THE RADIOSENSITIVITY AND SYNCHRONY OF

OEDOGONIUM CARDIACUM

THE RADIOSENSITIVITY OF HAPLOID AND DIPLOID *OEDOGONIUM CARDIACUM* AND STUDIES ON THE SYNCHRONY OF *OEDOGONIUM CARDIACUM*

By

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SCOPE AND CONTENTS:

The γ-radiosensitivity of haploid and diploid Oedogonium cardiacum cells was measured and compared to other cell lines. With the doubling of the chromosome complement, the Do value doubled, but the extrapolation number decreased four-fold. A general conclusion was drawn from the results that at all doses of γ-radiation, the diploids were more resistant than the haploids. A new radiation technique was used and compared to that used routinely in the laboratory. The further use of the technique was not recommended since the data obtained with the diploid line only was not as reliable as one would like.

The degree of synchrony of Oe. cardiacum zoospore cultures was measured using cell division as the biological end-point and a mathematical expression, the percent phasing, as the index of synchrony. It was intended that this research problem be secondary to the radiation studies. The percent phasing values were determined for cells growing in two inorganic media and in the presence of an inhibitor, hydroxyurea.

However the degree of synchrony was not improved beyond that of the routine laboratory procedure. Attempts to improve the size of the synchronous populations collected also proved unsuccessful.

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PREFACE

This thesis describes work carried out at the Ontario Cancer Treatment and Research Foundation, Hamilton Clinic, in association with the Department of Biology, McMaster University, from May 1966 to June 1968.

The aims of the investigations were to measure and compare the radiosensitivity of haploid and diploid female Oedogonium cardiacum, and to assess any change in the degree of synchrony of Oedogonium cardiacum zoospore cultures under different conditions.

The studies on radiosensitivity were undertaken for two reasons. First, it would provide a quantitative analysis of any difference in radiosensitivity between haploid and diploid Oedogonium cardiacum. Second, it would serve as a means of evaluating a new radiation technique just introduced by Howard (1) which might provide larger cultures of zoospores for determining radiation response characteristics especially in the region of low survival.

The synchrony study was carried out to try to improve the degree of synchrony of Oedogonium cardiacum zoospore cultures, and to obtain larger populations of Oedogonium cardiacum zoospores than usually collected without sacrificing any degree of synchrony.

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CHAPTER I

INTRODUCTION

It is a commonly held hypothesis in radiobiology that cell killing by ionizing radiations is primarily a consequence of energy deposition in the nucleus (2-5). On the basis of most of the evidence from the literature the specific target within the nucleus is thought to be the genetic material (4). If the hypothesis is accepted that the chromosomes are the primary sites of radiation damage, then it is logical to suspect that a variation in chromosome number would affect the radiosensitivity of a cell. Several papers, including an extensive review (4), have been published regarding the influence of chromosome number on radiosensitivity. In this chapter, the salient features for higher and lower plants, insects, amphibians and mammalian cells will be reviewed.

Sparrow, Cuany, Miksche and Schairer (6) studied the influence of polyploidy on the tolerance of chronic X-radiation of five Chrysanthemum species with chromosome numbers of 18, 36, 72, 90 and 198 and of ten Sedum species with chromosome numbers ranging from 20 to 68. From their data they reported that the tolerance, as measured by growth inhibition, was found to increase with polyploidy. Since polyploidy results from the reduplication of the genome, they suggested that genetic redundancy was responsible for this increase in tolerance with polyploidy. Sparrow (3) has further correlated this increase in tolerance with

nuclear (*) and chromosome volumes (**).

Ichikawa and Sparrow (7) studied the loss of proliferative capacity in the stamen hairs of a polyploid series of Tradescantia species following X-irradiation. They reported that the extrapolation number η ($\overset{*}{*}$) remained about the same with the increase in chromosome complement from diploid to 12-ploid, while the D_{37} values ($\overset{**}{**}$) increased 7-fold. However, when they calculated the absorbed energy in keV per chromosome at D_{37} ($\overset{***}{**}$), the 7-fold variation in D_{37} values tended to disappear. On this basis they concluded that all species had similar radioresistance, and this they considered as evidence against the widely accepted idea of an influence of genetic redundancy on radioresistance.

Conger and Johnston (8) studied the effects of polyploidy on sensitivity to X-radiation using a mixed population of genetically identical haploid and diploid Tradescantia microspores. They reported that the diploid cells had almost twice as many chromosome aberrations

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(*) The nuclear volume was determined by assuming that the nucleus was a sphere, measuring its diameter, and then calculating from the equation $V = 4/3 \quad r^3$.

(**) The chromosome volume was determined by dividing the interphase nuclear volume by the chromosome number.

($\overset{*}{*}$) The extrapolation number η is the number obtained relative to 1.0 when the exponential portion of a survival curve, obtained when log percent survival is plotted as a function of dose, is extrapolated back to zero dose.

($\overset{**}{**}$) The D_{37} value represents the dose required to reduce the surviving fraction to 37%.

($\overset{***}{**}$) From the D_{37} values reported in Roentgens, the energy absorbed per chromosome was based on an average value of 32.5 ev per ion pair and 1.77 ionizations per $\mu 3$ of wet tissue, per Roentgen.

as the haploid following a fixed dose of X-radiation. However, since there are twice as many chromosomes per cell in the diploid, they concluded that the aberration frequency per chromosome was the same in the diploid as in the haploid.

Mortimer (9) studied the survival curves of haploid and diploid Saccharomyces cerevisiae with X-rays and reported that the haploid cells were more sensitive than the diploids. Further, he reported that the haploid cells exhibited an approximately linear survival curve on a semilogarithmic plot indicating exponential inactivation, and the diploid cells exhibited sigmoid survival curves. From the results he suggested that a single event, a recessive lethal, was required to inactivate a haploid, whereas in the diploid where the genetic information is redundant two such recessive lethals at homologous sites, or a dominant lethal event, was required. However, results in complete contrast to those described above were reported by Mortimer (9) from studies of survival curves in S. cerevisiae with higher levels of ploidy, namely diploid, tetraploid and hexaploid. He reported LD_{10} values (*) for the diploid, tetraploid, and hexaploid cultures of 47.5, 30 and 16.8 kilorads respectively which shows a progressive increase in radiosensitivity with increasing ploidy. In comparison to the data of Conger and Johnston discussed previously, Mortimer accounted for his results by assuming that lethality in higher ploidies was associated mostly with chromosome aberrations of the dominant lethal type.

(*) The LD_{10} value is the dose necessary to reduce viability to 10%.

