DNA Repair in *Dictyostelium discoideum* Exposed to Pesticides and Ultraviolet Light

by

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Abstract

The cellular slime mold Dictyostelium discoideum is a good organism for studying the excision repair process. The fungicide benomyl is known to have some unusual mutagenic properties; for this reason, we studied its effects on excision repair in the slime mold. By doing a series of survival studies, we first corroborated the existence of genetic repair in the organism. Later, we were able to show a dose dependent decrease in cell viability by benomyl following the inducement of genetic damage with ultraviolet light. Other studies under starvation conditions provided some evidence that the nutrient state of the cell has an influence on genetic repair. A cell division study in buffered saline shows a 17.9% cell increase over a 2.5 hour period.

Acknowledgements

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Introduction

The widespread use of commericial pesticides has necessitated an increase in research efforts aimed at understanding the biochemical interactions these compounds have with living systems. One such commericially available compound, benomyl, is known to have some unusual mutagenic properties in the bacteria Salmonella typhimuruim (1), and is thus suspected of exerting some type of influence on genetic repair. The objective of the research reported here has been to investigate the possible influences this compound might exert on excision repair in the cellular slome mold Dictyostelium discoideum.

D. discoideum is a good research subject for several reasons. This organism has a fairly rapid reproductive cycle; it can be grown and maintained with relative ease, and at low cost; it is a eukaryotic organism. D. discoideum has the added advantage of being one of the most radioresistant organisms known, indicating the existence of an efficient genetic repair system. Finally, the slime mold displays a differential life cycle consisting of vegetative and developmental phases; this attribute lends itself to the study of cellular differentiation processes.

Review of the Literature

Dictyostelium discoideum

The life cycle of Dictyostelium discoideum (Dd) can be divided into roughly three stages. In the first stage, Dd exists as a free-living amoebae (10μ in diameter) which feeds on bacteria. As the supply of bacteria becomes exhausted, a complex series of biochemical events initiates the second stage, aggregation. The signal to start aggregation is extra-cellular cAMP secreted by certain initiator cells; in response to the cAMP, the amoebae begin streaming together into aggregates of about 10^5 cells. As the ameobae are chemotactically attracted to the cAMP center, they in turn begin the secretion of extra-cellular cAMP, which ultimately results in a cAMP gradient. As aggregation progresses, the amoebae finally form a psuedoplasmodium, or grex capable of migration over the substrate. The grex usually migrates for several hours and stops, marking the beginning of the third stage, culmination. In culmination, Dd sends up a stalk, and eventually differentiates into a fruiting body. The fruiting body contains spores in an apical capsule; under suitable conditions, the spores are released

from the fruiting body and germinate.

In this project a mutant strain of Dd, Ax-2, was used in place of the wild type described above. The Ax-2 amoebae are capable of growth and reproduction on a specially defined liquid medium (2), devoid of bacteria. The Ax-2 amoebae have a generation time of about 9 hours, which is longer than that of the wild type; the organism is normal with respect to the other phases of the life cycle.

The amoeboid stage of Ax-2 contains a cell cycle composed of the following phases: a 1-2 hour S phase of DNA synthesis; a 4 hour G_2 phase preceding mitosis; a 1-2 hour M phase during which cell division occurs. The salient feature of this cell cycle for our purposes was the cessation of growth and synthesis at a point in G_2 following periods food deprivation (3). Under conditions of starvation, the amoebae continue through the cell cycle until the middle of G_2, where cell growth and DNA synthesis stops as the cells prepare to aggregate. By placing the Ax-2 amoebae in nutrient-free media, we hoped to stop the semi-conservative synthesis of DNA.

UV Effects and Genetic Repair

The primary photoproducts formed in DNA by ultraviolet light in the 250 nm range are pyrimidine dimers. The majority of the dimers produced are of the thymine-thymine type, although some cytosine-cytosine and thymine-cytosine dimers are formed. The mechanism for the formation of dimers involves the removal of a water molecule to form a cyclobutane ring bewteen the adjacent thymine bases. There is evidence that indicates that dimer formation in long pyrimidine tracts is cooperative, due to the denaturing effect each dimer has on the helix; a single dimer has a straightening effect which places adjacent regions in a conformation favoring dimer formation. Dimerization represents a substantial local distortion of the DNA helix.

The following types of genetic repair mechansims have been demonstrated in eukaryotic organisms: 1.) photoreactivation, 2.) excision repair, or dark repair, 3.) postreplication repair. Photoreactivation, which has been demonstrated in Dd, is an error-free mechanism involving one enzyme (PRE), which simply cleaves the cyclobutane ring adjoining adjacent bases; PRE is inactive, unless vitalized by light in the far-UV region (370 nm) (5). Postreplication repair, as the name suggests, occurs one replication cycle after the lesion has formed and involves the replacement of as many as 800 bases by de novo synthesis. The postreplication repair medhanism is non-specific for the type of damage

it can repair, and may be error prone.

