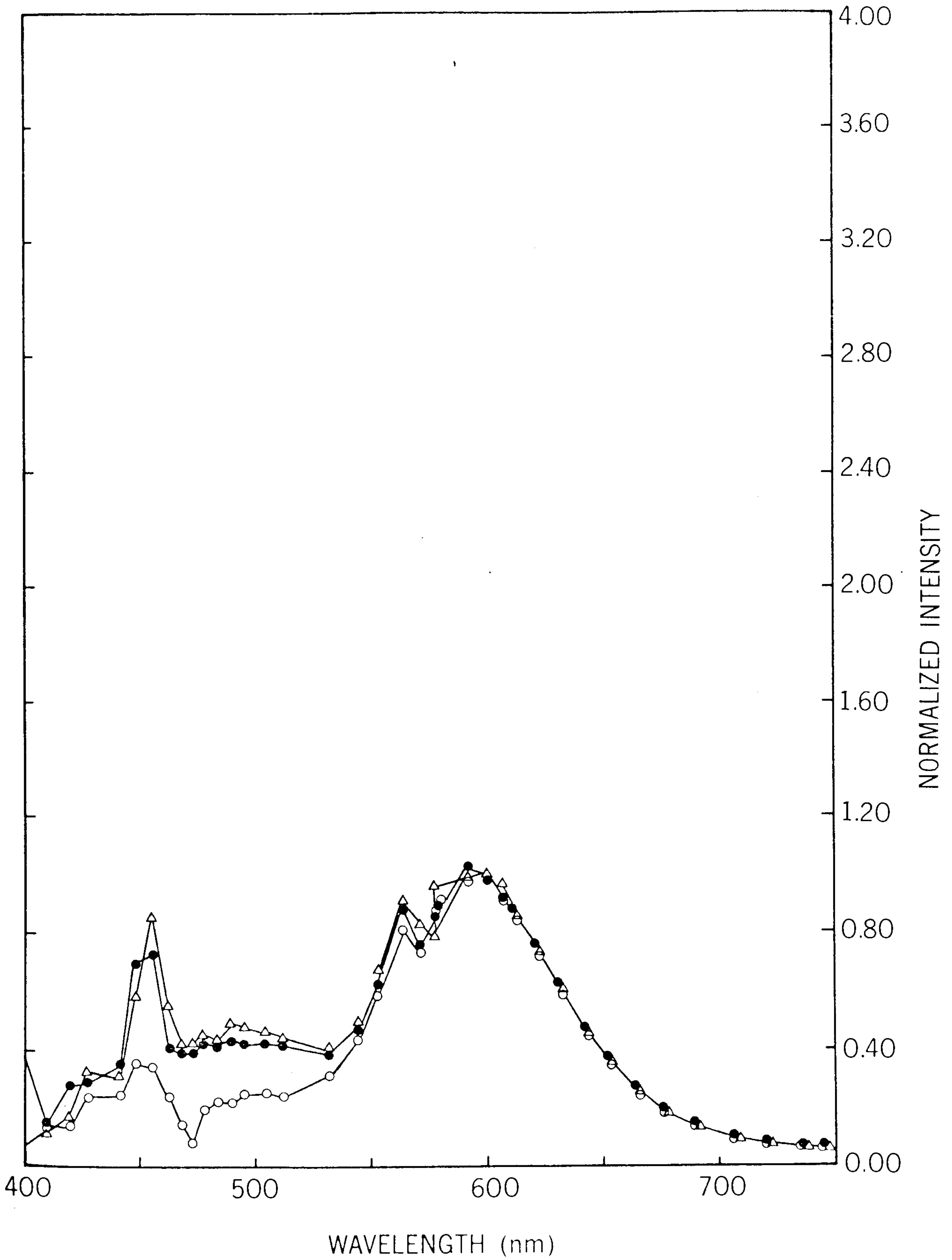
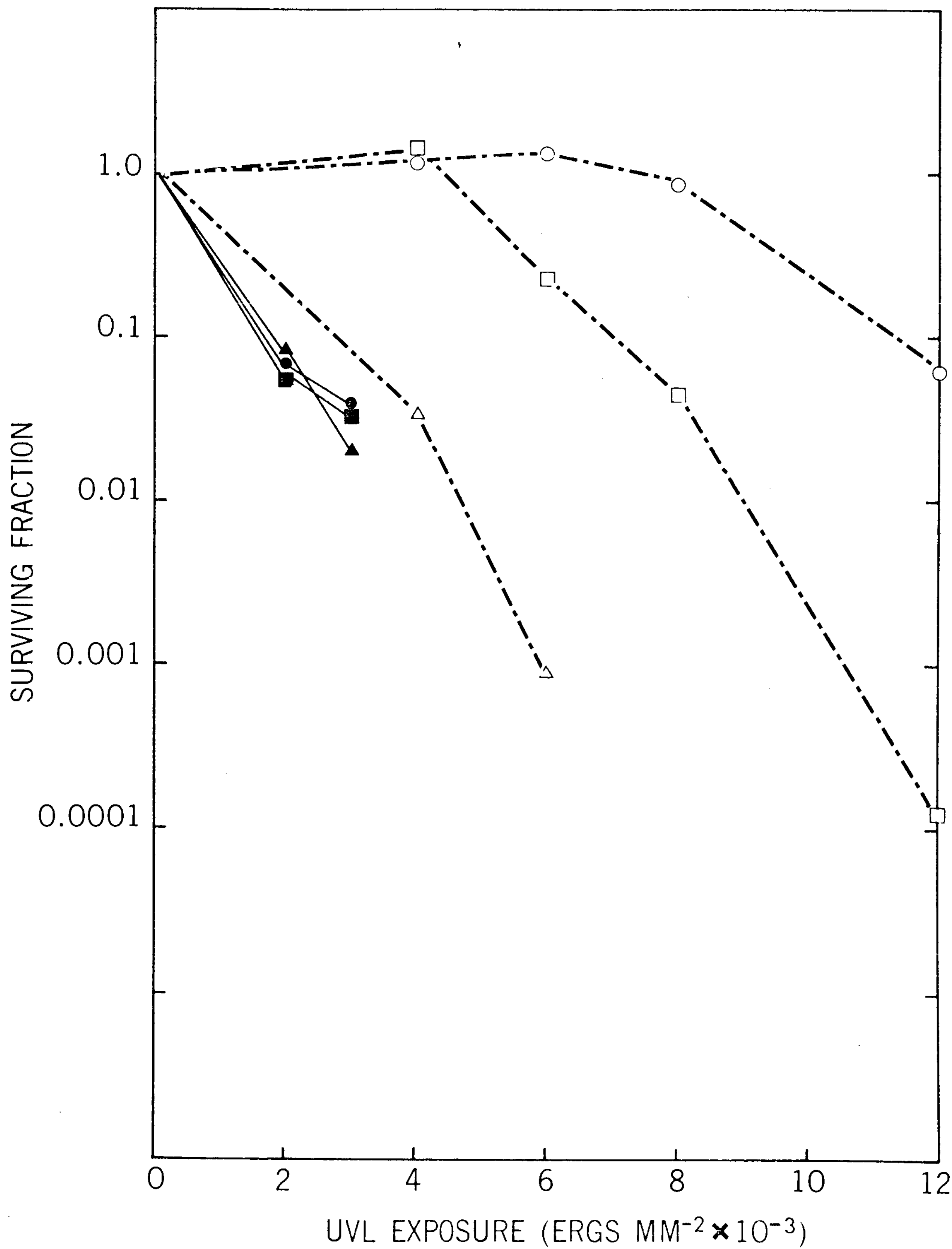

4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
NORMALIZED INTENSITY
400
500
600
700
WAVELENGTH (nm)

Figure III-6. Effects of AO on PR: contact with AO vs light filtering by AO. UVL-irradiated and plated organisms were kept either under PR conditions (open symbols) or NPR conditions (closed symbols). Aliquots were plated on Bc (○—○, ●—●), on Bc which was incubated under Bc + 15 μg/ml AO plates (i.e. AO filters) (□—□, ■—■), and on Bc + 15 μg/ml AO (△—△, ▲—▲).


1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
0
2
4
6
8
10
12
UVL EXPOSURE (ERGS MM$^{-2}$ × 10$^{-3}$)

50% reduction in survival over organisms exposed to AO only as a filter.

It is possible that this difference was due to a concentrating of the drug in the cells; those cells containing the drug would then have a greater concentration of AO than in the surrounding agar, and would perhaps be screened more effectively from PR light. This theory was tested by using different concentrations of AO (15 and 60 μg/ml) in plates used as filters. The survival rate on plates exposed to PR under 60 μg/ml AO filters was intermediate between that on plates under 15 μg/ml filters and that plated in medium containing 15 μg/ml AO (Fig. III-7).

If the reduction of PR survival by AO was due to light filtering, the effect should be overcome by the use of greater intensities of PR light with the same concentration of AO. The experiment was done both with AO in contact with the irradiated cells, and with AO used only as a filter. First, instead of increasing light intensity, both AO concentration and light intensity were reduced; sheets of opal plexiglass were used as neutral density filters to reduce PR light intensity. As shown in figure III-8, both "contact" and "filter" AO had less effect on PR survival at higher light intensities; this reduction in effectiveness appeared to be of the same order whether AO was used as a filter or was placed in contact with the organisms. Further attempts at overcoming the effect of AO on PR were made using a high-intensity Dicrolite lamp (intensity approximately 4 times that of the Cool White fluorescent tubes). The effects of AO on PR survival under this lamp were compared with the effects under Cool White fluorescent tubes, as shown in Table III-1. To help compensate for the apparent concentrating of AO by the

Figure III-7. Effects of AO on PR: contact with AO vs light filtering by two concentrations of AO. UVL-irradiated cultures were plated and placed under PR conditions. Plating and incubation were done as follows: ●—● plated on Bc, no filter; o—o plated on Bc, incubated under 15 μg/ml AO filter; △—△ plated on Bc, incubated under 60 μg/ml AO filter; ▲—▲ plated on 15 μg/ml AO, no filter.