Davies (10) studied the ɣ-ray sensitivity of haploid and diploid cell stages of Chlamydomonas reinhardii and reported that the diploids could be either more or less resistant than the haploids depending on the stage of the cycle. From survival curves described for haploid and diploid lines in the early stages of the life cycle, he found little difference in the Do values (*), but the extrapolation number n increased significantly from haploid to diploid. A number of interpretations were offered by Davies to explain the greater resistance of the diploids and are summarized briefly now, but presented in more detail in the discussion. The first interpretation related the different response of the diploid to its capacity to repair primary damage. A second interpretation was that different types of lesions were induced in the two cell types. A third interpretation, for which he reported no evidence, was that different concentrations of protective substances existed in haploid and diploid forms.

Davies referred to two other interpretations given in the literature to explain the increase in radioresistance with ploidy but dismissed their applicability to the Chlamydomonas system. He contended that Mortimer's findings (9) with yeast, that haploids were inactivated by recessive lethal events and diploids by dominant lethals or homologous recessive lethals, did not apply to Chlamydomonas because the survival of these haploids was sigmoidal and not exponential. He also contended that Sparrow's findings (3) with higher plants, that radio-

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(*) The Do value represents the dose required to reduce the surviving fraction to 37% along the exponential portion of a survival curve when log percent survival is plotted as a function of dose.

sensitivity was related to chromosome volume, did not apply because the chromosome volumes in Chlamydomonas were the same for the haploids and diploids.

Mortimer and von Borstel (11) reported that doubling of the chromosome complement in Mormoniella sperm, from haploid and diploid males, doubled the sensitivity to X-radiation when dominant lethality was the criterion. This is consistent with Mortimer's previous work (9) with yeast in which he accounted for the increase in sensitivity from diploid to hexaploid by assuming an increase in production of dominant lethal events. Mortimer and von Borstel concluded that doubling the chromosome number in Mormoniella sperm had effectively doubled the target size.

Clark (12) used natural haploid male and diploid female Habrobracon wasps to study the influence of polyploidy on the lethal response of individuals exposed to X-radiation at different stages in development. He reported that during the early cleavage stages of the fertilized egg, the diploids were slightly more sensitive than the haploids. However, he reported that the differences changed with development, the diploids becoming relatively less sensitive in the larval and pupal stages. The response in early development was similar to that found with irradiated sperm of Mormoniella (11) and the response in late development was similar to that found in higher plants (6). Also, the results reported for Habrobracon were similar to those with Chlamydomonas (10) in that the relative radiosensitivities of haploids and diploids were different at different stages of development. Clark explained the differences in terms of the relative importance of

dominant and recessive lethal mutations and concluded that the diploid, in the late development, appeared to minimize the production of dominant lethal damage.

A comparison of the x-ray sensitivity of haploid and diploid embryos of the toad Xenopus laevis by Hamilton (13) revealed that at all survival levels it was necessary to give the diploids twice the dose given to haploids to kill the embryos. He reported LD_{50} values (*) of 1275 rads for the haploid and 2550 rads for the diploid, and suggested the difference could be related directly to the chromosome number. However, he pointed out that evidence from radiation studies on salamander and frog embryos reported in the literature made it difficult to ignore the possibility of cytoplasmic damage contributing to the mortality of amphibian embryos.

The influence of polyploidy on the lethal response in irradiated mammalian cells has been examined in both in vivo and in vitro systems using a number of cell types. A variety of different results has been obtained and these are summarized as follows. Puck (14) studied the x-ray survival curves of an aneuploid HeLa S_3 cell line, having a modal chromosome number around 78, and reported Do values and an extrapolation number almost twice those obtained from survival data on newly established diploid cell lines. He concluded that the increased resistance of the aneuploid line was simply a function of its genetic duplication.

Studies by Lockart, Elkind and Moses (15) on three sublines of HeLa S_3 cells throw some doubt on the above conclusion. These authors

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(*) LD_{50} is the dose required to kill 50% of the cells.

reported no correlation in these lines since an increase in modal chromosome number from 68 to 78 did not confer an increase in resistance to the lethal effects of x-rays.

Studies reported by Till (16) on five lines of mouse L cells with modal chromosome numbers ranging from 53 to 109 showed no difference in Do value between the lines. Nias and Ockey (17) exposed HeLa cells in culture to continuous low-dose-rate irradiation (30 rads per day) for a period of 12 months and reported that during this period the modal chromosome number was reduced from about 60 to about 52, but was not accompanied by a change in radiosensitivity.

On the other hand, there are a number of experiments on other mammalian cell lines which do show a marked influence of polyploidy on the radiation response. Revesz and Norman (18) studied the effects of polyploidy on the radiation response using a hyperdiploid Ehrlich ascites tumour with a modal chromosome number of 46 and a derived hypertetraploid subline having a modal chromosome number of 92. They reported that the proliferative capacity of the two lines showed that the hypertetraploid was considerably more resistant than the hyperdiploid, and suggested that genetic redundancy was the reason.

A difference in the relative response of near-diploid and near-tetraploid Ehrlich ascites tumour cells to X-radiation was noted by Silini and Hornsey (19). They used a near-diploid line with a modal chromosome number of 46 and a near-tetraploid line with a modal chromosome number of 96 and measured the survival of these cells, irradiated in vitro, by determining the ratio of tumour takes from irradiated and unirradiated cells injected subcutaneously into young mice. They

reported that, though the Do values were the same, the extrapolation number increased from 2.4 to 10 with increased ploidy. They concluded that the near-diploid cells had a greater capacity to repair sub-lethal damage.

Similar results were reported by Berry (20) with diploid and tetraploid mouse lymphocytic leukemia cells which were irradiated with x-rays in the donor animals and assayed in the same way as the Ehrlich cells. He found that the extrapolation number doubled from 1.6 to 3.1 with a doubling of ploidy and attributed it to the diploid cell containing twice the number of targets. This implies that genetic redundancy is responsible for the resistance of the diploids.

A comparative study of the radiosensitivity of Ehrlich ascites cell clones of a hypo-diploid and hypo-tetraploid, with modal chromosome numbers of 42 and 84 respectively, was made by Bhaskaran (21) who reported that the Do values were about the same, but the extrapolation number doubled for the hypo-tetraploid line.

Dose-survival studies were made by Bedford and Hall (22) using x-rays with two sublines of Chinese hamster cells cultured in vitro which had a preponderance of cells with 23 chromosomes and with 46. They reported that the Do values were similar, but the extrapolation number increased 3 to 4-fold with the higher ploidy. They speculated that genetic redundancy might account for the reduction of radiosensitivity with increased ploidy.

It is apparent from the review, just presented, of the influence of polyploidy on radiosensitivity in a variety of different cell types that there is no simple pattern common to all the cell systems studied.