The third repair mechanssm, excision repair, is a non-specific system involving at least 4 enzymes in eukaryotic cells. The excision mechanism in initiated when a specific endonuclease detects a distortion in the helix due to some form of DNA damage (e.g. pyrimidine dimer, protein adducts, cross-links); an endonuclease makes a strand incision near the lesion causing an oligonucleotide of as many as 100 bases to be displaced. The excision of the lesion-containing oligonucleotide is completed by a 5′-3′ exonuclease, whose cut is usually made many bases away from the lesion. The replacement and sealing of the excised segment is accomplished with a polymerase and a ligase. The excision step and the base replacement step probably occur simultaneously. Excision repair appears to be largely error-free; although, Nishioka and Dudley (6) have postulated the existence of an error-prone excision repair system in E.coli. The existence of such an error-prone system in eukaryotic cells has not been conclusively demonstrated.

Benomyl

Benomyl (methyl-1-(butylcarbomyl)-2-benzimidazole carbamate) is a commonly used systemic fungicide, which has been shown to have mutagenic activity (3) in bacteria. Talpaert et al (7) reports a significant decrease in DNA synthesis and mitotic activity in the fungus Fusarium oxysporum by the primary benomyl metabolite MBC (methyl-2-benzimidazole carbamate). The most convincing evidence for the the supposition that benomyl influences genetic repair comes from Kappas et al.; they suggest that MBC acts as a unusual purine analogue, which promotes the misrepair of gaps in newly synthesized DNA. The structure of MBC is compatible with the theory that MBC is incorporated into DNA as a non-pariing purine analogue with a large aryl group, which passes through the replicating fork during semi-conservative synthesis and causes the formation of gaps in the daughter strands in a manner similar to that of dimers (8). It is possible that these gaps in the newly synthesized DNA are misrepaired by an error-prone pathway, analogous to the one suggested by Witkin (8).

Work on the effects of pesticides on genetic repair in D. dictyostelium is limited to a doctoral dissertion by R. J. Bushway (9). As far as we know, the effect of benomyl on repair in Dd has not been investigated outside of our research effort.

Materials and Methods

In all studies conducted in this project, the Ax-2 strain of Dictyosteluum discoideum was used as the experimental organism. The experiments with Dd were all performed during the amoeboid, or cellular stage of the life cycle; the amoebae in all cases were used during the phase of logarithmic cell grow, which extends to a maximum concentration of about 2.5×10^7 cells/ml (10). Throughout the research, samples taken from stocks in the stationary phase were transferred to fresh culture media to maintain the cell supply. To avoid the formation of aberrant cell forms in the stock, these transfers were carried out no more than seven times. For many experiments, the cells were grown up from supplies of frozen spores. In all procedures involving amoebae in growth media, we used aseptic technique. The cells for all experiments were grown in an Environ-Shaker (160 rpm) maintained at a temperature of 23°C.

UV Survival Studies

Ax-2 amoebae were grown to a concentration of approximately 5×10^6 cells/ml in 20 mls of liquid growth media defined by Ashworth and Watts (2). Immediately prior to irradiation, the cells were centrifuged at 1200 rpm for 5 minutes, washed, and resuspended in 20 mls of ice cold phosphate buffered saline (PBS). This washing procedure was carried out a total of three times to remove any adhering nutrient media. The cells were suspended a final time in sufficient cold PBS to bring the concentration to 2.0×10^6 cells/ml. To irradiate the ameobae, a 4 ml aliquot of the suspended cells were placed in a sterile irradiating dish, which had been previously packed with ice along the outer compartment. A stirring flea kept the cells suspended through out the UV exposure to maintain an even distribution in the dish. The UV source was a Sylvania 15-watt germicidal lamp (253.7 nm); the fluence density for all studies was approximately 15×10^2 μw-cm^2. The UV dosage was always measured as a function of the exposure time; in all studies these exposure times were 0,10,30, and 60 seconds. Following each exposure period, a .1 ml aliquot of amoebae was removed from the irradiating dish and placed in cold PBS dilution tubes. A series of dilutions were carried out such that the final tube contained about 2.14×10^3 cells total, i.e.viable and inviable. The cells for each UV dosage were spread plated on .1 ml aliquots of E. coli B/r in PBS. All plating was done in triplicate. The plates were allowed to incubate for 3 days at 23°C in total darkness. Following incubation, the plates were counted for plaque formations

(clear spots) in the agar at approximately 24 hour intervals until the sixth post-irradiation day. The percentage survival at each dosage was calculated from the number of surviving cells (plaques), as compared with the zero UV dosage control. From this data the survival curves were plotted.