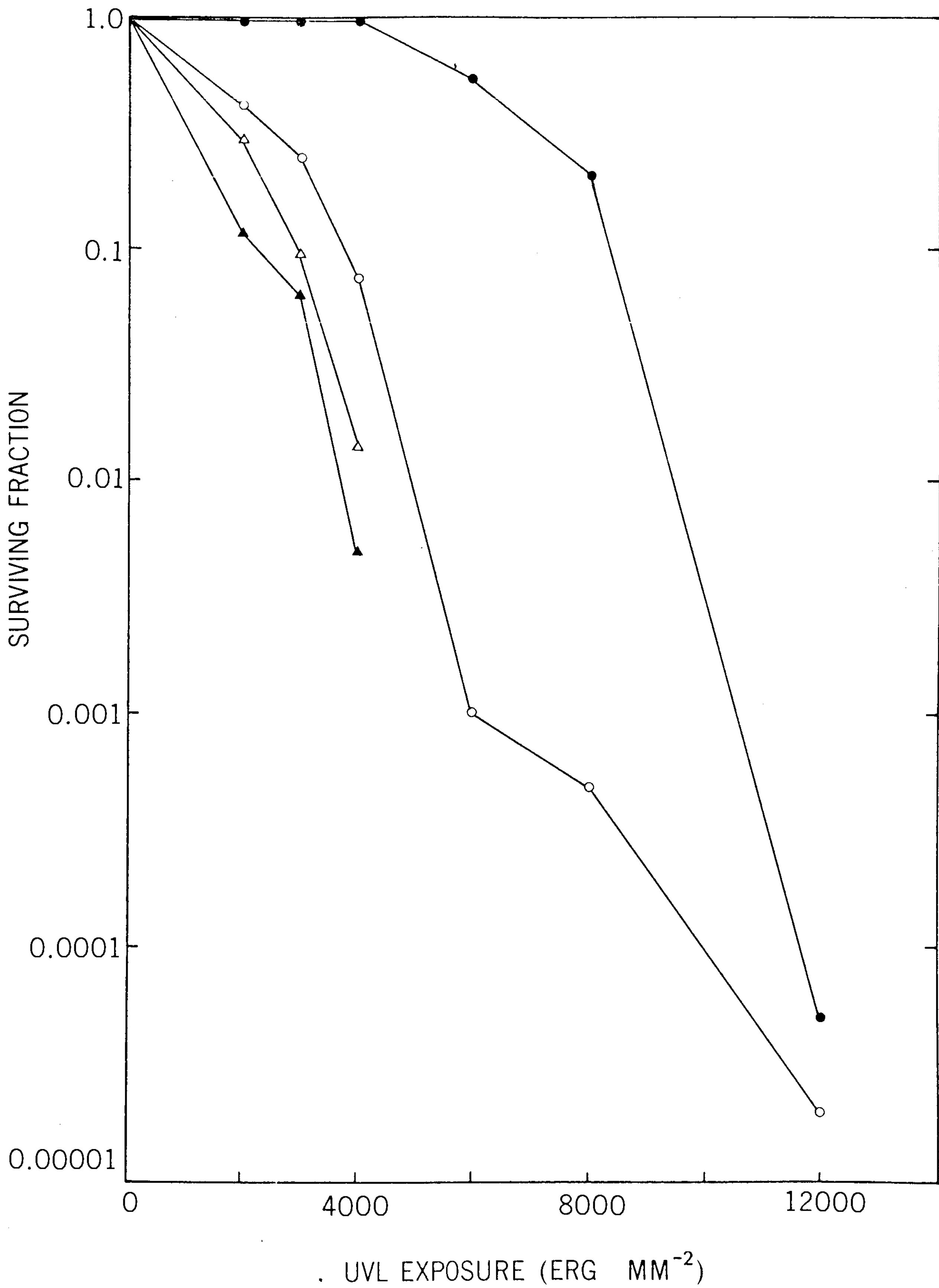
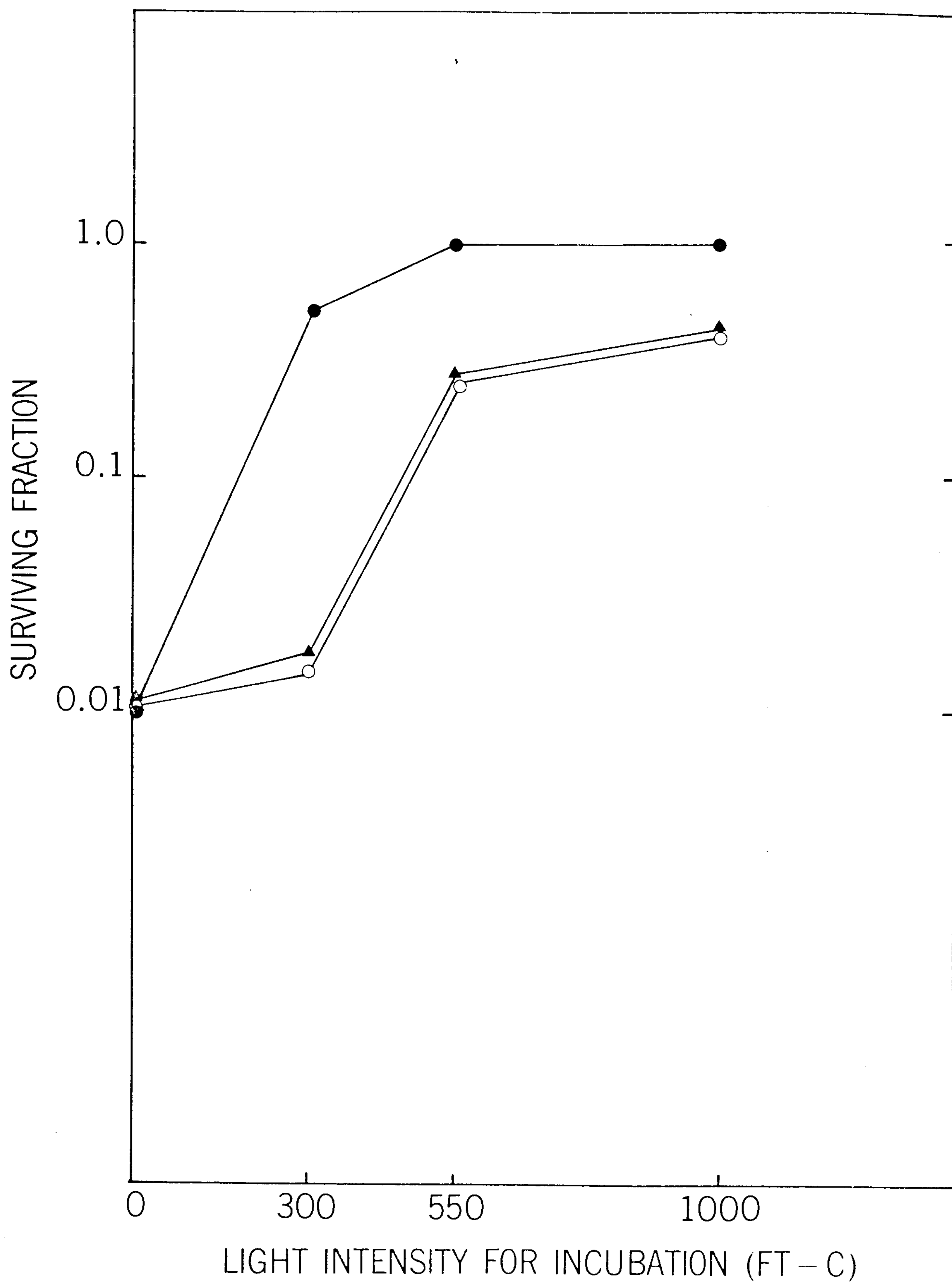

1.0
0.1
0.01
0.001
0.0001
0.00001
SURVIVING FRACTION
0
4000
8000
12000
UVL EXPOSURE (ERG MM$^{-2}$)

Figure III-8. Overcoming PR light filtering effect on AO with increasing light intensity. Organisms were exposed to 6000 ergs $mm^{-2}$ UVL, plated, and placed under various light intensities (achieved with the use of opal plexiglass) for growth. Aliquots were plated on Bc (●—●), on Bc + 15 μg/ml AO (▲—▲), and on Bc incubated under 10 μg/ml AO filters (o—o). All points in this figure are normalized against an unirradiated control. This figure represents an average of two experiments.



SURVIVING FRACTION
1.0
0.1
0.01
0
300
550
1000
LIGHT INTENSITY FOR INCUBATION (FT – C)

Table III-1. PR under high light intensity (Dicrolite lamp) and low light intensity (Cool White fluorescent lights); effects of AO in plating medium or as a PR light filter. A culture irradiated with 6000 ergs $mm^{-2}$ UVL was plated in the dark. Plates were then exposed to either Dicrolite (approx. 4000 ft-c) or Cool White fluorescent (approx. 1000 ft-c) lights for 2 hr. Growth was completed under standard growth conditions. Bc plates were used as filters on all those plates not under AO filters in order to eliminate differences in PR light intensity reaching the organisms.

| UVL dose (ergs $mm^{-2}$) | Plate type | % survival under Cool White fluorescent lights | % survival under Dicrolite lamps |
|---|---|---|---|
| 0 | Bc | 100 | 100 |
| 0 | Bc + 5 μg/ml AO | 100 | 87 |
| 6000 | Bc | 80 | 100 |
| 6000 | Bc + AO filter (Bc + 60 μg/ml AO) | 1.3 | 15 |
| 6000 | Bc + 5 μg/ml AO | 18 | 17 |

cells, the concentration of AO in the filter plates was increased 12-fold over that in the contact plates. Even with this concentration difference, the effect of AO when used as a filter was overcome to some extent, while no such overcoming of its effect was noted with AO in contact with the organisms. Direct comparisons of survival under the two light intensities were made difficult because of apparent photodynamic killing by the AO under the influence of the higher light intensity.

The phenomenon of the decay of PR ability is completely masked by the presence of AO in the plating medium, as illustrated in figure III-3. PR is prevented, reducing all survival to the NPR level.

C. Effects of Caffeine:

Caffeine also has been reported to interfere with dark repair, but not, apparently, in the same manner as does acriflavine (Cleaver, 1969). In _E. elegans_, post-UVL survival was somewhat reduced by the presence of caffeine in the plating medium, in both PR and NPR situations (Fig. III-9). There is a small but reproducible reduction of NPR survival at all dose levels; PR survival is reduced only at higher dose levels (greater than 4000 ergs $mm^{-2}$). The possibility of a filter effect, as with AO, could be discounted here, as the absorption spectrum of caffeine is in the UV range, peaking at 270 to 275 nm (Fig. III-10); and the shortest wavelength emitted by the lights used for PR is about 300 nm.

The decay of PR ability is affected differently by caffeine than by AO (Fig. III-3). The decay rate on caffeine-containing medium is increased over that found on Bc; although the slope changes at about 24 to

Figure III-9. Post-UVL survival in E. elegans: effect of caffeine. UVL-irradiated cultures were plated and placed under either PR conditions (open symbols) or NPR conditions (closed symbols). Plating was done on Bc (o—o, •—•) and on Bc + 2 x $10^{-3}$ M caffeine (o--o, •--•).

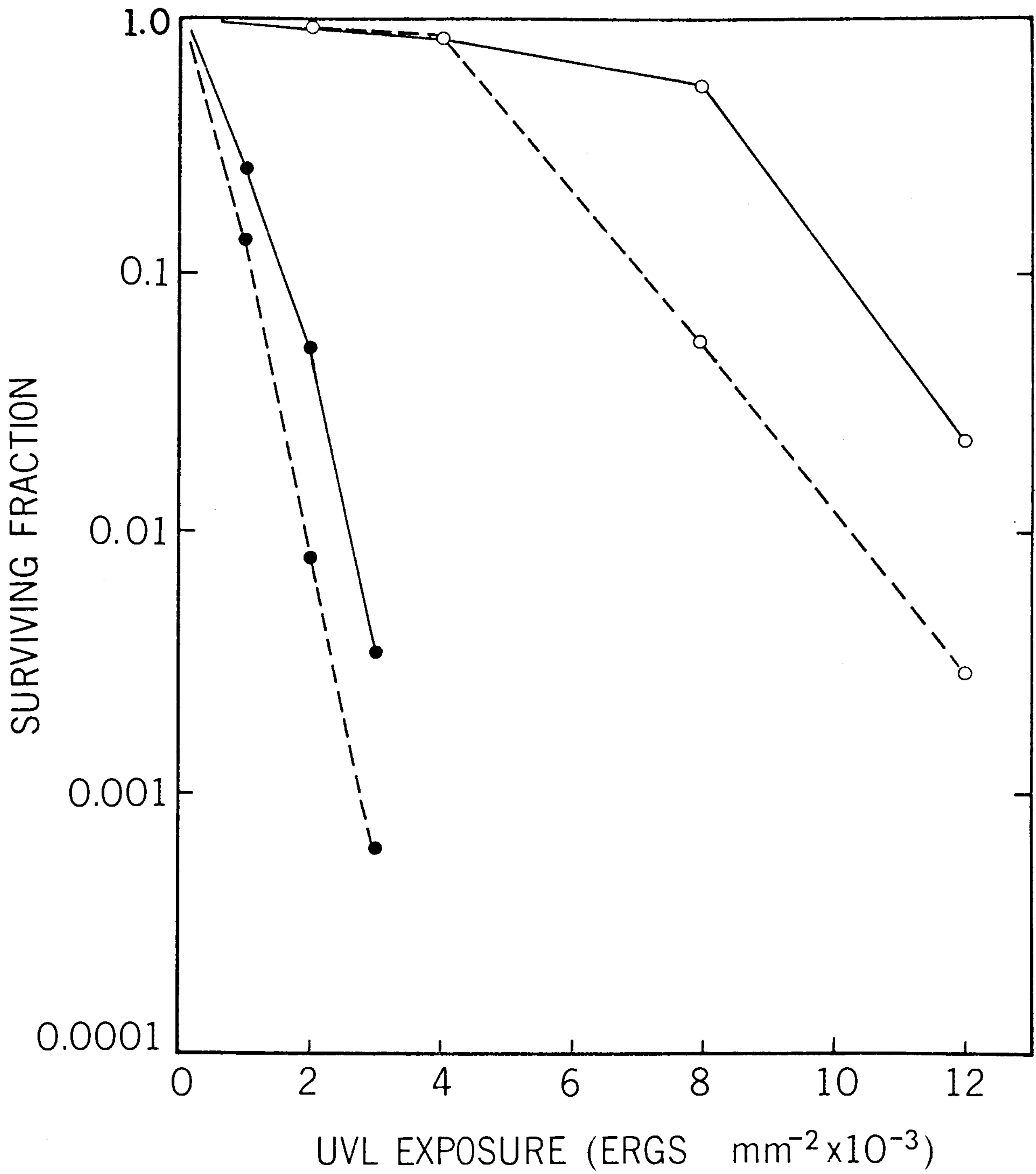
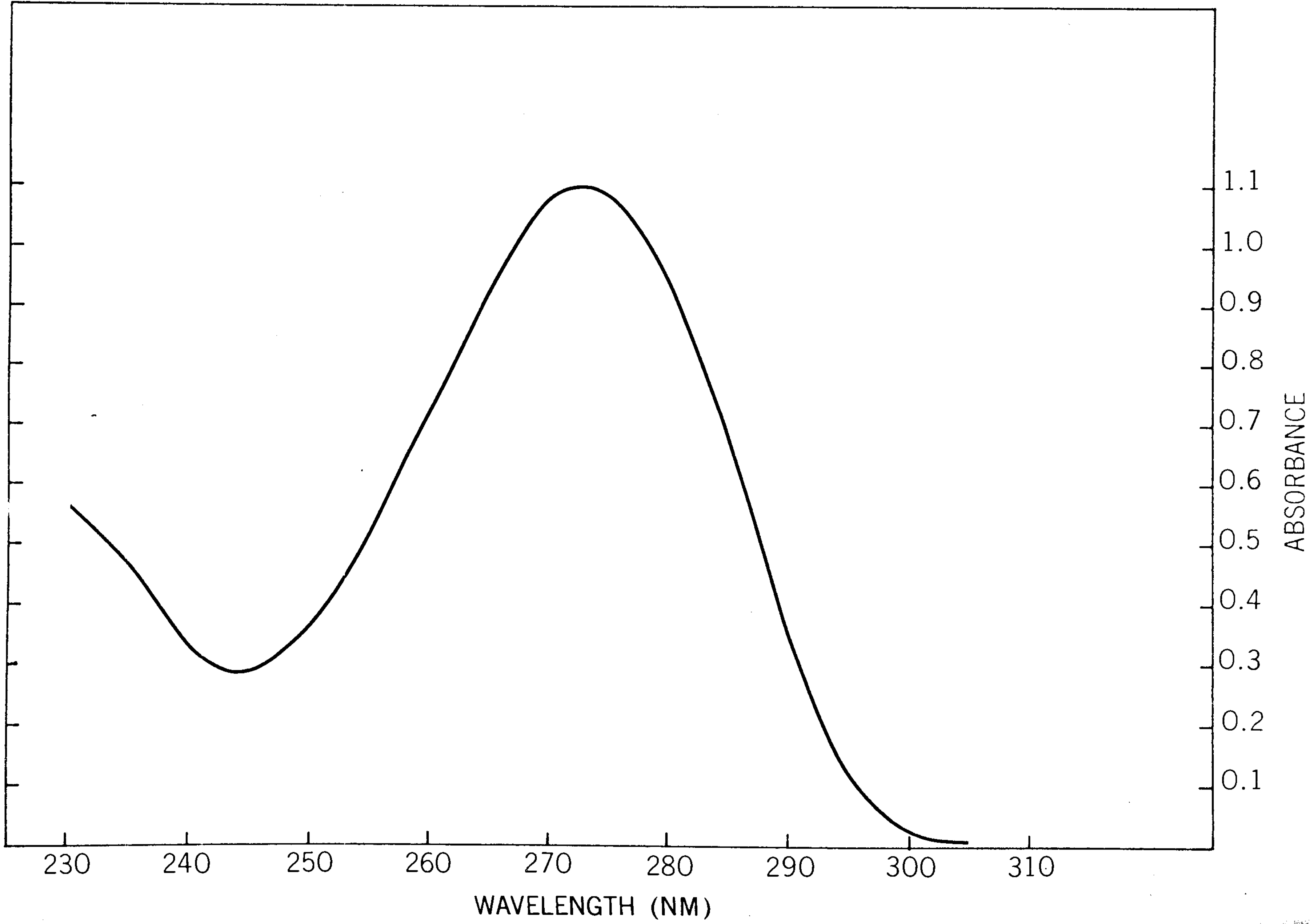

1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
0
2
4
6
8
10
12
UVL EXPOSURE (ERGS mm$^{-2}$ x10$^{-3}$)

Figure III-10. Absorption spectrum of caffeine ($10^{-4}$ $\underline{M}$ aqueous solution). Absorbance was measured in a Bausch and Lomb Spectrophotometer, using a 1-cm path length.