One obvious difficulty is that investigators have not used the same biological end-point as the criterion of radiation response. For example, cell lethality, growth inhibition and chromosome aberrations were some of the end-points selected. Another difficulty is the lack of a uniform index of radiosensitivity since investigators used Do, η and LD_{50} values and chromosome aberration frequency as examples.

If the hypothesis, previously mentioned in the introduction that cell lethality is a consequence of radiation-induced chromosome damage, is accepted, then polyploidy could in some instances confer resistance and in other circumstances could increase sensitivity, depending on the type of damage which is important and the degree of ploidy involved. The data reported by Sparrow et al. (6) for the Sedum and Chrysanthemum species of higher plants showed an increase in resistance with continually increasing ploidy, and this is the type of result to be expected if chromosome deletions are the important cause of death. With yeast, on the other hand, Mortimer (9) reported no further resistance due to polyploidy beyond the diploid level, and it seems that the results are best interpreted on the basis that chromosome bridges and not deletions are the prime cause of lethality.

The variety of results that have been obtained with mammalian cells presents a confusing picture and no clear-cut pattern emerges. However, the review presented in this chapter indicates that Silini and Hornsey (19), Berry (20), Bhaskaran (21), and Bedford and Hall (22) were essentially in agreement in that the survival parameter η increased with increasing ploidy. This finding was also reported for certain of the plant species studied, namely Chlamydomonas (10) and the haploid and

diploid in lines of yeast (9).

The lack of correlation between radiation response and chromosome number in mammalian studies by Lockart et al. (15), Till (16), and Nias and Ockey (17) was interpreted by Bedford and Hall (22) who postulated that these widely aneuploid cells were monosomic for a portion of their genome which was vital to the maintenance of reproductive integrity.

It is difficult to draw a firm conclusion from the literature regarding the increased radioresistance conferred by polyploidy, but there is a profusion of opinion that suggests that genetic redundancy plays the most important role, but not necessarily so in every biological system.

The purposes of the experiment described in the following chapters were to measure any difference in radiosensitivity between haploid and diploid Oedogonium cardiacum and to evaluate a new radiation technique modified from Howard (1) in studies with haploid Oe. cardiacum. The standard radiation technique used in this laboratory has been described previously by Horsley and colleagues (23-25, 33). Essentially the two procedures differ in that Horsley et al. induced zoosporogenesis before irradiation and then irradiated the zoospores, whereas Howard irradiated the filaments and then induced zoosporogenesis. A potential advantage in the Howard approach is that larger populations of zoospores might be obtained to assess survival especially at low survival levels, since a separate culture is used for each dose. It was anticipated that in these investigations survival could be extended to a decade lower than that reported by Howard. On

the other hand, a possible disadvantage might be the inability of identifying the stage of the cell cycle in which the cells are irradiated.

CHAPTER II

MATERIALS AND METHODS

A. Morphology of "Oedogonium cardiacum"

A brief description will be given of the morphology and life cycle of Oedogonium cardiacum and of those features which have made it a useful experimental system for the radiobiologist. Detailed accounts of its morphology and life cycle have been reported by Ohashi (26), Smith (27), Lewin (28), Fritsch (29), and Hoffman (30). The details have been summarized in previous theses by Parker (31) and Wilfong (32).

The order Oedogoniales of the class Chlorophyceae comprises three genera and approximately 350 species of filamentous green algae. The filaments of Oedogonium cardiacum are composed of cylindrical, uninucleate cells (80 x 20μ) aligned end to end. The cells contain peripheral reticulate chloroplasts with pyrenoids of starch at the interstices, a single central haploid nucleus, and large central vacuoles. The fresh-water Oedogonium cardiacum cells divide mitotically to produce unbranched filaments in which the basal cell is modified to form a holdfast for substrate attachment.

Oedogonium cardiacum is endowed with a unique type of cell division. Immediately preceding prophase a hemicellulose ring begins to form, encircling the lateral walls of the cell very near to its distal end. By telophase the ring is fully developed, and transverse

cytokinesis occurs midway between the ends of the cell. A stretching of the cylinder of new wall material ensues giving rise to the lateral walls of the distal daughter cell. When the elongation has been completed, the transverse wall is laid down separating the two cells from each other. The ring is retained as a cap near the end of the distal daughter cell, and provides a record of completed divisions as the filament grows.

Reproduction in Oedogonium cardiacum can be either sexual or asexual. In asexual reproduction, phototactic, multiflagellate zoospores are produced singly within any cell of the filament. The cell wall splits at the apical end to release the zoospore. The zoospore swims around, usually for a short period of time, and finally comes to rest with the anterior end down. The flagella disappear and a holdfast develops in the region representing the region of the original anterior of the zoospore. Filamentous growth is then initiated by division of the zoospore.

The induction of zoosporogenesis in the laboratory provides a means of obtaining a culture of single cells. It has been reported (33) that if zoospores are induced and harvested in a short time interval, they will divide synchronously to produce two-celled filaments. Factors affecting zoospore formation in Oedogonium have been reviewed and investigated in detail by Hoffman (30). He concluded that the induction of zoosporogenesis was governed by two main factors: light and the level of free carbon dioxide in the medium. The way in which these factors were manipulated in this laboratory will be discussed in Chapters II D and VI C.

Oedogonium cardiacum is heterothallic (*) and sexual reproduction is of the oögamous type (**). Each oögonium contains a single large oöspore and each antheridium (***) two motile sperms. The fertilized egg develops into a thick-walled diploid zygote which is liberated by rupture of the oögonial wall. Following a resting period which may last for several months, the zygote undergoes meiosis and liberates four haploid zoospores which give rise to unisexual male and female plants.

Hoffman (34) reported that it was not at all uncommon for the zygote, failing to undergo meiosis, to produce a diploid strain. Also, he reported that an even more common source of diploid strains was by the somatic doubling of the chromosome number. He distinguished diploid filaments by their relatively large diameter and a chromosome number about twice that of normal haploid cells. The artificial induction of diploidy by colcemide treatment has been reported by Banerjee, Horsley, and Pujara (35). Their evidence was also based on filament diameter and chromosome number (36). The present author has observed that the diploid strain grew vigorously in culture, but the filaments did not attain the same length as the haploid strain. This was possibly due to filament breakage which may have been caused by more frequent zoosporogenesis in diploid stock cultures or fragile cell

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(*) Heterothallic refers to algae in which the male sex organs are produced on one filament and the female sex organs on another.

(**) Oögamous refers to sexual reproduction involving unlike gametes.

(***) An antheridium is a male sex organ in which sperms are produced.

walls.

In this laboratory, stock cultures were grown from unisexual innocula and thus sexual reproduction did not occur. Therefore, the genome was not altered by genetic recombination. The female strain was used exclusively for the present investigative purposes because it exhibits a healthier growth than the male.