A total of six survival studies were carried out, each designed to show what effect one or more experimentally controlled parameters had on cell viability. The survival studies consisted of the following control parameters, as they were performed in chronological sequence: 1.) the effect of UV light alone, 2.) the effect of UV light and benomyl, 3.) the effect of UV light under starvation conditions, 4.)the effect of UV light and benomyl under starvations. The common denomitor in all cases is UV irradiation, which was the agent primarily responsible for the damage and/or death of the cells. In each study every effort was made to duplicate the technique used in the first studies so that certain data comparisons could made.

Benomyl Plating

To determine how benomyl influences amoebae viability, we conducted survival studies, in which the cells were incubated post-irradiation on benomyl impregnated agar plates. The procedure followed in these studies was identical to all other survival studies, except with regard to the preparation of the BPM agar. To make the plates, stock solutions containing 17.5 mg and 35 mg benomyl in 10ml of nanograde acetone were prepared (9). From these stock solutions, 1.2 ml aliquots were withdrawn and mixed with 418.8 ml volumes of liquid BPM agar at approximately 50°C to produce agar solutions of 5 ppm and 10 ppm in benomyl. This liquid media was used to pour plates having a total volume of about 35 mls. The plates were stored in the dark at 23°C and used no later than 24 hours after solidification to minimize the decomposition of benomyl. The control plates, consisting of 0 ppm benomyl, were made with nanograde acetone alone.

To insure that any influence on viability observed in this experiment was due to an interaction with the DNA repair mechanism, and not a toxicity effect of the fungicide, we plated non-irradiated amoebae on agar of 5 ppm and 10 ppm benomyl for comparison with non-irradiated amoebae on plates completely lacking the fungicide. If the effect on survival was indeed the result of an influence on repair, there would be no loss in viability bewteen these unirradiated plates.

Cell Growth in PBS

The original design of the project involved measuring excision repair in the presence and absence of benomyl through the incorporation of labelled

thymidine following the inducement of genetic damage with UV radiation. To facilitate the measurement of thymidine incorporation, it was necessary to find some means of inhibiting semi-conservative DNA synthesis; in doing so, we would be cutting out the background thymidine incorporation of DNA synthesis which would invariably accompany the incorporation due to repair. As previously mentioned, we decided the best method of accomplishing this would be suspension of the amoebae in non-nutrient media until growth and replication ceased.

To ascertain how long log. phase Ax-2 amoebae would continue to divide in nutrient-free media, we placed stocks of amoebae in PBS and counted the cells at two hour intervals over a six hour period. The cells used in this experiment were at an initial concentration of 1.5×10^6 cells/ml. The cells were kept in a shaker at 23 C through out the experiment. All counts were done in duplicate with hemocytometers. At the fourth hour and the sixth hour the sample volumes had to be agitated to break up clumps of cells, which were interfering with the accuracy of the counts.

Thymidine Uptake in PBS

To determine how long DNA synthesis continues in nutrient-free PBS, i.e. under starvation conditions, we monitored the uptake of tritiated thymidine by amoebae in PBS. This experiment was designed to reveal two things: 1.) the time in PBS required to stop semi-conservative DNA synthesis, 2.) the amount of label or nucleotide that is used in DNA synthesis.

The amoebae required in this experiment were grown to a concentration of at least 4×10^6 cells/ml. As in previous experiments, the cells were washed three times with room temperature PBS before suspension at a concentration of 2×10^6 cells/ml in 23 mls of PBS. Prior to the addition of label, a 1 ml background sample was taken and placed in 4 mls of 10% TCA/5% acetone . The cell protein in the sample was allowed 30 minutes for thorough precipitation; the precipitate was then mixed thoroughly and filtered through a Whatman glass fibre filter under vacuum suction. The precipitation on the filter was washed three times with 5 ml portions of cold TCA/acetone. All subsequent samples were treated in an identical manner. Following the addition of .1 ml of tritiated thymidine (S.A. 60 Ci/mmol), samples were withdrawn at the following times immediately after addition of label: 1,2,3,3.5,4,4.5, 5,5.5, and 6 hours. All samples were allowed to dry overnight in their

scintillation vials before the addition of Aquasol scintillation fluid. All counts were done on a Beckman LSC 250 scintillation counter. All phases of this experiment were done in duplicate.

To increase the yield of TCA precipitate, two modifications were added to the procedure above when the experiment was repeated. These modifications were: 1.) increasing the cell concentration to $2x10^7$ cells/ml in the sample flasks, and 2.) adding 1 ml of bovine serum albumin (BSA) to a 1ml aliquot of sample cells and 2 mls of 10% TCA/5% acetone in the precipitation step. These changes were designed to increase the entrapment of labelled DNA by increasing the amount of protein precipitated.

Results and Discussion

Survival Studies Under Non-Starvation Conditions

The survival curve obtained from irradiation under non-starvation conditions gives evidence for the repair of sub-lethal damage in Ax-2. The broad shoulder extending from exposure times of zero to thirty seconds indicates the organism was, in general, able to successfully repair the damage sustained in this dosage region. Further evidence for repair comes from the correlation bewteen plaque formation time and dosage. It was observed that plaque formation was delayed at the higher dosages, which is consistent with the dose-dependent cell division delay observed by Deering (10). As the amount of damage to the DNA is increased, the organism requires longer periods of time for repair and the resumption of cell division. Since all subsequent survival studies were performed with the same basic procedure, this curve gave us a reference standard for comparing the effects of the variables in other studies.