1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
230
240
250
260
270
280
290
300
310
WAVELENGTH (NM)

to 30 hours, as with Bc, the final survival level is reduced to less than 50% of that found on Bc plates.

D. Effect of other drugs on UVL sensitivity:

Another drug used in an attempt to affect repair processes in E. elegans was iodoacetate ($5 \times 10^{-7}$ M, incorporated in the plating medium); it has been reported to interfere with dark repair (Elder and Beers, 1965). However, its presence, at the highest concentration not affecting the number of input cfu, had no effect on either PR or NPR survival. Deoxyadenosine ($5 \times 10^{-5}$ M) was also used, in the same way, as it is a nucleic acid synthesis inhibitor (Klenow, 1962); thus it was possible that it could interfere with repair replication. It had no discernible effect on post-UVL NPR survival in E. elegans; however, it slightly reduced survival under PR conditons (at UVL doses greater than 4000 ergs $mm^{-2}$) when cultures were drug-treated prior to UVL for at least 24 hours.

E. UVL and the Biology of E. elegans:

Work presented in this study was done with cultures in late logarithmic phase, growing in enriched medium (Bc) at 32°C; post-UVL survivorship levels are taken from cultures grown under these conditions. Work by Wentworth (1970) and by Kemp and Wentworth (1971) has shown that post-UVL survival in E. elegans is reduced by slightly less optimal conditions (i.e. conditions producing slower growth), such as lower pre- and post-irradiation temperatures, minimal growth medium, or "starvation" in phosphate buffer prior to UVL irradiation.

We have also found a change in apparent UVL sensitivity with the time of day - that is, with the stage in the life cycle of the organism. As previously described, each cell in a 16-celled coenobium divides until it is 16-celled; then this mulberry coenobium breaks down to form 16 new coenobia. This breakdown stage appears to occur at noon or in the early afternoon - about 6 to 8 hours after the start of the daily 16-hour light period. In the morning, as might be expected, E. elegans was somewhat less sensitive to UVL than in the afternoon as a result of the presence of many mulberry coenobia in the culture. These coenobia may themselves have been more UVL-resistant because of the extra cells to contribute to survival of the coenobium as a whole. They also provided higher apparent survival in the culture, as the UVL treatment seemed to induce premature breakdown, thus producing a higher cfu count in the irradiated samples (at low doses, the cfu level was sometimes higher than that of the unirradiated control). However, the increase in survival due to a certain level of mulberry coenobia has not been quantitatively predictable; little success has been achieved in attempting to equate a certain level of mulberry coenobia in a culture (by microscope counts) with a level of survival.

Although it seems likely that a high number of cells in a coenobium would result in a greater resistance to UVL, it is not known just what the relationship is between the number of cells and survival. In a 16-celled organism, how many of the cells must be inactivated by the UVL to eliminate the coenobium as a colony forming unit? In an attempt to answer this question, cultures were cloned both before and after UVL treatment.

In this way counts could be taken of the number of coenobia which emerged from non-irradiated and from irradiated coenobia. These experiments, however, were unsuccessful due to the extreme variability of the results.

## DISCUSSION - UVL

### A. Effect of Lighting Conditions:

The very small "shoulder" region on the NPR post-UVL survival curve (Fig. III-1) suggests a relatively inefficient dark repair mechanism in E. elegans (Davies, 1966, 1967; Kemp and Wentworth, 1971). The shoulder region in the NPR survival curve reported here is somewhat smaller than that reported by Wentworth (1970). There are two possible reasons for this difference: first, the dark incubation time used by Wentworth for NPR conditions was 24 hours rather than the 48 used in this study, so that some PR may have occurred, raising his observed survival levels; second, his media and growth conditions were slightly different from those used here, so that the condition of his cultures in terms of metabolic activity and growth stage were probably quite different. Kemp and Wentworth (1971) reported the presence of a shoulder region more comparable to that shown here; in this case, culture conditions were very similar to those reported here, except that their cultures were harvested at a slightly earlier stage of growth.

It should be mentioned here that there may be another reason, not related to a repair mechanism, for the observed "shoulder" in the NPR survival curve. As already noted, breakdown of multiple coenobia may

be induced by UVL irradiation. And, although the number of multiple coenobia in experimental cultures was very low, it was at times as high as 5 percent of the total coenobial population. The breakdown of one or two of these per 100 coenobia, at low doses of UVL, would be sufficient to cause a small shoulder on the UVL survival curve.

E. elegans possesses a very efficient PR mechanism, as evidenced by the fact that 100% recovery can be obtained with UVL dose levels as high as 6000 or 7000 ergs $mm^{-2}$. The dose modifying factor of 0.2 is computed at the 10% survival level, as it is not constant over the whole inactivation range; if it were constant, the PR and NPR inactivation curves would extrapolate to the same point on the ordinate (Jagger, 1958). Constant dose modifying factors, though, seem to be the exception rather than the rule (Jagger, 1958); this suggests, as might be expected, a change of conditions within the cell with increasing UVL dose. There presumably comes a dose level of UVL irradiation where the rate of PR is governed not primarily by the amount of light, but by the limiting number of PR enzyme molecules. Thus a change occurs in the factors involved in PR rate determination, and a resulting change in the dmf is observed.

Complete reciprocity of time and light intensity was not found in the PR response, although it is evident from figure III-2 that PR is dependent upon both of these parameters. However, allowing for the ± 10% plating error and realizing that the survival curve slopes change as maximum PR is approached, fairly close reciprocity can be calculated. It is evident from the final survival of the lowest-intensity curve that, at this light intensity, there is not sufficient time for maximum PR to

occur before any remaining damage is "fixed" and can no longer be photoreactivated. Presumably this fixation may be due to the occurrence of DNA synthesis. From the curves shown in figure III-2, it appears that such fixation is initiated within 2 to 3 hours following UVL irradiation. This may be an indication that incubation of UVL irradiated coenobia in visible light shortens the UVL-induced lag from that found during incubation in the dark (Fig. III-3). Under dark post-UVL incubation conditions, PR ability does not completely decay until about 30 hours after irradiation; thus the post-UVL growth lag appears to be much longer under dark conditions. Growth and metabolism are slower in initiation and execution under these conditions - naturally enough, as E. elegans is a photosynthetic organism, and while photosynthesis is not required for growth on enriched medium, the presence of light under standard growth conditions presented here induces growth about 1/3 more rapid than that in the dark.

B. Effects of Acridine Orange:

The lack of effect of AO on NPR survival in E. elegans is good evidence that this organism possesses no NPR excision repair. This is consistent with the results of other work done with this organism, such as the finding of a lack of liquid holding recovery (Kemp and Wentworth, 1971). The small shoulder in the NPR survival curve indicates that there is a small amount of repair taking place in the dark by means of some mechanism not affected by AO. Acriflavine (which gives the same results in our system as does AO) has been found to interfere in several systems with the classical dark repair (Witkins, 1961; Davies, 1966; Singh, 1968; Cleaver, 1969).

Its mode of action has been specifically linked with the excision of pyrimidine dimers (Setlow, 1966 b). It has never been reported to interfere with PR (probably because of its light absorption qualities); from this, and from knowledge of the mechanism of PR (Jagger, 1958) and of the DNA-acridine complex (Lerman, 1961, 1963), it is possible that AO has no effect upon the monomerization of pyrimidine dimers by light. What, then, is the mechanism of action of AO on PR in _E. elegans_?

A possible mechanism is one that does not chemically affect the PR process but acts by simply absorbing the wavelengths of light required for PR. The major wavelengths required for PR in _E. elegans_ are not yet known; however, action spectra for purified PR enzyme from other plant systems show peaks at 4047 A for bean sprouts (Saito and Werbin, 1969) and 4358 A for _Anacystis nidulans_ (Saito and Werbin, 1970). _In vivo_ PR action spectra for other systems range from just over 300 nm to about 480 nm (Jagger, 1958; Jagger _et al._, 1970). Any irradiation of wavelength shorter than 300 nm tends to inactivate the organism, and no PR ability has been found for wavelengths greater than 500 nm (possibly light at these longer wavelengths is not absorbed by any PR enzyme). Since from figure III-5, the main light absorption effect of AO appears to be the removal of the peak at 450 to 460 nm (a finding consistent with work done by Zanker, 1952, in which he showed an absorption peak of 455 nm at the concentration used in these experiments), perhaps this is a critical wavelength for PR in _E. elegans_; it is certainly within the range suggested by reports of PR in other systems. The amount of total intensity reduction by the presence of AO is not in itself sufficient to

reduce PR survival (see Appendix II; cf. Fig. III-2).

However, as outlined in Results - UVL, it appears that AO may interfere with PR neither solely by light absorption nor by chemical interference, but by a combination of the two working simultaneously. Light absorption (an AO plate used as a light filter) alone, even at high concentrations of AO (Fig. III-7), did not reduce PR to the level reached when cells in contact with AO (at a much lower concentration) are exposed to PR light. Attempts to overcome the filter effect by means of increasing light intensity showed, however, that both "contact" and "filter" reductions of PR were overcome to approximately the same extent, suggesting that the effect of AO on PR survival is due mainly to light filtration by the AO (Fig. III-8). However, when light intensity was increased further with the use of a high-intensity Dicrolite lamp, the influence of the AO filter was overcome to some extent by the higher intensity, while AO in contact with the organisms (at 1/12 the concentration of the filter) maintained its effect at both intensities. Thus if this effect of AO on PR is due to selective concentration of AO in the cells, with subsequent absorption of PR light wavelengths, the concentration gradient from within the cell to the surrounding medium must be greater than 12-fold. Again, this suggests the possibility of a chemical effect of the AO in interfering with PR. The photodynamic killing action of AO under high light intensity makes the exact numbers obtained slightly uncertain; however, since this effect appears to be approximately the same on both irradiated and unirradiated cultures (Table III-1), the conclusions drawn above are not invalidated.

The elimination of PR and thus the masking of its decay by AO (Fig. III-3) is consistent with effects on PR and NPR survival found for AO (Fig. III-4). PR is completely prevented, and no effect on NPR survival is demonstrated. This is further supporting evidence for the lack of NPR excision repair in E. elegans, but does not aid in solving the problem of the mechanism of AO action on PR.

All evidence so far suggests strongly that the effect of AO on PR survival is due to light absorption, but the possiblity of chemical interference still cannot be completely ruled out. It has not yet been determined what type of chemical interference might be involved; this could be an area for further research.

C. Effects of Caffeine:

Caffeine reduces survival of irradiated E. elegans under both NPR and PR conditions. Reduction occurs at all dose levels under NPR conditions, and only at higher doses under PR conditions. In other systems, caffeine has been reported to interfere with dark repair mechanism, both excision repair (Witkin, 1961; Harm, 1967; Rauth, 1967; Cleaver and Thomas, 1969), and another type of dark repair not involving the excision of pyrimidine dimers (postulated for mouse L cells by Cleaver, 1969). There has also been a report of interference with PR in E. coli by caffeine (Harm, 1970, 1971).

A possible explanation for the effects of caffeine on PR in E. elegans is a change in the major class of UVL-induced DNA lesions at higher UVL doses, requiring another, more caffeine-sensitive repair mechanism.

As mentioned previously, the main source of the biological effects of UVL irradiation appears to be pyrimidine dimers; these lesions have been shown to be eliminated by PR (Setlow, 1966 a), dark repair (Setlow and Carrier, 1964), and by short-wavelength reversal (Setlow and Setlow, 1962). The possibilities for another class of repairable lesions are somewhat speculative, as UVL irradiation causes a fairly wide range of molecular changes in DNA (Jagger, 1958; Setlow, 1966 b), and not all of the biological effects of these are known. The two most likely possiblities are: guanine-cytosine dimers, which, though no thymine is present, are found to be photoreactivable (Rupert, 1964); and hydrates of cytosine and uracil, which, according to Ono, Wilson and Grossman (1965), cause DNA coding changes. These hydrates are heat-reversible, but so far have not been shown to be affected by an enzymatic repair system (Setlow, 1966 b).