The useful features of this species from a radiobiological point of view are its ease of culture and availability of large populations of synchronous zoospores (33) with a relatively short generation time. Since it is a eukaryote it has chromosomes and mitotic divisions typical of those of higher organisms. Another useful quality is that each zoospore gives rise to a filamentous colony and the number of cells in a filament can be determined unambiguously by counting under a microscope. Due to the cap that is retained by the daughter cell following cell division, the filament provides a record of completed divisions. This cell line has been used extensively in this laboratory to study, for example, the effects of ionizing radiation on free amino acids (37), the induction of giant cells by X-rays (38), radiation-induced division and mitotic delay (39), and radiosensitivity during the cell cycle (40).

B. Stock Cultures

Female haploid and diploid cultures of Oedogonium cardiacum, supplied by Professor R.C. Starr from the Culture Collection of Algae at Indiana University (41), were routinely subcultured in the laboratory. The cultures are listed in Table 1 together with data describing

TABLE 1

Cultures of Oedogonium cardiacum (41)

SPECIES	ISOLATOR	NUMBER	REMARKS
Oe. cardiacum Wittr.	Christensen	LB40	female, haploid
Oe. cardiacum Wittr.	Starr	LB847	female, diploid of LB40

their origin.

The non-axenic cultures were maintained in a 1:12 (v/v) biphasic soil (*) - water medium in 50 ml test tubes fitted with cork stoppers that had been bored and plugged with cotton. Each tube was boiled for one hour (or autoclaved at 15 psi for 15 minutes) on two successive occasions about a week apart. When there was no discernible turbidity of the supernatant, the tubes were inoculated with a few washed Oedogonium cardiacum filaments. As needed, the liquid phase of the cultures was replenished with soil extract medium (see Appendix A).

Initially, the cultures were maintained at room temperature under a 12:12 hour light:dark regimen on an illuminated (**) culture rack. After inoculation, the tube cultures were exposed to a light intensity (***) of 200-240 foot-candles. Once growth was established, the light intensity was reduced to 50-100 foot-candles.

C. Radiation Source and Dosimetry

A perspex cylinder (14 x 5 cm) was bored out to support a glass stock-culture tube (15 x 2.5 cm) during the irradiation period. The wall of the cylinder was 1.2 cm thick to allow for the radiation build-up. This set-up yielded an essentially uniform dose across the diameter of the tube. All the radiations were carried out at room temperature,

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(*) Garden soil, obtained from Waterdown, Ontario, was dried, cleaned, powdered, and mixed.

(**) Sylvania "Gro-Lux" fluorescent lamps.

(***) General Electric type 213 light meter.

at 80 cm F.S.D. (*), and with a 30 x 32 cm field (**).

The radiation source was a Theratron 80 ^{60}Co therapy unit (Atomic Energy of Canada Limited) emitting gamma rays of an average energy 1.25 Mev. In the initial experiments (May 1966), the dose rate absorbed to the centre of the biological specimen was 118.9 rads/minute and the rate decayed to 97.5 rads/minute in the concluding experiments (November 1967).

The exposure was measured by placing the ionization chamber of a Baldwin-Farmer Sub-standard Dosemeter in a water-filled culture tube placed at a position corresponding to that of a culture tube of Oedogonium cardiacum filaments. A reading was taken with the ionization chamber in the centre of the culture tube as represented by "B" in Figure 1. The doses for positions "A" and "C" in Figure 1 were estimated from measured depth-dose curves prepared by the Physics Department of the Hamilton Cancer Clinic.

D. Irradiation and Post-irradiation Procedures

The irradiation and post-irradiation procedures were carried out as follows (Fig. 2). The cultures selected for irradiation were at least two months old and exhibited a dense, dark green growth. They were assumed to be in the stationary phase of growth in which mitotic divisions are infrequent and metabolic activity reduced. This assumption

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(*) F.S.D. (focus to surface distance) is the distance from the focus of the cobalt source to the surface of the perspex cylinder.

(**) The jaws of the radiation unit were opened to the fullest extent. The field bounded by the jaws measured 30 x 32 cm.

FIGURE 1

The letter "B" represents the placement of the ionization chamber of a Baldwin-Farmer sub-standard dosemeter to measure the γ-radiation dose. The letters "A" and "C" represent positions at which doses were estimated from measured depth-dose curves.

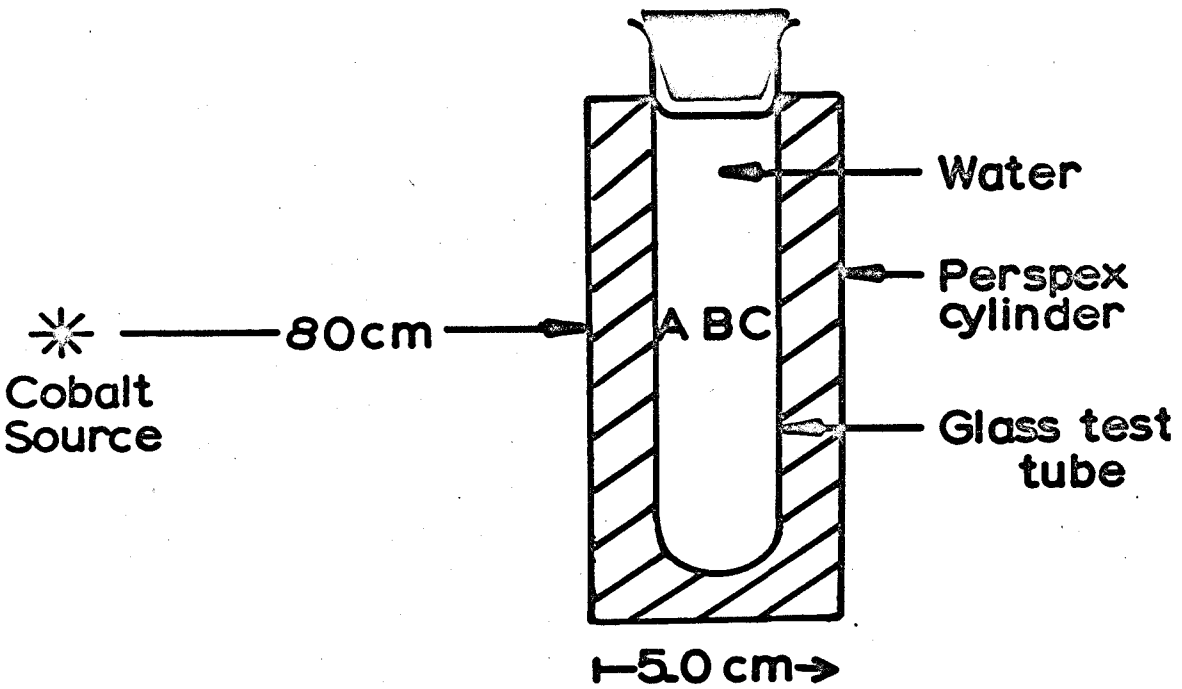

Water
Perspex cylinder
80cm
A B C
Cobalt Source
Glass test tube
5.0 cm

FIGURE 2

Diagrammatic representation of the procedure used for the irradiation of haploid and diploid Oedogonium cardiacum.