The survival study with benomyl impregnated agar was successful in establishing the influence of MBC on cell survival. The gradual elimination of the low dose repair shoulder as the concentration of benomyl was increased indicates a reduction in the organism's ability to repair its genetic damage at normally sub-lethal levels. It is important to note that a significant reduction in viability was observed bewteen the 0 ppm, 5 ppm, and 10 ppm benomyl plates in the absence of UV radiation, suggesting the possibility of a toxicity effect on some cellular pathway unrelated to repair. Alternatively, one could view the decreased viability as an increase in lethal mutagenicity, which is consistent with the hypothesis that benomyl exerts its influence through an error-prone

repair pathway. One important observation made in this experiment was a dose dependence on benomyl, not previously observed in bacteria (1). This observation, however, does not tend to prove or disprove the theory since specific comparisons bewteen prokaryotic and eukaryotic systems cannot be made with certainity. Another interesting phenomenon observed in this experiment was an exaggerated dose-dependent cell division delay as compared with the one observed in the survival study sans benomyl. This could indicate that MBC is causing an increase in the number of helix lesions and thus lengthening the recovery time.

Survival Studies Under Starvation Conditions

The survival study of Dd after 3 hours in PBS does not conclusively tell whether starvation conditions alone are influencing cell survival following UV irradiation. To determine what effect, if any, the imposition of a starvation period prior to irradiation was having on repair, we simply compared the relative shapes of the survival curves. The curve for the starved cells shows a less pronounced "repair shoulder" indicating that perhaps some interference with the repair mechanism is occuring. Nonetheless, there is not a radical change in shape bewteen curves that one would expect if there was a definite influence. The reliability of comparing these survival curves, even though virtually identical technique was used, is suspect because of limitations in the instruments and procedure used in these experiments. It is conceivable that the differences bewteen the curves may be due to an inherent lack of sensitivity in the testing procedure.

The survival curves obtained under starvation conditions with benomyl agar incubation in the post-irradiation period shows a more marked departure from the analogous curve obtained under normal nutrient conditions. At the high benomyl concentration (10 ppm), there was a significant decrease in cell viability; the change in viability was less drastic at 5 ppm benomyl. Due to the time limitations, we were unable to duplicate the initial results of this experiment to our complete satisfaction, so that caution must be used in interpreting the results. Furthermore, it must be pointed out that the rate of benomyl decomposition is extremely temperature-dependent above 25°C (11); even the rate and duration of stirring the agar/benomyl mixture could cause a significant amount of decomposition. To obtain reproducable results for this experiment, one must use great care in the preparation of the agar plates. Although the absolute reliability of these experiments is

questionable, I believe that the nutrient state of the cell has an adverse effect on repair and thus survival.

Cell Growth Study

The cell growth curve obtained for amoebae in PBS yielded results that are in good agreement with some the reported literature values. We obtained a 17.9% increase in the number of cells at the end of 2.5 hours, compared with an increase of 20% after a comparable period of time reported by Katz et al (3). Moreover, our study indicates that all cell division has stopped by the end of three hours. One would expect that the semi-conservative synthesis of DNA should also stop after a slightly longer period of time.

Thymidine Uptake Study

At the time of this writing, the results obtained from the thymidine labelling of DNA were unsatisfactory. The data we have obtained thus far is erratic and at best shows only a very vague resemblance to the trend expected. Since this experiment is essentially a recapitulation of the cell growth study, it is expected that we should obtain a trend resembling that of the cell growth curve. Actually, DNA synthesis continues for a slightly longer period of time because the amoebae pass through the mitosis phase and into G_2 before shutting down normal cell activity. By this, we would expect to see continued thymidine uptake until 3, or perhaps 3.5 hours before cessation occurs.

Conclusions

From the work done here, it seems likely that benomyl does have some effect on excision repair in the cellular slime mold. Moreover, this influence appears to be a dose-dependent response. Although we were not able to establish the nature of the interaction, we have laid much of the groundwork for further investigation.