Another, perhaps more plausible, explanation for the effect of caffeine on the survival levels of E. elegans following UVL irradiation is that caffeine interferes with some repair-like process during regular DNA synthesis (S phase), such as the by-passing of dimers suggested by Cleaver (1969). Thus can the small consistent reduction of NPR survival by caffeine be explained. This hypothesis also explains the shoulder region in the NPR survival curve, and the lack of effect by AO on this survival. The effect on PR can also be accounted for if it is assumed that the cell's PR mechanism is rapid enough that few UVL-induced lesions remain in the DNA at the time of the next S phase, until fairly high doses of UVL are used (since a definite time interval is required for the PR of each lesion; Harm, 1970). At some point the PR mechanism would not be

able to repair all repairable lesions before *S* phase began; the number of lesions remaining at the beginning of synthesis would increase from this point with increasing UVL dosage. If the cell possessed a caffeine-sensitive mechanism for by-passing pyrimidine dimers during DNA synthesis, results similar to those shown in figure III-10 would be expected. It is assumed that a certain proportion of pyrimidine dimers and/or other lesions remaining in the DNA during *S* phase would cause errors in replication, and thus result in mutation and/or lethality.

A possible mechanism for the by-passing of pyrimidine dimers is suggested by the results of Rupp and Howard-Flanders (1968). Using an *E. coli* mutant defective in excision repair, they found evidence that when DNA synthesis is proceeding and encounters a thymine dimer, a gap is left in the daughter DNA strand. Synthesis continues on the other side of the lesion; the gaps left are later either filled or eliminated, since viable daughter cells are produced. They suggest that rather than the gaps being filled with random nucleotides (which, according to them, would lead to high rates of mutation and lethality), they are eliminated by a process of recombination, leaving at least one daughter strand with the full complement of genetic information. Caffeine would be very likely to interfere with this type of "recombination repair", as there is evidence that caffeine interferes with the enzymatic linking of two segments of DNA (Cleaver, 1969).

The reduction in post-UVL survival by caffeine over the whole PR decay curve (Fig. III-3) is presumably due mainly to an interference with

NPR survival, as suggested by the low final survival in the presence of caffeine. The mechanism for this interference has been discussed above. PR survival would not be affected significantly under conditions illustrated by figure III-3 (UVL dose = 3000 ergs $mm^{-2}$), for it has been shown (Fig. III-10) that caffeine has no apparent effect on PR survival until UVL doses greater than 4000 ergs $mm^{-2}$ are used. This result, then, is consistent with the effects of caffeine on post-UVL survival as shown by other experiments.

The possibility must be borne in mind when considering the effects of these drugs on post-UVL survival, that they may simply be causing further damage to the DNA. They do not reduce the number of cfu in an unirradiated culture, but perhaps UVL-induced damage makes the DNA more susceptible to damage by other agents - in other words, a synergism occurs between the effects of the UVL and of the drug. However, this cannot be determined at present; and the more likely interpretation is probably that which is more consistent with the literature, and which attributes survival reduction by AO and caffeine to interference with DNA damage repair mechanisms.

D. Effects of Other Drugs on UVL Sensitivity:

The hypothesis of a lack of excision repair in _E. elegans_ is further supported by the response of the irradiated organism to iodoacetate, deoxyadenosine, and mitomycin C. Any or all of these should reduce the cell's capability for excision repair, but none had any effect on NPR post-UVL survival. The tendency for mitomycin C to reduce PR survival

at higher dose levels, giving similar results to those found with post-UVL treatment with caffeine, suggests that the efficiency of the cell metabolism as a whole is reduced by this compound, and thus the ability to synthesize DNA past pyrimidine dimers is reduced.

### E. UVL and the Biology of E. elegans

The great variety of life stages in a culture of E. elegans at any given time, and the difficulties involved in obtaining a synchronous culture, are the main factors reducing its attractiveness as an experimental organism (although Ken Lee, in our laboratory, has recently been successful in producing fairly well-synchronized cultures). There is some factor (or factors) other than coenobial size or life cycle stage which is introducing variability into experimental results. The relationship of UVL sensitivity to, simply, the biology of the organism, is still largely a mystery. We do not know whether changes in UVL sensitivity are due to differing numbers of cells per coenobium, to phases of culture growth, to timing of the light:dark cycle, to different stages in the cell cycle, or to any or all of these parameters. There is also much yet to study of intercellular interactions within each coenobium: How independent is each cell from its neighbours? How many cells must be killed by UVL to inactivate the coenobium as a colony forming unit? Can each coenobium be considered a true multicellular organism? Methods used in this study were not sufficient to answer these questions; this is an area for further research.

# CHAPTER IV

## INTRODUCTION - DRUGS

If *E. elegans* can repair DNA damage induced by UVL, does it have the capacity to repair lesions induced by other agents? Or, with the use of other agents, can UVL-induced lesions be "mimicked", inducing repair of the damage by DNA repair mechanisms? These questions prompted the use of the drugs ethyl methanesulfonate (EMS) and 4-nitroquinoline-1-oxide (NQO) to inactivate *E. elegans* and to induce repair, hopefully to produce a clearer picture of the repair mechanisms present in the organism.

It has been found that the lesions induced in DNA by EMS (a monofunctional alkylating agent) are repairable in *E. coli* by some mechanism different from that which repairs UVL-induced DNA damage (Bohme and Geissler, 1968). Thus the use of this drug as an inactivation agent may help to indicate the specificity of AO and caffeine, by comparing their effects on post-UVL and post-EMS survival.

The carcinogen NQO has been found to complex with the DNA molecule (Malkin and Zahalsky, 1966; Matsushima *et al.*, 1967), subsequently causing single-strand scissions, or "nicks" in the DNA (Sugimura *et al.*, 1968). Since the first step in NPR excision repair (see Introduction - General) is thought to be the enzymatic removal of the dimer, leaving a single-strand scission to be recognized by the next enzyme in the repair sequence, it was thought that NQO might mimic this first step and its damage be repaired by the excision repair mechanism. NQO has been reported to cause unscheduled DNA synthesis in mammalian cells (Stich and San, 1970), sug-

gesting that repair of NQO-induced lesions is possible. The use of this drug, then, was originally intended as another means of characterizing the repair mechanisms in E. elegans. The responses of E. elegans to both of these drugs, however (particularly to NQO), became of such interest that more time was devoted to them than was strictly required for a comparison with the response to UVL. The results reported in this chapter should help to elucidate the kinds of responses of E. elegans to various inactivating agents (as a means of comparison with other systems), the versatility of DNA repair processes in E. elegans, and the specificity of AO and caffeine as inhibitors of DNA repair.

MATERIALS AND METHODS - DRUGS

Cultures were harvested in late logarithmic phase, using the same methods as described in Materials and Methods - General. The resuspended culture (in phosphate buffer) was transferred to a sterile 25-ml Erlenmeyer flask, which, if dark treatment conditions were desired, was wrapped in aluminum foil. Treatment with EMS was carried out by adding 8 M liquid EMS directly to the culture, giving a final concentration of 0.2 M EMS. The flask was then placed on a shaking platform at $22 \pm 1°C$, and aliquots taken for survival assays at appropriate times. Dilution (10-fold) in sterile phosphate buffer was sufficient to stop the EMS reaction for the time it took to plate the sample following dilution.

Treatment with NQO was done in a similar manner, except that the NQO was taken from a stock solution of $10^{-2}$ M NQO in 95% ethanol. An

aliquot of this solution was diluted in phosphate buffer, and an appropriate amount of this latter solution was added to the coenobial suspension. Concentrations of NQO ranging from $10^{-7}$ M to $10^{-4}$ M were used, although the most frequently used was $10^{-6}$ M NQO.

Survival was assayed by the pour plating technique; incubation conditions were the same as those used for post-UVL incubation (i.e. "dark" conditions here correspond to NPR conditions used following UVL; "light" conditions correspond to PR conditions). If caffeine or AO was used, it was incorporated into the plating medium in the same way as was done for post-UVL treatment with these drugs.

RESULTS - DRUGS

Treatment with 0.2 M EMS produced a characteristic survival curve, with little effect for the first 40 minutes or so of treatment, followed by an abrupt change to exponential inactivation (Fig. IV-1). Plating EMS-treated cultures on medium containing 2 x $10^{-3}$ M caffeine had little effect on survival (Fig. IV-1); plating the same cultures on medium containing 15 μg/ml AO reduced survival somewhat, but with varying effectiveness and at varying times in different experimental cultures; figure IV-1 is an average of 3 experiments. Changing the treatment and post-treatment lighting conditions (i.e. from light to dark) had no discernible effect on inactivation by EMS.

Treatment of E. elegans with NQO also produced a characteristic survival curve (Fig. IV-2), though of quite a different nature from that

Figure IV-1. Survival of _E. elegans_ following treatment with EMS: effects of AO and of caffeine. Cultures were treated in phosphate buffer with 0.2 _M_ EMS: samples were taken for plating at intervals. Aliquots were plated on Bc (o—o), on Bc + 2 x $10^{-3}$ _M_ caffeine (□—□), and on Bc + 15 μg/ ml AO (▲—▲). All plates from this and other drug-treatment experiments were incubated in the light at 32 ± 1°C until colonies were countable.

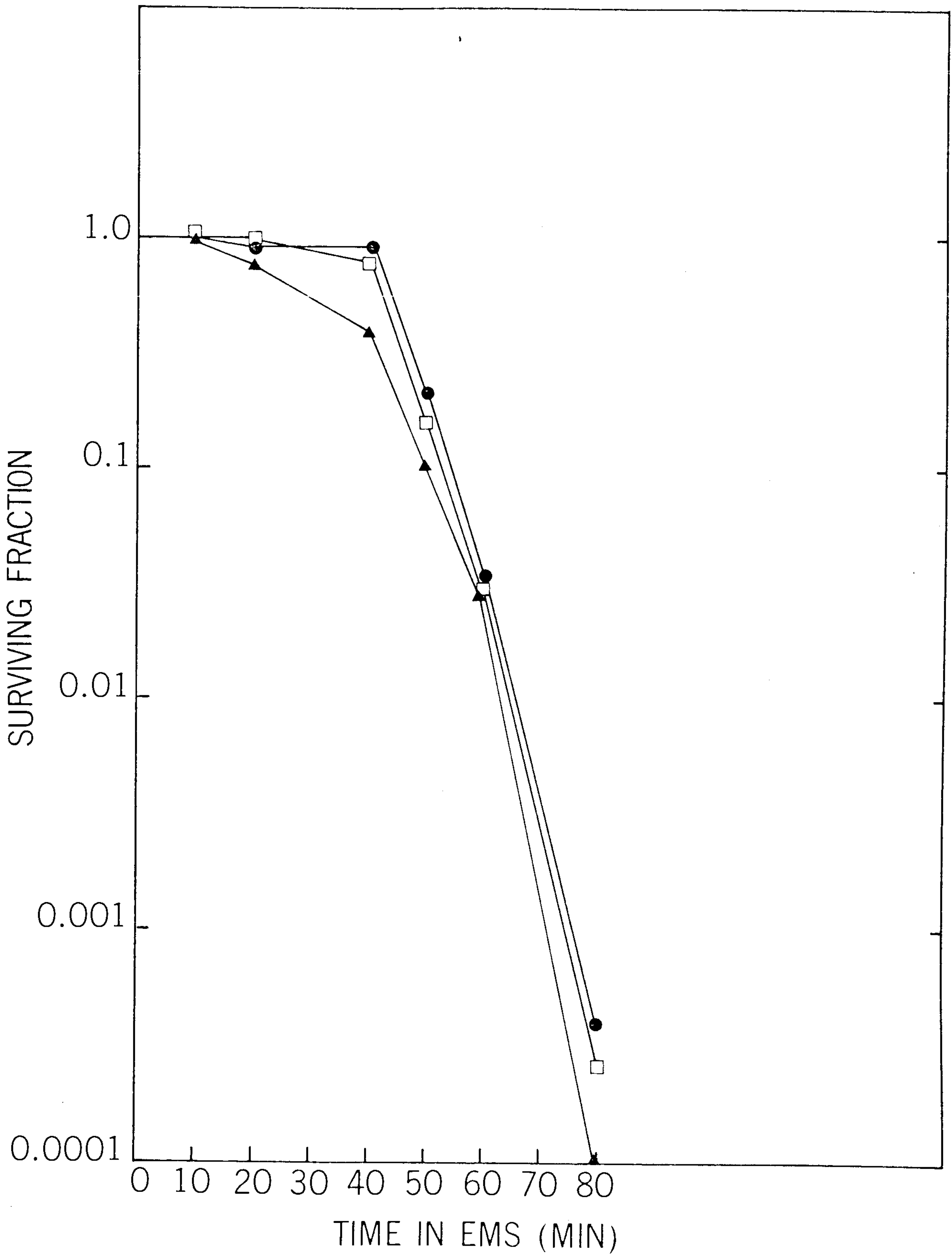
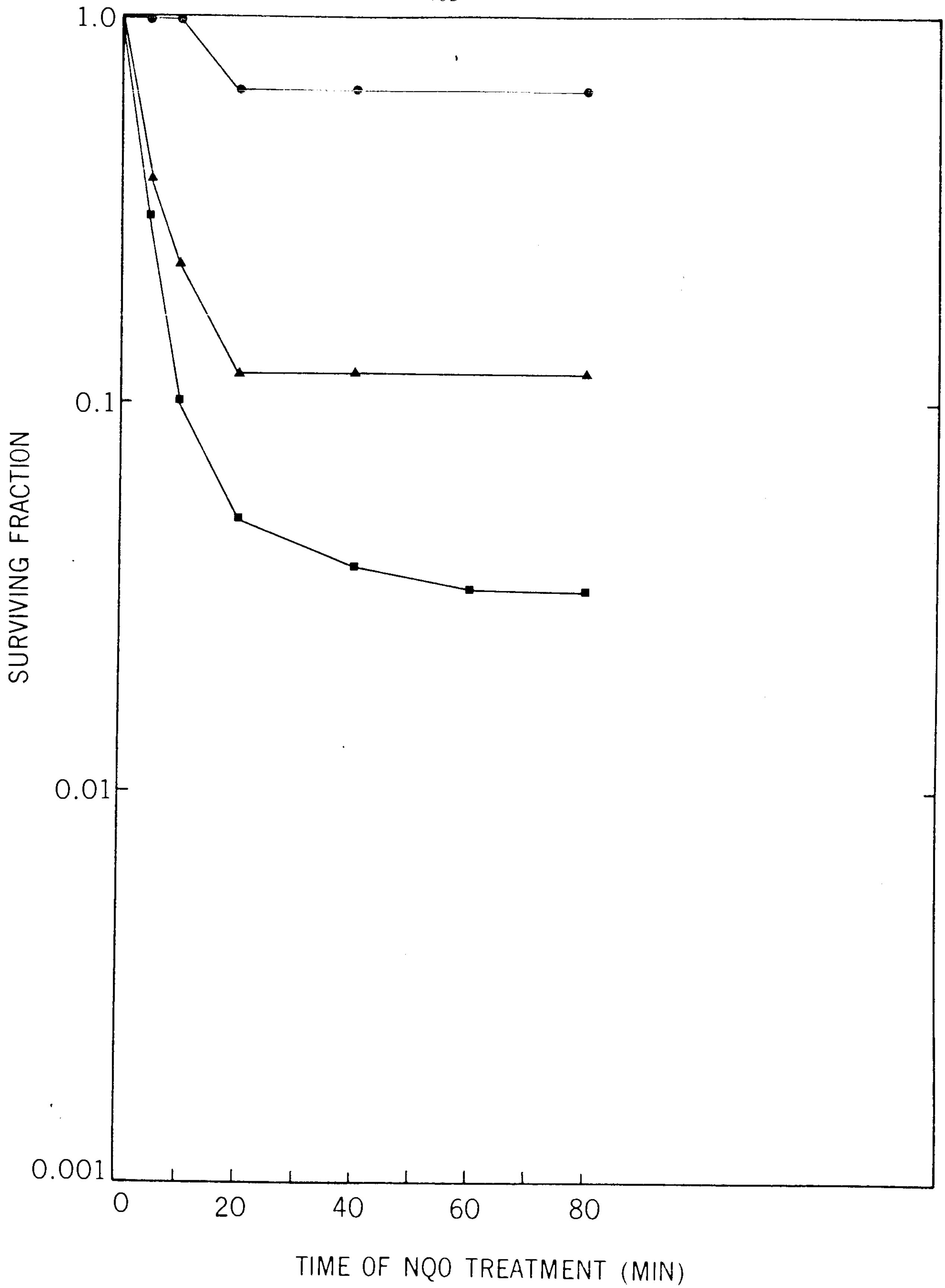