A. Stock cultures of filaments were illuminated by fluorescent light.

B. Stock cultures, supported by a perspex cylinder, were irradiated with γ-rays.

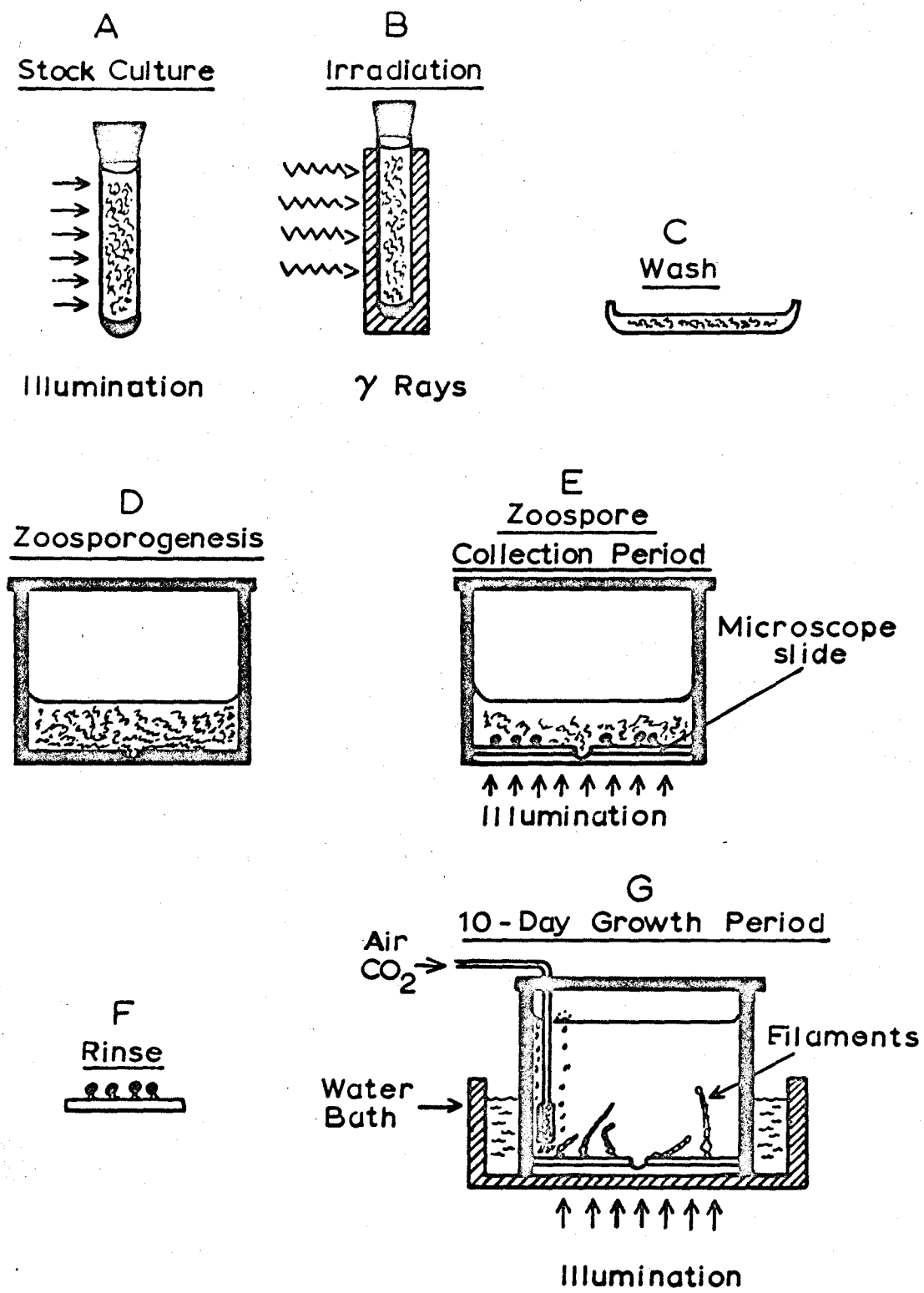
C. The filaments were removed and washed in distilled water.

D. To induce zoosporogenesis, the filaments were transferred to dilute soil extract medium in a blackened vessel.

E. To collect the zoospores, the filaments were transferred to a similar vessel but with a transparent floor on which glass slides had been placed. The vessel was illuminated from below to attract the photo-tactic zoospores to the slides for attachment.

F. When a sufficient number of zoospores had attached, the slides were removed and rinsed with a stream of distilled water.

G. The zoospores were set to grow for 10 days in Molisch medium bubbled with a mixture of air and carbon dioxide. The growth vessel was placed in a water bath and illuminated continuously from below.


A
Stock Culture
B
Irradiation
C
Wash
Illumination
γ Rays
D
Zoosporogenesis
E
Zoospore
Collection Period
Microscope
slide
Illumination
F
Rinse
G
10-Day Growth Period
Air
CO_2
Filaments
Water
Bath
Illumination

was based on gross observation of the cultures which indicated that beyond the second month there was a cessation of further growth. The irradiation procedure was modified from that described by Howard (1) in previous studies of Oedogonium cardiacum in that the cultures were irradiated directly in the stock-culture tube supported by a perspex cylinder rather than transferred to a tube of distilled water. To determine the radiosensitivity, the filaments were induced to sporulate and the proliferative capacity of the zoospores was determined after ten days' growth.

To induce zoosporogenesis, the filaments were removed from the culture tubes within an hour after irradiation and transferred to 400 ml of 1:3 soil extract (*) - distilled water medium in a blackened perspex vessel (12 x 17 x 16 cm). The vessel was placed in the dark at room temperature for about 60 hours.

To harvest or collect the zoospores, the vessel was removed from the dark and its contents transferred to a similar vessel but having a transparent floor. This vessel was illuminated (**) from below at an intensity of 150-200 foot-candles. Eight glass slides were placed on the floor of the vessel to collect the swimming zoospores after their liberation from the filaments. Since the zoospores are phototactic, they swim toward the slides and attach by forming holdfasts. When a sufficient number of zoospores had attached, they were removed and rinsed with a stream of distilled water, and transferred to a similar

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(*) See Appendix A.