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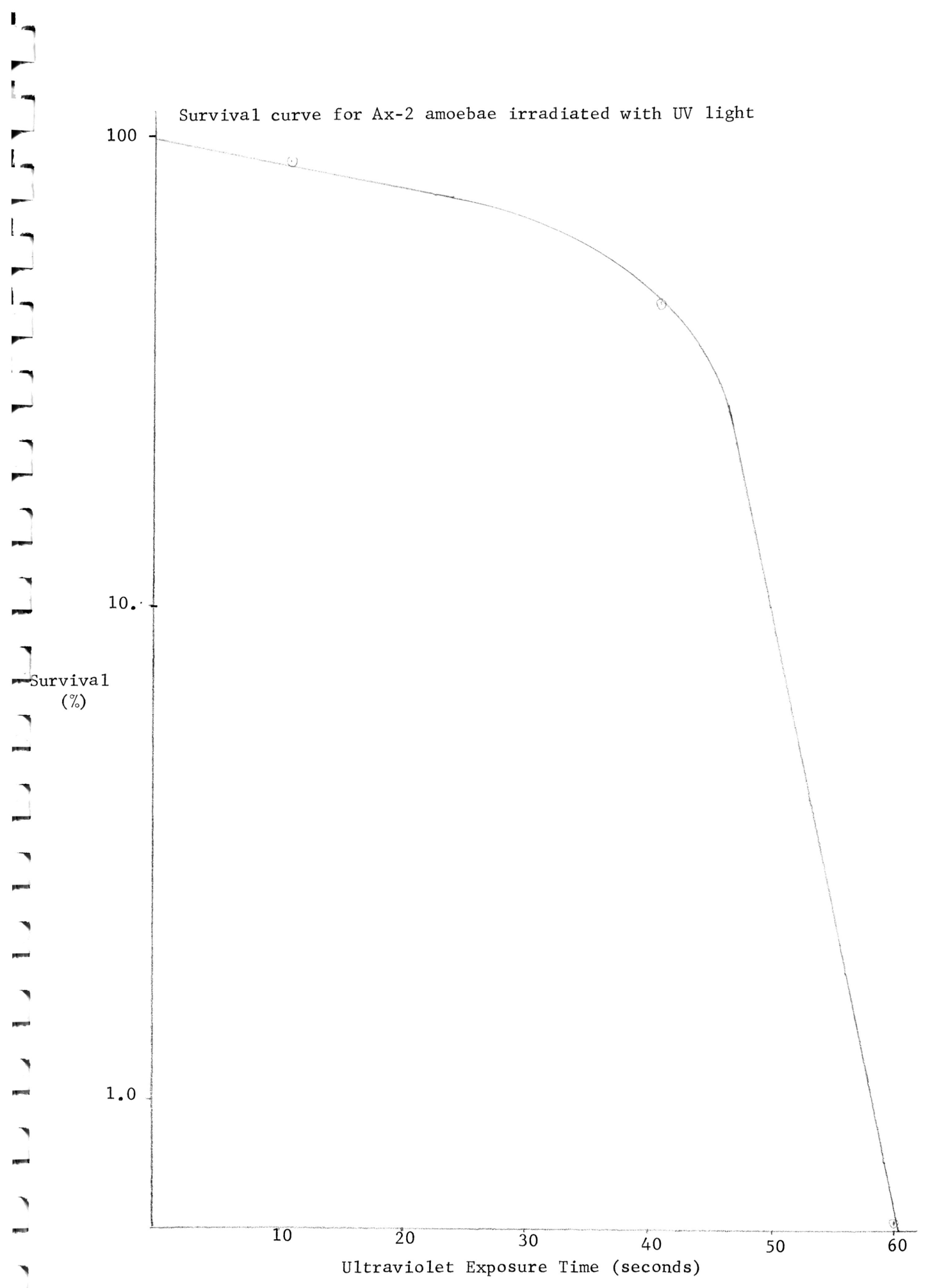
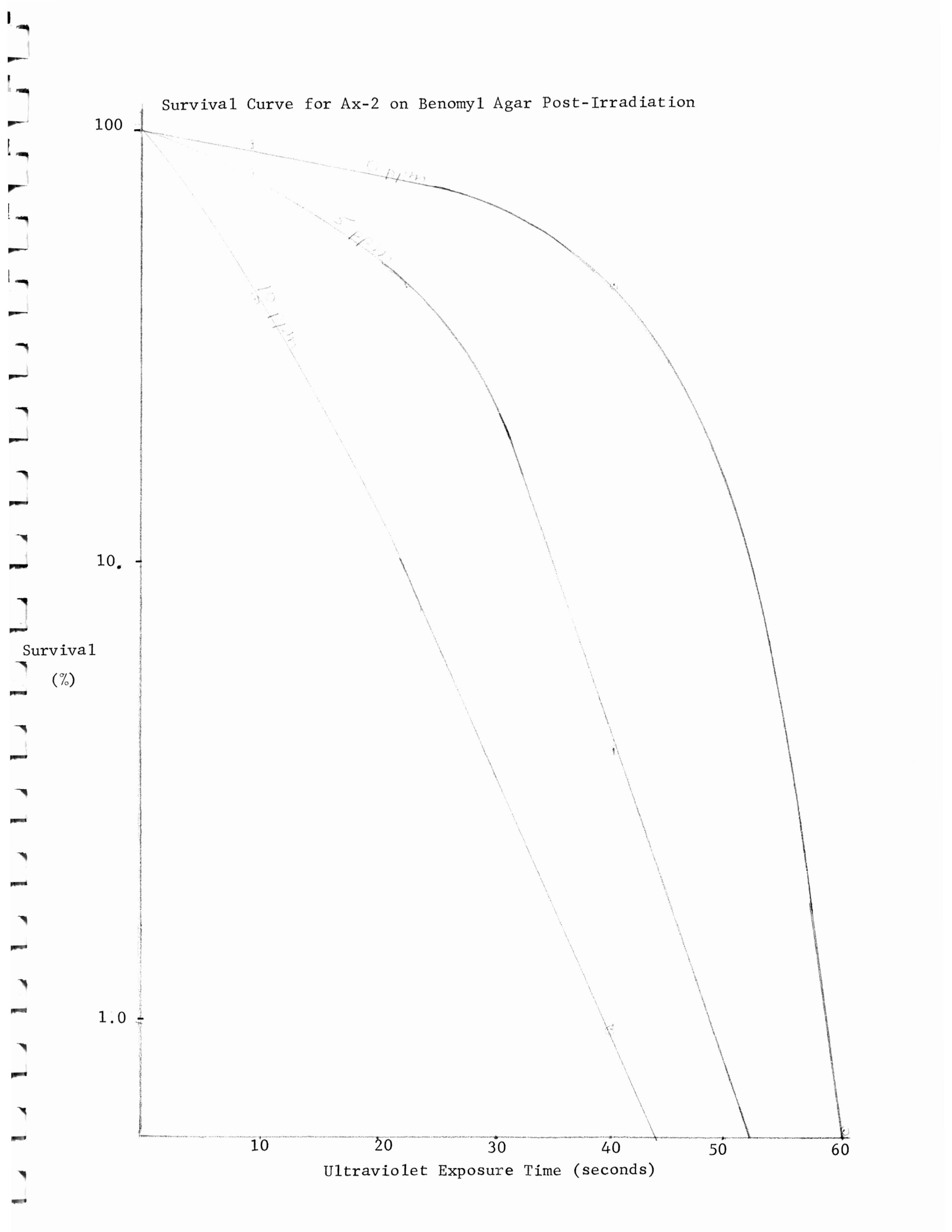