1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
0
10
20
30
40
50
60
70
80
TIME IN EMS (MIN)

Figure IV-2. Survival of E. *elegans* following treatment with NQO. Cultures were treated in phosphate buffer with $5 \times 10^{-7}$ M NQO (○—●), $8 \times 10^{-7}$ M NQO (▲—▲), and $10^{-6}$ M NQO (■—■). Samples were taken at intervals during the treatment and plated on Bc.


1.0
0.1
0.01
0.001
SURVIVING FRACTION
0
20
40
60
80
TIME OF NQO TREATMENT (MIN)

found with EMS. The shape of the curve did not vary with NQO concentration; only the final survival level varied. The final plateau in survival occurred 20 to 60 minutes after NQO treatment was begun (Fig. IV-2). This plateau was notdue, as proposed by Stich (personal communication), to inactivation of the drug in aqueous solution; a 5-hr-old solution (kept under the conditions used for treating cultures with NQO) is just as effective as a fresh one (Fig. IV-3). The presence of cells in the solution, however, does reduce the lethality of the NQO. If a $10^{-6}$ M NQO solution is used to treat a culture, producing a normal inactivation curve, the supernatant from this suspension has no lethal effect when used to treat a second culture. No effect is seen on the second culture to be treated until the concentration of NQO for the first treatment is raised to about $10^{-4}$ M; this concentration produces greater than 99.99% inactivation in the first culture, and about 50% inactivation in the second. If this is due to a high absorption of the drug by the cells, then the presence of this high drug concentration does not block further NQO action; addition of a fresh solution to a treated culture produces as much lethality as it does to an untreated culture (Fig. IV-4). It was thought that the loss of lethality of the NQO after a period of treatment time could be due to the presence in the coenobial population of an NQO-resistant sector. However, alternating NQO treatments with growing of the treated culture (in Bc, under standard growth conditions) in an attempt to produce an NQO-resistant strain of E. elegans met with no success. The cultures grown up from NQO-treated coenobia were just as susceptible to NQO as the parent culture, through four successive cycles of treatment and growth (Table IV-1).

The survival level is fairly closely dependent upon NQO concentration. If a series of different concentrations is used, with the same exposure time

Figure IV-3. Survival of *E. elegans* following treatment with fresh and 5-hr-old aqueous solutions of NQO ($10^{-6}$ *M*): a test of stability of NQO in aqueous solution. A culture was treated with $10^{-6}$ *M* NQO for 5 hr, with samples taken at intervals for plating (o—o). A control was run simultaneously in which the organisms were omitted, and which had an NQO concentration of $10^{-6}$ *M*. A second culture was then treated for 15 min with this control solution, and survival assayed (▲—▲).

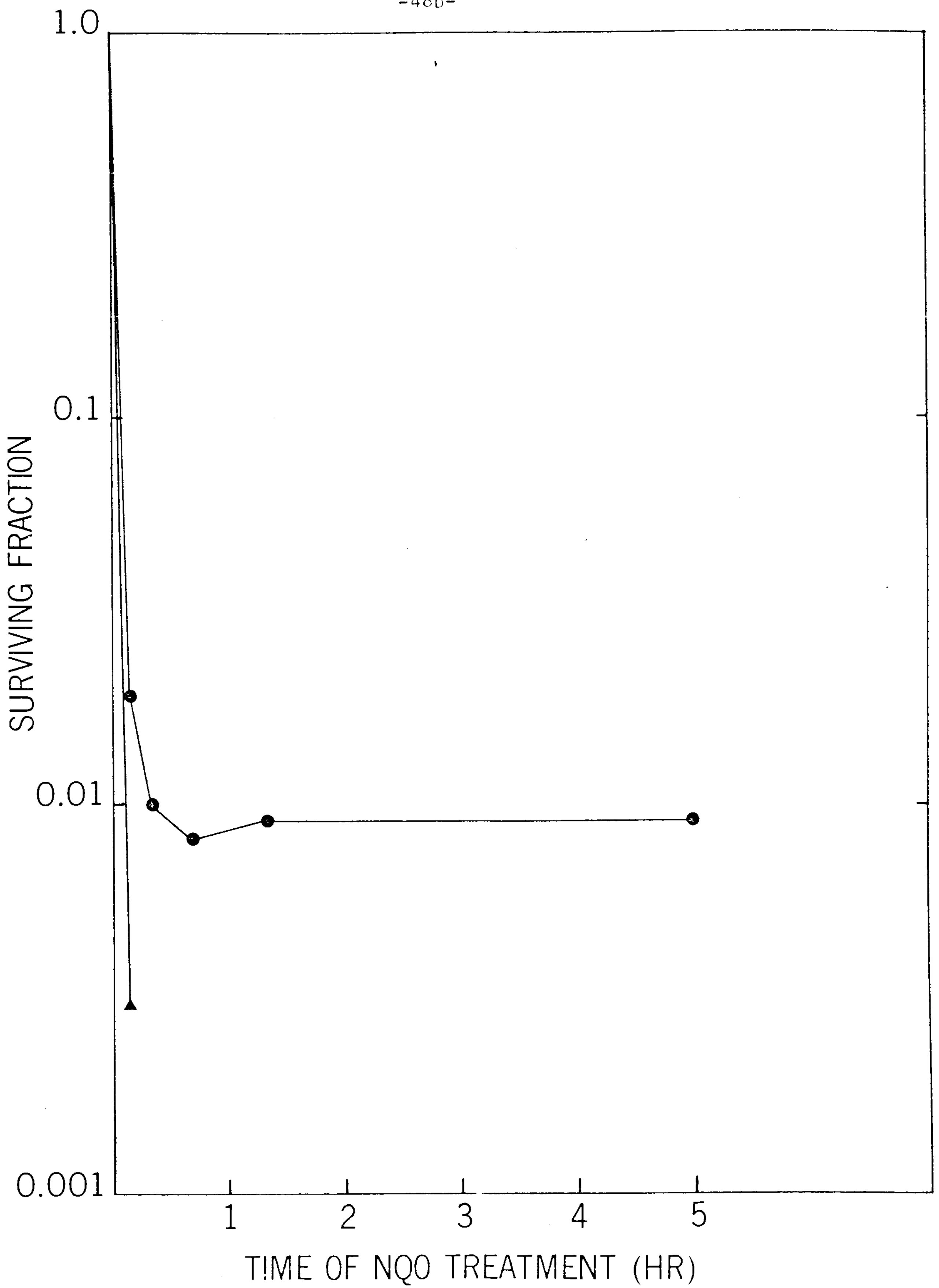
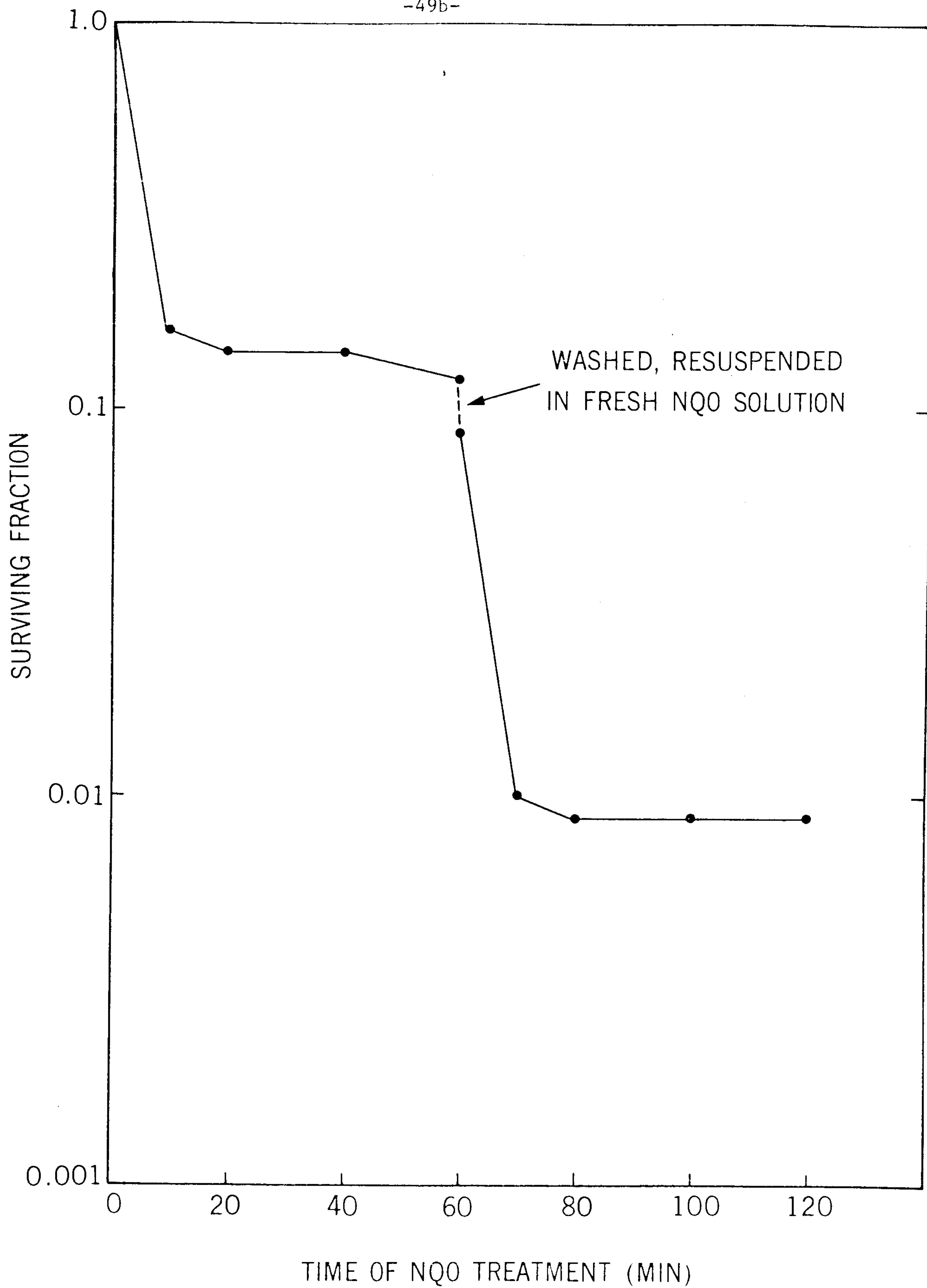

1.0
0.1
0.01
0.001
SURVIVING FRACTION
1
2
3
4
5
TIME OF NQO TREATMENT (HR)

Figure IV-4. Survival of _E. elegans_ following two successive NQO treatments. A culture was treated for 1 hr with $10^{-6}$ _M_ NQO; samples were taken at intervals and plated on Bc. The culture was then resuspended in a fresh solution of $10^{-6}$ _M_ NQO, and the same procedure followed.