(**) General Electric warm-white fluorescent lamps.

vessel containing about 1.5 litres of Molisch inorganic medium (see Appendix A). The vessel was placed in a transparent water bath (about 23° C) illuminated continuously from below with 300 foot-candles of fluorescent lighting (*). The medium was bubbled slowly with a mixture of 3% CO_2 + 97% air during the subsequent growth period for optimum growth of the cells.

Cell survival, as measured by the loss of proliferative capacity, was determined by fixing the filaments in Carnoy's solution following a 10-day growth period and examining them under the microscope. Following the criterion adopted in previous work (23), zoospores which grew into filaments with more than 12 cells were designated as survivors, and those with 12 cells or less as non-survivors. Although this criterion was reported for the haploid strain, its application has been extended here to the diploids as well.

(*) General Electric warm-white fluorescent lamps.

CHAPTER III

RESULTS

A. Dosimetry

The placement of the ionization chamber in a water-filled culture tube for dose determination and the two positions at which doses were estimated from measured depth-dose curves have been shown previously in Figure 1. The doses determined are reported in Table 2. The dose absorbed across the tube varied by 13.6%. At the centre of the tube the absorbed dose was 88.9% of the peak build-up dose and the dose-rate in May 1968 was found to be 91.3 rads/minute. The exposure measured in Roentgens was multiplied by the conversion factor 0.965 to give doses in rads. The monthly dose-rates for investigations carried out preceding May 1968 were estimated from tables of ^{60}Co decay. The dose-rates decayed from 118.9 rads/minute in May 1966 to 97.5 rads/minute in November 1967.

B. Survival Curves

Tables 3 and 4 show the results of an experiment in which haploid and diploid Oedogonium cardiacum filaments were irradiated over a dose-range extending to 8000 rads at a dose-rate of 97.5-118.9 rads/minute. The data are summarized in Figure 3. The x-axis indicates the dose of γ-radiation in kilorads and the y-axis indicates the percent survival of the cells on a logarithmic plot. The zero-dose survival has been

TABLE 2

Dose Determination

Distance from Front Surface of Perspex Cylinder (Centimetres)	Percent Absorbed Dose (Rads)
1.25 (point A in Fig. 2)	108
2.50 (point B in Fig. 2) (*)	100
3.75 (point C in Fig. 2)	93

(*) Doses given on the graph (Fig. 3) were measured at point B.

TABLE 3

Survival of haploid Oedogonium cardiacum cells at various doses

Dose (rads)	Percent Survival
0	100.0 (*)
842	92.3 ± 0.8
1229	83.5 ± 1.2
1660	65.0 ± 0.8
2045	55.1 ± 1.5
2525	42.6 ± 1.4
2871	35.2 ± 1.5
3331	17.8 ± 0.8
4207	6.1 ± 0.7
5042	3.4 ± 0.5
5365	2.3 ± 0.4
5816	0.72 ± 0.11
6706	0.31 ± 0.10
7382	0.17 ± 0.07

(*) Zero-dose survival of 97.8% was set equal to 100 and all other survival values corrected by multiplying by $\frac{100}{97.8}$.

TABLE 4

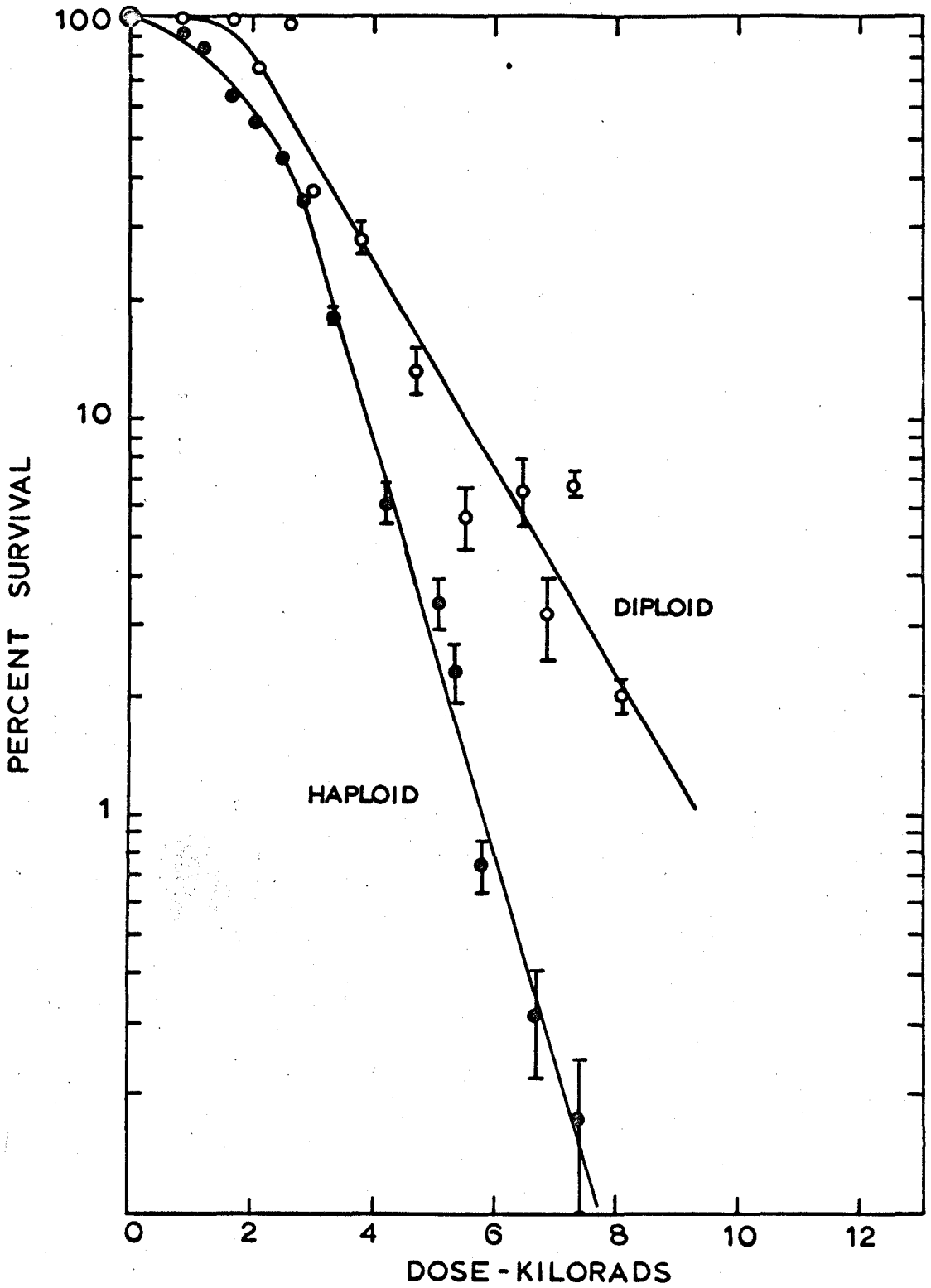
Survival of diploid Oedogonium cardiacum cells at various doses

Dose (rads)	Percent Survival
0	100.0 (*)
858	100.0
1716	100.0
2125	75.0 ± 0.7
2574	97.4 ± 0.5
2970	36.8 ± 0.9
3820	28.2 ± 2.6
4670	13.3 ± 1.7
5520	5.6 ± 1.0
6440	6.6 ± 1.3
6864	3.2 ± 0.8
7290	6.8 ± 0.5
8080	2.0 ± 0.2

(*) Zero-dose survival of 81.0% was set equal to 100 and all other survival values corrected by multiplying by $\frac{100}{81.0}$.