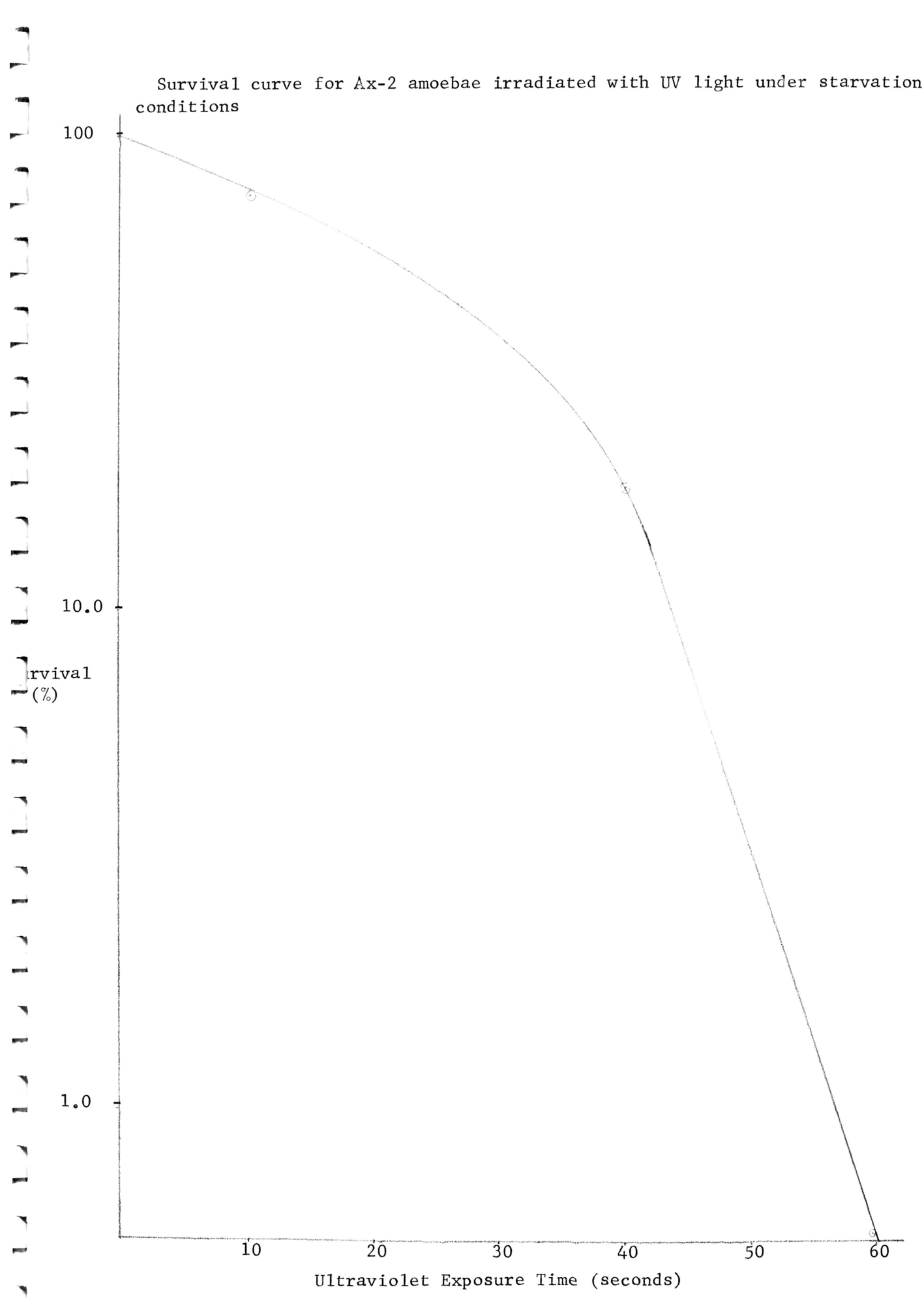
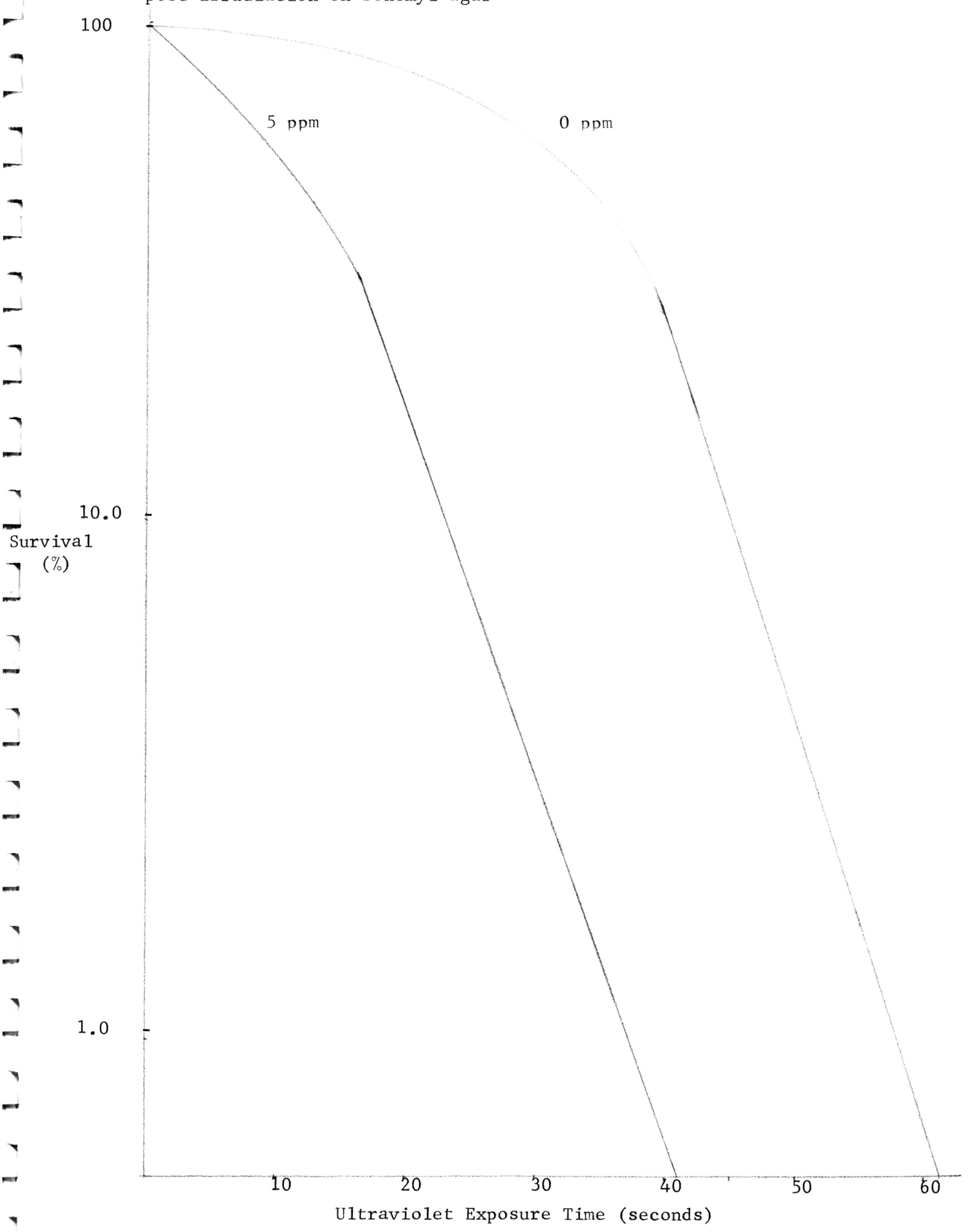