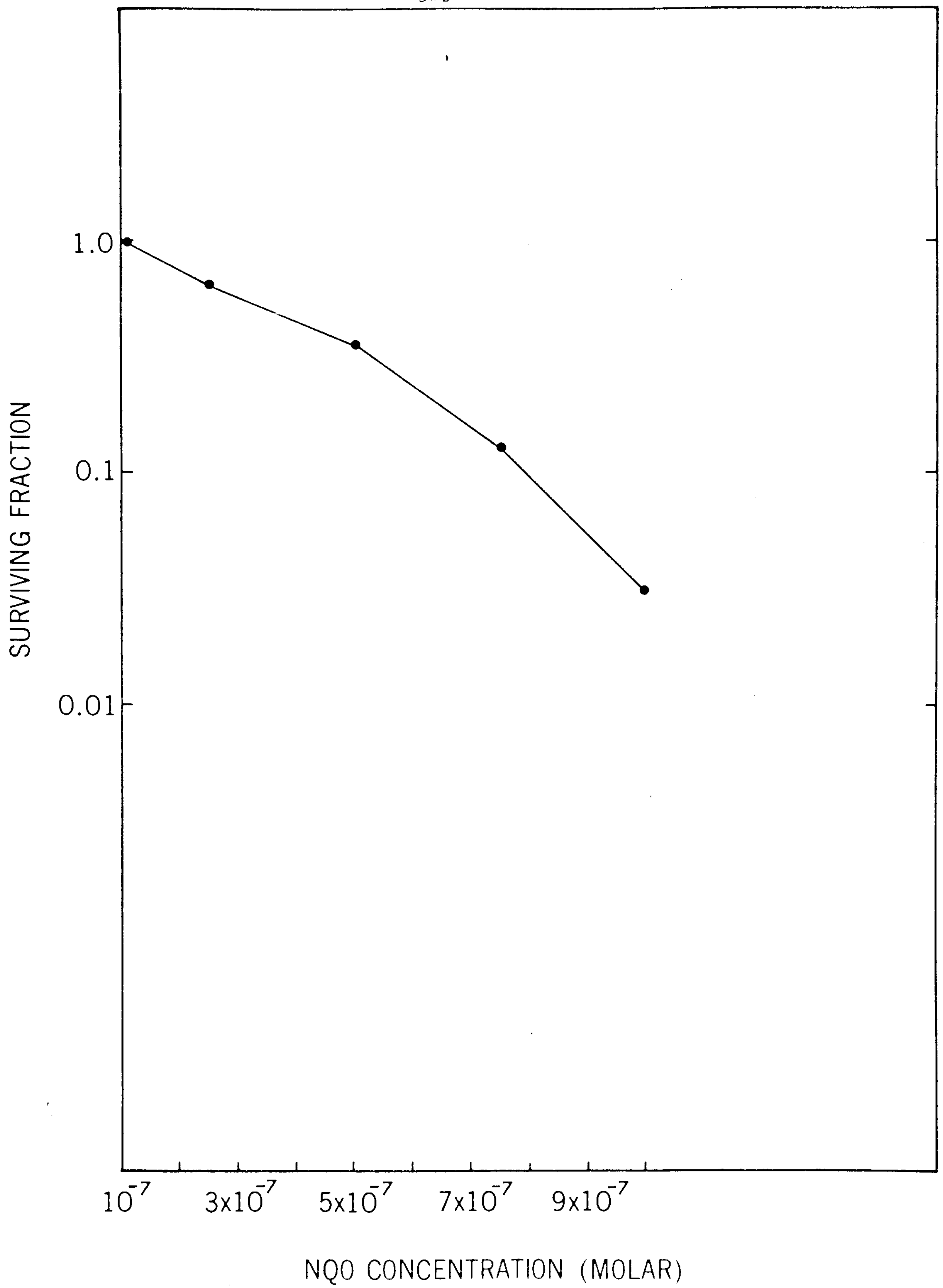


1.0
0.1
0.01
0.001
SURVIVING FRACTION
WASHED, RESUSPENDED
IN FRESH NQO SOLUTION
0
20
40
60
80
100
120
TIME OF NQO TREATMENT (MIN)

Table IV-1. Survival of E. elegans after successive treatments with NQO. The survivors of a 60-min treatment with $10^{-6}$ M NQO were allowed to grow under standard conditions for 2 days, when the treatment was repeated. A total of 4 NQO treatments was given.

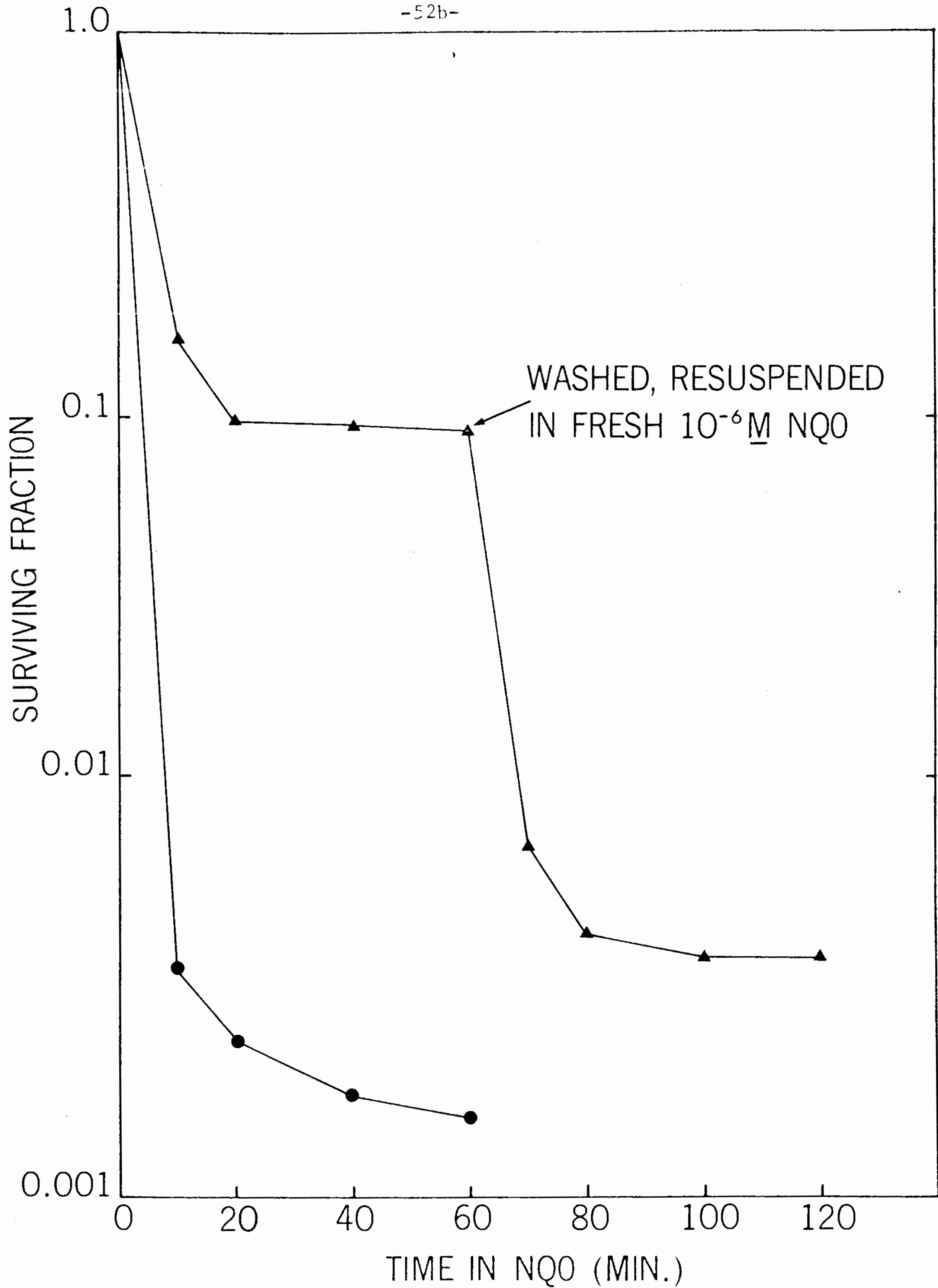
| Treatment # | Original cfu/ml | Post-treatment cfu/ml | % Survival |
|---|---|---|---|
| 1 | $8 \times 10^4$ | $1.4 \times 10^4$ | 17.5 |
| 2 | $1.6 \times 10^5$ | $1.1 \times 10^4$ | 6.9 |
| 3 | $7.4 \times 10^4$ | $1.3 \times 10^4$ | 18.0 |
| 4 | $1.2 \times 10^5$ | $1.8 \times 10^4$ | 14.7 |

Figure IV-5. Survival of E. elegans following treatment with several concentrations of NQO: a dose-dependent survival curve. A culture was divided into 5 aliquots and treated with 5 different concentrations of NQO for 30 min. All were assayed for survival (on Bc plates) at the end of this period. The curve given in this figure is the average of 3 such experiments.



1.0
0.1
0.01
SURVIVING FRACTION
$10^{-7}$
$3x10^{-7}$
$5x10^{-7}$
$7x10^{-7}$
$9x10^{-7}$
NQO CONCENTRATION (MOLAR)

Figure IV-6. The effects of NQO concentration on survival levels: two successive treatments with $10^{-6}$ M NQO *vs* one treatment with 2 x $10^{-6}$ M NQO. A culture was divided into 2 aliquots; one was treated with 2 x $10^{-6}$ M NQO for 1 hr (o—o), while the other was treated with $10^{-6}$ M NQO for 1 hr, washed, and resuspended in fresh $10^{-6}$ M NQO for 1 hr (▲—▲). Survival was assayed by plating on Bc at intervals during the treatment. The curves given in this figure are the average of 2 such experiments.



1.0
0.1
0.01
0.001
SURVIVING FRACTION
WASHED, RESUSPENDED
IN FRESH 10⁻⁶ M NQO
0
20
40
60
80
100
120
TIME IN NQO (MIN.)

for each concentration, a dose-dependent survival curve results (Fig. IV-5). This concentration dependence seems to hold true for successive doses, as well, as shown in figure IV-6; the survival level after two successive 1-hour treatments with $10^{-6}$ M NQO is close to that shown after a single 1-hour treatment with $2 \times 10^{-6}$ M NQO.

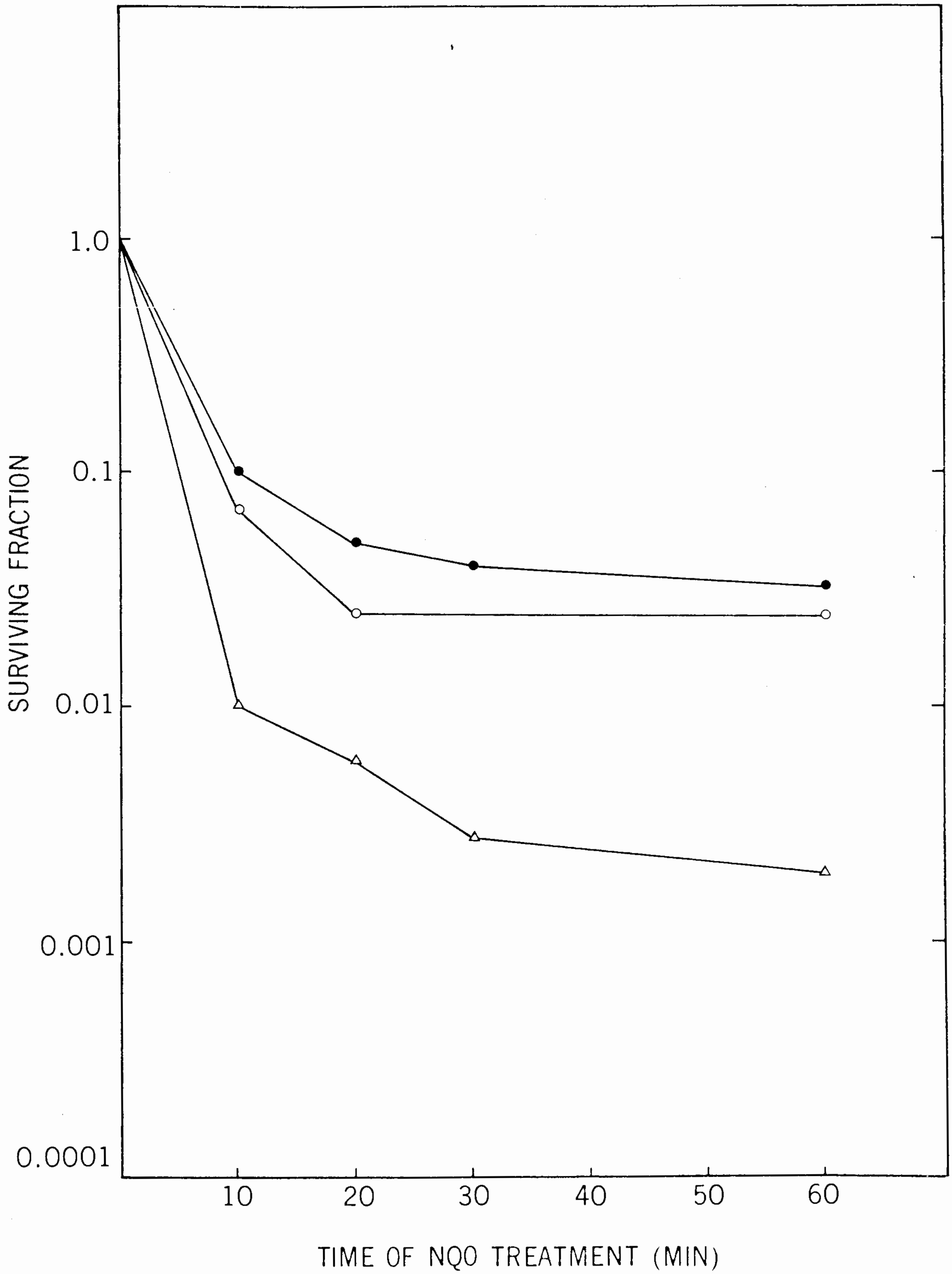
To try to discover if NQO-damaged DNA may be repaired in E. elegans, various post-treatment conditions were used. No effect was found with a change in lighting; thus there appears to be no photoactivated repair for this type of damage. The presence of caffeine ($2 \times 10^{-3}$ M) had little effect on post-NQO survival; however, the presence of AO (15 μg/ml) in the plating medium reduced survival in NQO-treated cultures (Fig. IV-7). Figure IV-7 shows the results of a single typical experiment; although the amount of reduction of survival by AO was variable, there was always a substantial reduction.