FIGURE 3

Survival of haploid and diploid Oedogonium cardiacum cells at various doses of γ-radiation. Standard deviation has been indicated by vertical bars.


100
10
1
PERCENT SURVIVAL
DIPLOID
HAPLOID
0
2
4
6
8
10
12
DOSE - KILORADS

normalized to 100% and the other survival values corrected accordingly, based on the plating efficiencies in unirradiated controls of 97.8% for the haploids and 81.0% for the diploids. Each survival curve has an initial shoulder region followed by a linear fall off, and can be fitted satisfactorily to the equation,

$$S = 1 - (1 - e^{-D/Do})^{\eta}$$

where S = surviving fraction of zoospores

D = dose (rads)

Do = dose (rads) required to reduce survival to 37% on the exponential part of the curve.

η = extrapolation number.

Each point in Figure 3 represents the irradiation of one or more stock cultures and, after a 10-day growth period, the scoring of an average of 1700 filaments for survival. The criterion of survival was the attainment of a filament of more than 12 cells during the growth period. At some doses a variable proportion of the collected zoospores failed to divide and died at an early age (see Appendix B), but this behaviour was unrelated to treatment. Such non-viable cells were omitted from the calculations. The standard deviation of the points is given by the expression $\sqrt{\frac{pq}{\eta}}$ (42) where p is the percent survival, q the percent non-survival, and η the number of filaments counted.

The survival parameters, η and Do, were obtained from the dose-survival curves in Figure 3 by regression analysis (*) and are presented in Table 5. In the analysis, nine experimental points on the exponential

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(*) Standard computer program.

TABLE 5

The Do values and extrapolation numbers for haploid and diploid Oedogonium cardiacum obtained by regression analysis.

Do		η	
Haploid	Diploid	Haploid	Diploid
836 ± 40	1660 ± 175	10.6 ± 3.5	2.7 ± 1.0

portion of the haploid curve and eleven points on the diploid curve were considered. The Do value doubled from 836.0 rads for the haploid to 1657.6 rads for the diploid, whereas, the extrapolation number decreased 4-fold from 10.6 for the haploid to 2.7 for the diploid.

CHAPTER IV

DISCUSSION

As mentioned in the introduction, one of the objectives of these investigations was to determine the X-radiosensitivity, as measured by the loss of proliferative capacity, of haploid and diploid Oedogonium cardiacum. The survival parameter, Do, doubled from haploid to diploid, whereas the extrapolation number decreased 4-fold. This apparently opposing nature of the two survival parameters with doubling of ploidy has not been reported previously in the literature. No regular pattern emerges from the literature regarding the variation of these two survival parameters as ploidy increased. For example, several investigators (10, 19-22) have reported an increase in η with only a negligible change in Do associated with increased polyploidy, whereas others (7) found little change in η but an increase in the Do value with ploidy. Further, two reports (9, 14) indicated that both η and Do increased with ploidy. Thus, the parameter Do, a measure of radiosensitivity, can increase with ploidy indicating that the cells are apparently less sensitive, and at the same time, η can vary independently in either direction or remain constant, but generally only one of the parameters changes.

From studies on the x-ray sensitivity of mouse L cells, Whitmore, Gulyas and Botond (43) have suggested that the measure of radiosensitivity depends in a complicated manner on both parameters, η and Do, and further

is dose dependent. The interplay between n and Do and how it affects survival curves was also pointed out in studies by Horsley and Pujara (44) where they showed that, by mathematical manipulation, an observed inflexion point in the survival curve was made to disappear. Because of the complexity of the survival parameters, the conclusion that best can be drawn from the results reported here is that at all doses of radiation the diploid line was found to be more resistant than the haploid.

From the literature reviewed in the introduction, there appears to be numerous examples of cell lines (6, 9, 10-14, 18-22) in which polyploidy played an important factor in modifying the radiation response, but in certain cells (7, 8, 15-17) polyploidy seemed to have little or no influence on the response. In much of the work with cells of different ploidy, chromosome number, unfortunately, has not been the sole biological variable. For example, from comparisons of nuclear volumes and interphase chromosome volumes of a number of closely related diploid and polyploid pairs of species of higher plants, Sparrow (3) reported that the nuclear volume in a tetraploid may be greater than twice that of the diploid, less than twice that of the diploid or even below that of the diploid; also, the interphase chromosome volume of polyploid nuclei may be greater, about the same as, or less than that of a closely related diploid. Further, Banerjee and Horsley (33) reported that nuclear volume even within the same cell line may vary at different stages of the cell cycles as shown for Oedogonium cardiacum.

Another variable, pointed out by Hamilton (13), is cell volume which he reported appears to be regulated by nuclear volume in such a

way that each cell line appears to have its own nucleo-cytoplasmic ratio. As examples, the size of yeast cells has been found to increase in proportion to ploidy (9) whereas the size of fertilized eggs of Xenopus (13) was the same for haploids and diploids.

A third biological variable is that the polyploid genome may not be an exact duplicate of the genome from which the polyploid was derived, and this is particularly evident from reports (15-17) on certain mammalian cell lines which exhibited no correlation between ploidy and radiosensitivity. In these cell lines, as pointed out by Bedford and Hall (22), the polyploids were widely aneuploid and possibly monosomic for a critical chromosome.

Yet an additional variable, though not biological in nature, is the particular index chosen as a measure of the radiosensitivity. For example, in a study of a polyploid series of Tradescantia species, Ichikawa and Sparrow (7) reported that there was a 7-fold variation in radiosensitivity when D_{37} was used as the index. However, when the absorbed energy in kev per chromosome at D_{37} was used as the index, they reported the radiosensitivity of all species to be similar.