Survival curve for Ax-2 amoebae irradiated with UV light
100
10.
1.0
Survival
(%)
10
20
30
40
50
60
Ultraviolet Exposure Time (seconds)

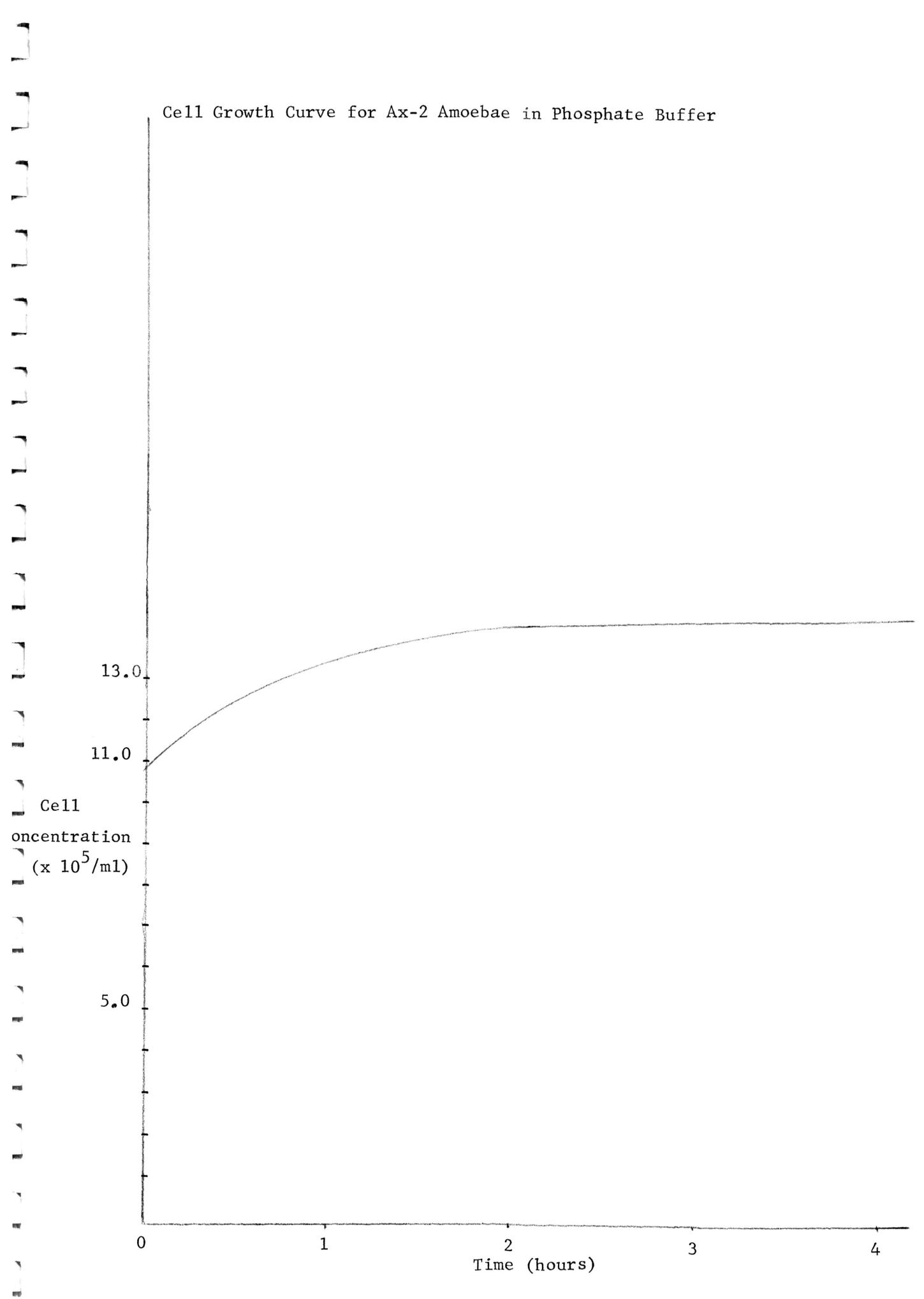

Survival Curve for Ax-2 on Benomyl Agar Post-Irradiation
100
10.
1.0
Survival
(%)
10
20
30
40
50
60
Ultraviolet Exposure Time (seconds)

Survival curve for Ax-2 amoebae irradiated with UV light under starvation conditions



Survival Curve for Ax-2 under starvation conditions--incubated post-irradiation on benomyl agar




Cell Growth Curve for Ax-2 Amoebae in Phosphate Buffer
13.0
11.0
5.0
Cell
oncentration
(x 10^5/ml)
0
1
2
3
4
Time (hours)