DISCUSSION - DRUGS

The drug EMS was used as an inactivating agent because its mode of action is quite different from that of UVL; thus any repair mechanisms acting on EMS damage to DNA would probably also be different in mechanism from those reactivating UVL-induced damage. The effect on this system of the drugs used to interfere with the repair of UVL-induced damage should indicate the specificity of action of those drugs - as well as indicating the presence of repair mechanisms for damage other than that caused by UVL.

The inactivation curves found with EMS treatment of E. elegans (Fig. IV-1) were somewhat atypical due to the large "shoulder" region; Loveless (1966) describes inactivation by alkylating agents as "instantaneous' inactivation", as treatment of bacteria or bacteriophage causes inactivation within 1 minute.

Figure IV-7. Survival of _E. elegans_ following NQO treatment: effects of caffeine and of AO. A culture was treated with $10^{-6}$ _M_ NQO for 1 hr, plating at intervals for survival. Aliquots were plated on Bc (●—●), on Bc + 2 x $10^{-3}$ _M_ caffeine (○—○), and on Bc + 15 μg/ml AO (Δ—Δ).


1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
10
20
30
40
50
60
TIME OF NQO TREATMENT (MIN)

The delay of inactivation of *E. elegans* may simply be due to time required for EMS to penetrate into the cells; or perhaps the number of alkylations and the degree of depurination must reach a fairly high level before the coenobium as a whole is inactivated. This latter reason is probably the more important, as EMS is a monofunctional alkylating agent, and as such only alkylates one base at a time (the predominant site of alkylation is N-7 of guanine: Loveless, 1966). Cross-linking does not occur, as it does with bifunctional alkylating agents; thus EMS is more potent as a mutagen than as a inactivating agent. However, as seen in figure IV-1, once the right conditions are reached in the cell, inactivation is rapid.

The interference of AO with survival after periods of EMS treatment may indicate some kind of repair process for alkylation. Loveless *et al*. (1965) observed a small amount of recovery following treatment of *E. coli* with the monofunctional 2-chloroethyl 2'-hydroxyethyl sulphide, but stated that it was only an indication, as attempts at recovery by the cell were swamped by continuing inactivation due to depurination. Bohme and Geissler (1968) report evidence for a repair mechanism for EMS damage, a mechanism associated with the *rec* function in *E. coli*.

The gap left in DNA following alkylation and depurination may provide a site for intercalation of the planar AO molecule (Lerman, 1963), either in the gap or between the disoriented bases on the opposite strand. Such intercalation could, like the insertion of a base analogue in such a position, disrupt subsequent DNA synthesis, resulting in mutation and lethality. Thus the effect of AO on EMS-treated cultures may be due to a kind of synergism between the effect of the two drugs rather than an interference with a repair mechanism.

This does not, of course, rule out the possibility of repair of EMS-induced damage; such repair was simply not detectable by methods used here.

The lack of marked effect of caffeine on EMS-induced inactivation suggests, perhaps, that caffeine has a fairly specific effect in E. elegans; caffeine may interfere only with the "recombination repair" hypothesized in Discussion - UVL, while AO has a more general effect. DNA damaged by almost any inactivating agent presumably will have irregularities in the molecule, and thus "gaps" between certain bases. These gaps would make the intercalation of AO that much easier; and the presence of the AO molecule in the DNA would disrupt DNA synthesis, as well as interfering with any sequential repair process (such as excision repair; Setlow, 1967) acting on the DNA molecule.

Kinetic studies of NQO inactivation have not been previously reported; however, the "leveling off" of inactivation (see Fig. IV-2) has been observed by Stich (personal communication), and ascribed by him to deterioration of the compound in aqueous solution. As described in the Results - Drugs section, experimental work shows that this is not so. An aqueous solution of NQO may be kept for several hours at 32°C, in the light, and retain all of its activity as an inactivation agent. The removal of its apparent activity was shown to be due to the presence of the organisms in the reaction mixture. The NQO seems to be absorbed very quickly by the cells and inactivated in some way. Perhaps, since NQO has been found to complex with DNA (Malkin and Zahalsky, 1966; Matsushima et al., 1967), the inactivation of the NQO molecule takes the form of irreversible binding to the DNA molecule. Another NQO inactivation mechanism

may involve its reduction in the cell to hydroxyamino quinoline oxide (HAQO), a process which has been shown to go on in mammalian cells (Sugimura *et al.*, 1968). This reduction product is just as effective as, if not more than, NQO, and is thought to be the proximate carcinogen in NQO treatments (Sugimura *et al.*, 1968); however, it is a very unstable molecule, and breaks down rapidly under aerobic conditions (Ishizawa and Endo, 1967).

If complexing of the NQO molecule with DNA does occur, it does not seem to block further action by fresh NQO. As can be seen from figure IV-4, addition of fresh NQO to an already treated culture (at the same concentration) produces a second inactivation curve almost identical to the first. Obviously there must be many sites available on the DNA for NQO action, whether this action involves irreversible complexing and "nicking" the DNA, or complexing, NQO reduction and nicking with subsequent HAQO breakdown, and release of an inactivated molecule.

There appears to be no sector resistant to NQO in the population of *E. elegans*; this was suggested by the two successive inactivations shown in figure IV-4, and confirmed by the failure to find any increase in NQO resistance in cultures grown out from those coenobia surviving an NQO treatment. Even when this treatment was repeated 3 more times (for a total of 4), with the same culture, no increase in resistance to NQO was observed. Another piece of supporting evidence for this conclusion is the observation that the final survival level is dependent upon the concentration of NQO (Fig. IV-5 and IV-6). There is some variability from culture to culture in susceptibility to NQO, but within one culture the

inactivation level seems to correspond only to NQO concentration.

A repair process for NQO damage is suggested by the response of the NQO-treated culture to the presence of AO. However, survival reduction by AO following either NQO or EMS treatment may be due, again, to photodynamic action on the DNA by AO - action made possible by alterations to the DNA by the other two inactivating agents. As with EMS-treated cultures, neither caffeine nor exposure to visible light had any effect on post-NQO survival. Thus, again, the action of caffeine in _E. elegans_ seems to be restricted to the interference with some process following UVL irradiation; and no photoactivated repair process is present to handle NQO-induced lesions. The single-strand scissions in the DNA molecule produced by NQO, however, would provide ample opportunity for the intercalation of AO. This would disrupt subsequent DNA synthesis, causing the observed decrease in survival by AO. This is not proof of a repair mechanism for NQO-induced damage; however, Kondo (1968) has found evidence for repair of nitroquinoline damage in _E. coli_ by some of the processes involved in NPR excision repair. Stich and San (1970) have reported that NQO causes unscheduled DNA synthesis in mammalian cells, a finding also suggestive of repair of NQO-induced lesions. As hypothesized above, though, unscheduled DNA synthesis may not occur in _E. elegans_, since there is much evidence for the lack of repair replication in this organism. The actual kind of repair process can only remain in question until DNA labelling and extraction techniques have been perfected for this organism, providing more information upon which to base a hypothesis.

# CHAPTER V

## DISCUSSION - GENERAL

This study is concerned with mechanisms present in *Eudorina elegans* which can reactivate damage caused to the DNA by various agents; these mechanisms are possibly also important as processes involved in mutation and recombination. If such repair were not possible, most organisms would not be able to survive the combination of conditions adverse to life on earth. But repair mechanisms have evolved to reduce lethality and mutation rates both in the dark, for those organisms never exposed to sunlight, and in the light. Photosynthetic organisms, which depend on sunlight directly as a source of energy, are exposed to large doses of UVL (Harm, 1969; Cleaver, 1970); for them to have survived and reproduced to the present day, an efficient light-activated repair process must have evolved soon after the advent of the first microorganisms on earth. This process, found in many organisms today, continuously repairs new UVL-induced lesions. Possibly the efficiency of this system has resulted in the loss of the ability for NPR excision repair in *E. elegans*, and perhaps in other photosynthetic organisms as well; for this ability may be considered redundant in such a system.

PR seems to be restricted in *E. elegans* to the repair of UVL-induced lesions; no evidence was found for PR of any drug-induced damage. There does seem to be another type of repair for some drug induced lesions;

but beyond the observations that the repair is not photoactivated and that it may be interfered with by AO, very little can be said about it.

A repair-like mechanism, not requiring light, was hypothesized for UVL-induced lesions left unrepaired at the onset of S phase; this mechanism, termed recombination repair (Rupp and Howard-Flanders, 1968), could presumably correct other types of lesions as well, although little evidence was found for its action on either EMS- or NQO-induced damage.

This study has expanded somewhat upon a part of the field of research dealing with DNA replication and conservation: that part concerned with the repair and reactivation of damaged DNA. It extends the knowledge gained from techniques used mainly with prokaryotic, unicellular, heterotrophic organisms to a eukaryotic, multicellular autotroph; thus the responses of this system may be compared directly with those observed in other systems. In common with most organisms studied, E. elegans has an efficient PR mechanism for reactivating UVL-damaged DNA; unlike most organisms studied, no evidence of NPR excision repair was found. This may prove to be the case with many photosynthetic organisms, as possession of a dark repair mechanism is not essential to survival if there is an efficient PR system making use of the abundant energy in sunlight. However, Davies (1966) has found evidence for dark repair in Chlamydomonas; thus data from many other organisms must be gathered before generalizations may be made.

The multicellular nature of E. elegans raised many questions and provided few answers. The investigations of intercellular interactions and their effect on UVL sensitivity met with no success; this is an area

which could prove very promising for further research, as the colonial organism represents a transition between unicellular and true multicellular systems. The heterogeneity of the system with respect to the number of cells per coenobium at any given time also presented a problem, as it is a source of variability for any kind of kinetic study.

There has been evidence presented for the presence of certain kinds of DNA repair mechanisms; but further characterization of these processes must wait until the biochemical techniques useful in this system have been worked out. The basis for the characterization of a repair system has been laid down with the demonstration in E. elegans of the presence of thymine dimers following UVL irradiation, and their elimination following PR (Kemp, Tsao and Thorson, 1972).

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Appendix I: Growth Media for *Eudorina elegans*.

Minimal Medium for *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

Stock Solution III (EDTA Solution) .................. 1.0 ml

Stock Solution IV (Ferrous Sulfate Solution) ......... 1.0 ml

Bring to 1000 ml with Pyrex-distilled water, pH 6.8 - 7.0

Stock Solutions:

I. Bristol's Stock Solutions (in Pyrex-distilled water)

| | | Molar Concentration | Final Concentration in Medium |
|---|---|---|---|
| A. | $NaNO_3$ | 0.294 | $2.9 \times 10^{-3}$ M |
| B. | $CaCl_2$ | 0.025 | $2.5 \times 10^{-4}$ M |
| C. | $MgSO_4.7H_2O$ | 0.0305 | $3.05 \times 10^{-4}$ M |
| D. | $K_2HPO_4$ | 0.043 | $4.3 \times 10^{-4}$ M |
| E. | $KH_2PO_4$ | 0.129 | $1.29 \times 10^{-3}$ M |
| F. | NaCl | 0.060 | $6.0 \times 10^{-4}$ M |

II. Gaffron's Trace Element Solution: to 1000 ml Pyrex-distilled water add:

$H_3BO_3$ .................................... 3.100 g

$MnSO_4.4H_2O$ ................................ 2.230 g

$ZnSO_4.7H_2O$ ................................ 0.287 g

$(NH_4)_6Mo_7O_{24}.4H_2O$ ......................... 0.088 g

II. Gaffron's Trace Element Solution (cont'd)

| | |
|---|---|
| $Co(NO_3)_2 \cdot 4H_2O$ | 0.146 g |
| $Na_2WO_4 \cdot 2H_2O$ | 0.033 g |
| $CuSO_4 \cdot 5H_2O$ | 0.125 g |
| KBr | 0.119 g |
| KI | 0.083 g |
| $Cd(NO_3)_2 \cdot 4H_2O$ | 0.154 g |
| $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ | 0.198 g |
| $VOSO_4 \cdot 2H_2O$ | 0.020 g |
| $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$ | 0.474 g |
| $Cr(NO_3)_3 \cdot 7H_2O$ | 0.037 g |

Adjust pH to approximately 7.5 with HCl, after autoclaving.

III. EDTA Solution: to 1000 ml Pyrex-distilled water add:

| | |
|---|---|
| EDTA | 50.0 g |
| KOH | 31.0 g |

IV. Ferrous Sulfate Solution: to 1000 ml Pyrex-distilled water add:

| | |
|---|---|
| $FeSO_4 \cdot 7H_2O$ | 4.98 g |
| $H_2SO_4$ (10 N) | 1.0 ml |

Complex (undefined) Medium for _Eudorina elegans_ (Bristol's Complete Medium)

Preparation of Medium:

| | |
|---|---|
| Bristol's Minimal Medium | 800 ml |
| Stock Solution V (Modified Euglena Medium) | 200 ml |

Stock Solutions:

V. Modified Euglena Medium (after Starr, 1964):

| | |
|---|---|
| Yeast extract (Difco) | 2.0 g |
| Sodium acetate | 1.0 g |
| Beef extract (Difco) | 1.0 g |
| $CaCl_2$ | 0.01 g |
| Stock Solution VI (Casamino Acid Solution) | 20 ml |
| Pyrex-distilled water | 980 ml |

VI. Casamino Acid Solution:

| | |
|---|---|
| Casamino acid hydrolysate (Difco vitamin free) | 100.0 g |
| Pyrex-distilled water | 1000 ml |

Add a 0.1 g of charcoal to decolorize. Filter; Millipore filter; autoclave.

Appendix II: Spectroradiometer readings (giving absolute intensities) from Cool White fluorescent lights with and without agar plates.

Figure i. Cool White fluorescent light without agar plate. The figure shows absolute intensity (▲—▲), and intensity normalized to 1.0 at 600 nm (+—+).

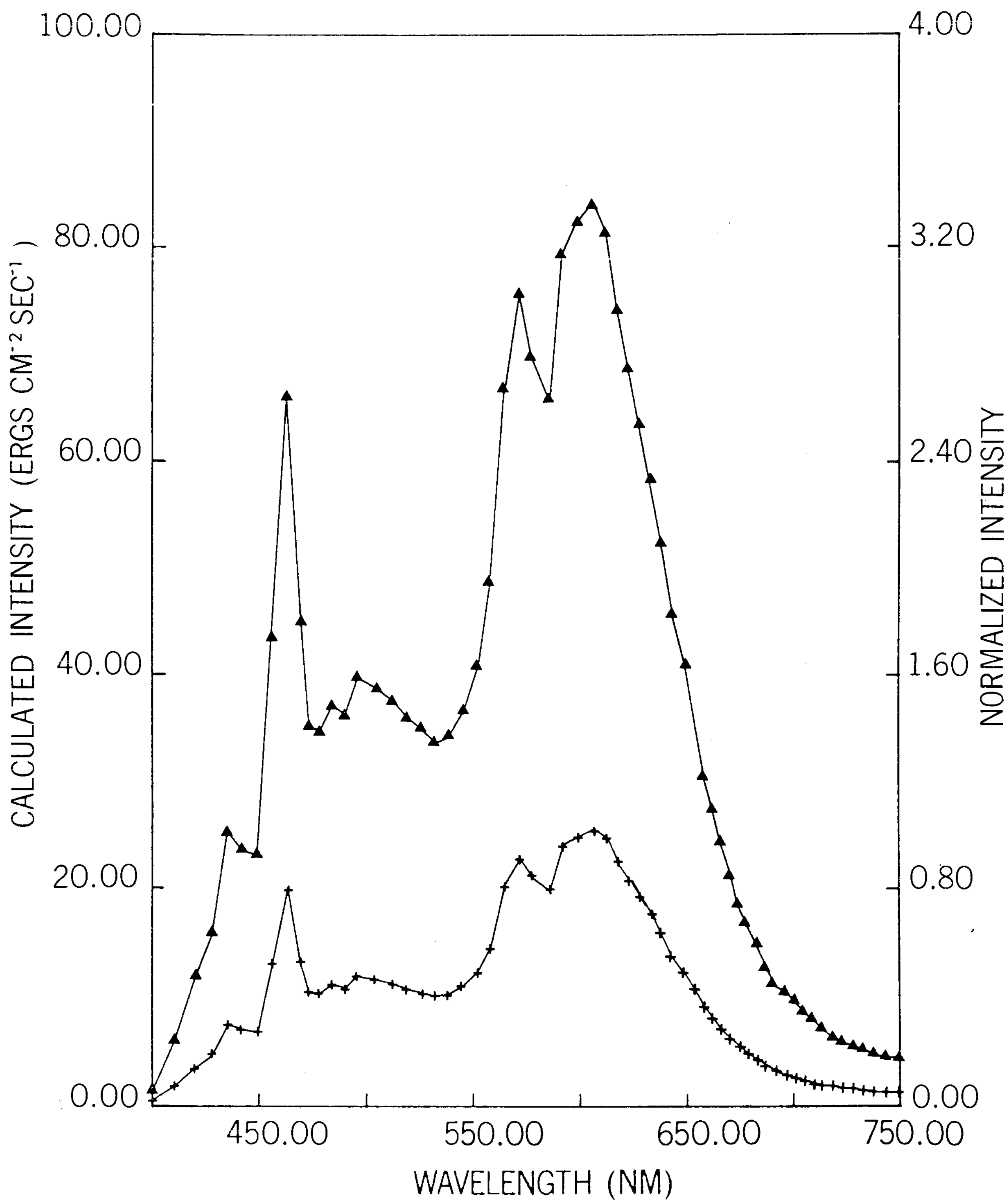
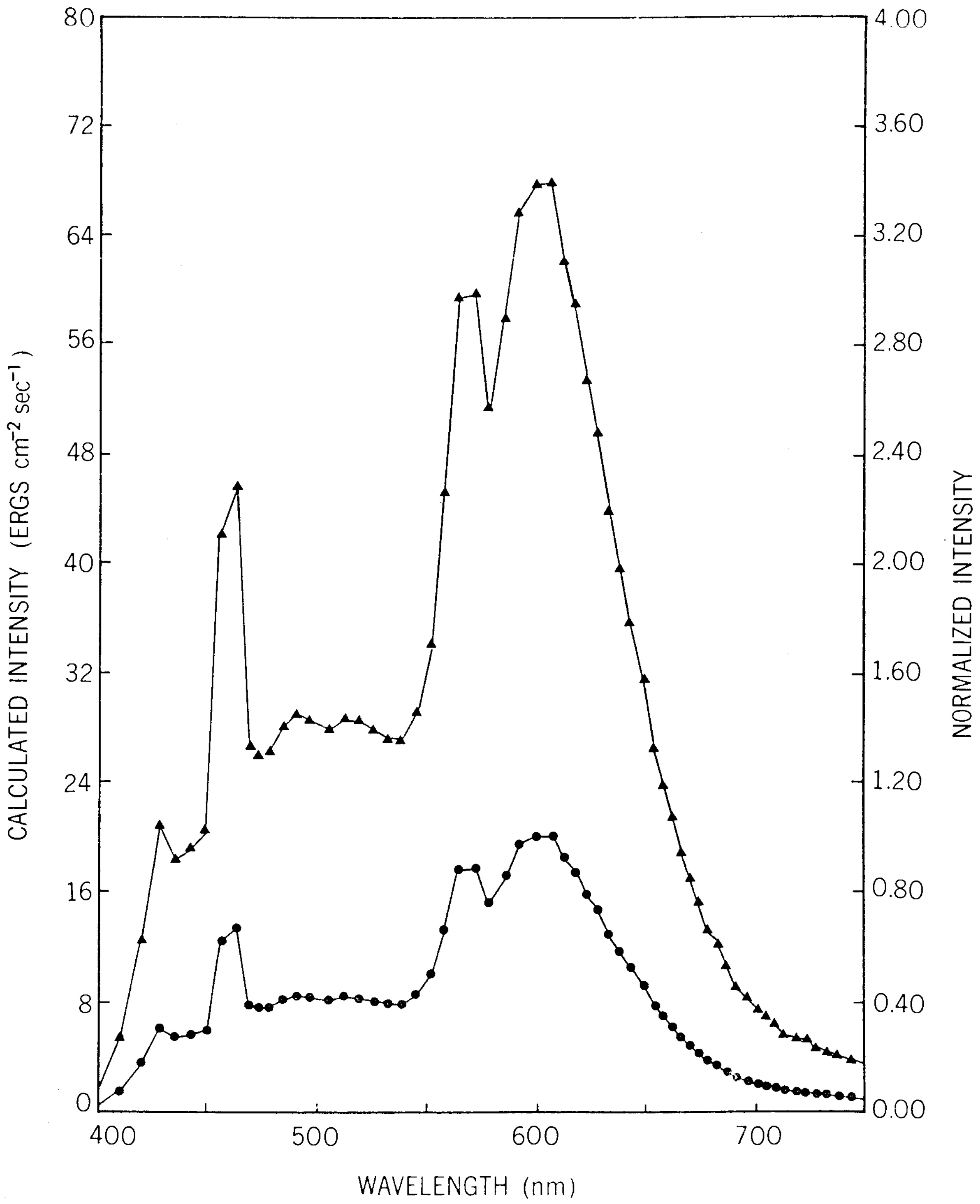
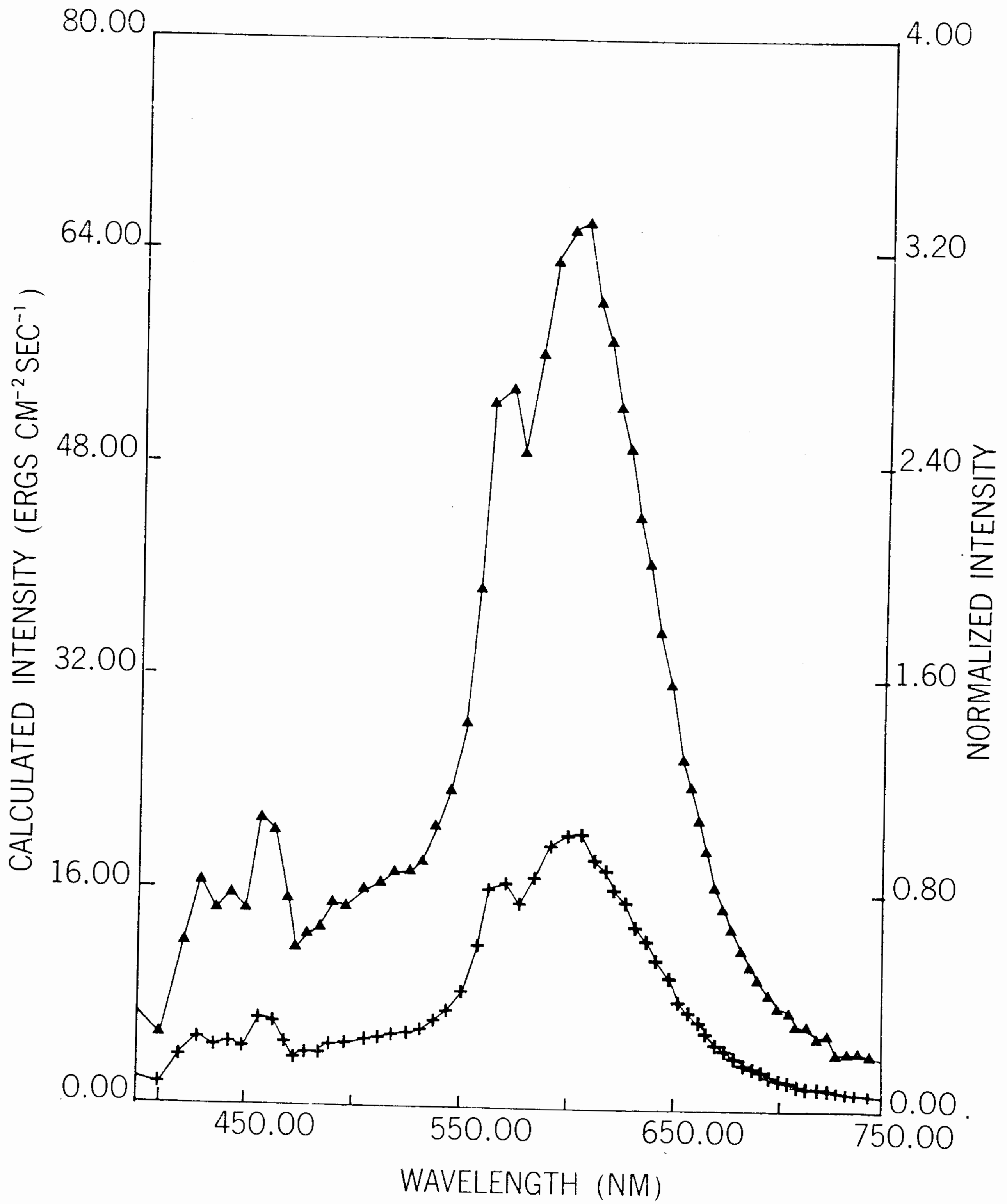

100.00
80.00
60.00
40.00
20.00
0.00
4.00
3.20
2.40
1.60
0.80
0.00
CALCULATED INTENSITY (ERGS CM-2 SEC-1)
NORMALIZED INTENSITY
450.00
550.00
650.00
750.00
WAVELENGTH (NM)

Figure ii. Cool White fluorescent light transmitted through Bc plate. The figure shows absolute intensity (▲—▲), and intensity normalized to 1.0 at 600 nm (o—●).



80
72
64
56
48
40
32
24
16
8
0
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
400
500
600
700
WAVELENGTH (nm)
CALCULATED INTENSITY (ERGS cm$^{-2}$ sec$^{-1}$)
NORMALIZED INTENSITY

Figure iii. Cool White fluorescent light transmitted through Bc + 15 μg/ml AO plate. The figure shows absolute intensity (▲—▲) and intensity normalized to 1.0 at 600 nm (+—+).



80.00
64.00
48.00
32.00
16.00
0.00
4.00
3.20
2.40
1.60
0.80
0.00
CALCULATED INTENSITY (ERGS CM$^{-2}$ SEC$^{-1}$)
NORMALIZED INTENSITY
450.00
550.00
650.00
750.00
WAVELENGTH (NM)

# CURRICULUM VITAE

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