In the reports from the literature that correlate radioresistance with polyploidy, several possible interpretations have been offered to explain the correlation. These interpretations were presented briefly in the introduction but will now be summarized and discussed in detail. The first interpretation is the existence of a protective effect due to the genetic redundancy of the diploid as suggested by Mortimer (9), Bedford and Hall (22), and others (13, 14, 18, 20). It is evident that since small deficiencies are usually lethal in diploid cells only when

both chromosome homologues carry the deletion, the relative contribution of this type of aberration to lethality will be high in a haploid cell and lower in a diploid cell, and lower still in a polyploid cell. Polyploidy may therefore act as a buffer against the expression of recessive lethal types of event and also against the larger simple deletions which may act as dominant lethals in the diploid state. On the other hand, it does not follow that polyploidy will have the same buffering effect on all the variety of structural changes which may behave as dominant lethals, particularly those aberrations which interfere with the mechanical separation of the chromatin at anaphase. Such appears to be the case in Mortimer's (9) findings of a decreased resistance of hexaploid yeast cells as compared with diploid cells. After conducting a series of genetic experiments, he concluded (45) that this dominant lethality in yeast was possibly due to chromatin bridge formation.

A second interpretation, suggested by Sparrow (3), related the changes in radioresistance at different levels of ploidy to variations in the interphase chromosome volume. In higher plants, he found that the interphase chromosome volume of polyploid nuclei could be greater, about the same as, or less than that of a closely related diploid. In a polyploid series of Tradescantia species, Ichikawa and Sparrow (7) found that a decrease in the average interphase chromosome volume was concomitant with increased polyploidy. As mentioned previously in the discussion the D_{37} values indicated a 7-fold variation in resistance among the species, but in terms of energy (kev) absorbed per chromosome at D_{37} the resistance was the same. They suggested that higher doses in Roentgens, shown by the changes in D_{37} values, were required to deliver

the same amount of energy in electron volts into the average polyploid chromosome. They concluded that the apparent increase in resistance expressed in Roentgens actually resulted from the smaller chromosome volume and not from the protective effect of genetic redundancy.

Three interpretations were reported by Davies (10) to explain the greater resistance of diploid cells of Chlamydomonas over haploid. The first was that the diploids have a more efficient capacity to repair primary damage. He indicated that it is becoming increasingly recognized that cellular repair mechanisms play an important role in determining the response to radiation of many biological systems. Davies referred to evidence in the literature suggesting that if the haploids and diploids differ in repair capacity, then their survival curves might well be expected to differ mainly in shoulder width, and this was the result reported for Chlamydomonas.

Referring to evidence in the literature, Davies suggested a second alternative that different concentrations of protective substances exist in haploid and diploid forms. The third alternative, for which Davies reported that there was no evidence available, was that different types of lesions were induced within the two cell types.

The conclusion that appears to emerge from the literature regarding the influence of polyploidy on radiosensitivity is that the sensitivity depends uniquely on the cell system being studied. Furthermore, there is no common agreement among the results indicating a change in survival parameters, n and Do, in the same direction with increasing polyploidy. In addition the explanations offered by various investigators are conflicting and there is no consensus of opinion as

to the reasons for any observed change in the measured radiosensitivity with the possible exception that genetic redundancy is an important factor not yet fully understood. The data reported in this thesis do not clarify the general picture. One could speculate that if recovery processes were completely understood this could be the key to understanding the conflicting results.

The second objective of the investigations reported here was the evaluation of the radiation technique modified from Howard (1) and comparison with the technique used routinely in this laboratory by Horsley and his colleagues (23-25, 33). Figure 4 shows a comparative outline of the two radiation techniques. With Horsley's technique zoosporogenesis is induced before irradiation to provide a collection of zoospores from which aliquots are taken for irradiation at various doses. In the Howard procedure samples of filaments are irradiated at various doses and then zoosporogenesis is induced and the zoospores collected. The collection period in Horsley's method cannot be extended to collect additional zoospores beyond a 90-minute limit since the degree of synchrony is likely to diminish below an acceptable limit. With Howard's approach the collection period can be extended to collect additional zoospores since the synchrony of the population is not important. The collection of more zoospores enables survival at higher doses to be determined with better statistical meaning. The actual number of zoospores collected was not determined, so a comparison of the two techniques on this particular basis is not possible. However, a comparison can be made on the basis of the average number of cells scored for the doses given. Horsley and Fucikovsky (23) reported

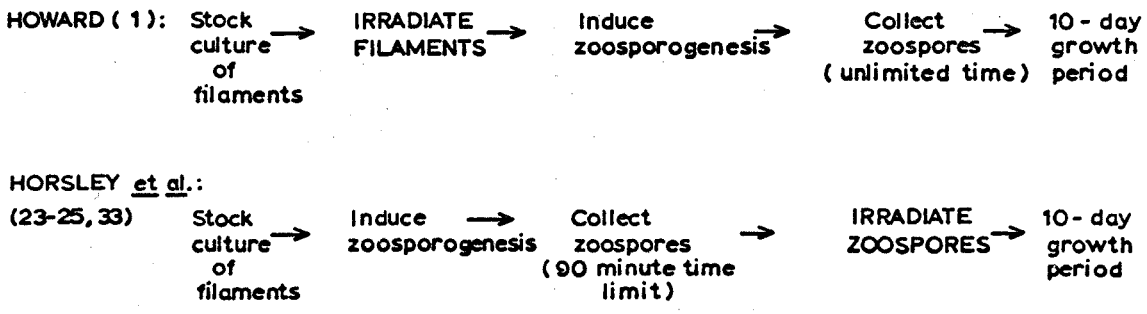

FIGURE 4

Comparison of the two radiation techniques which have been used in studies with Oe. cardiacum. The essential difference is that, in the Howard method, the cells are irradiated in the filaments, whereas in the Horsley technique, the cells are irradiated as zoospores.

scoring 200 cells per dose, Horsley et al. (40) 800 cells, and this author 1700 cells. Therefore, it seems that Howard's technique does enable a statistical analysis with larger numbers of cells.

The fact that the filaments were irradiated prior to zoospore induction suggests the possibility that radiation might damage the sporulation mechanism. Such damage could prevent a cell from producing or liberating a zoospore, or induce the production of a zoospore which is unable to swim or to attach to a microscope slide. If this were the case one would expect to find a decrease in the number of zoospores collected on the slides as the dose was increased. No correlation was found between these two factors. Howard (1) reported finding no effect on either the number or the visible morphology of the zoospores.

In the technique used by Horsley et al. (23-25, 33), the irradiation period must await the production of zoospores, the precise timing of which cannot be controlled. In Howard's method irradiation can be carried out whenever the radiation source is available and this proved to be a convenient asset.

Each irradiation, undertaken by this author, required the use of a separate stock culture of filaments, so the dose-survival curves are really a compilation of results from a number of independent cultures. This suggests that the physiological constitution of the cultures might not be uniform. However, the sigmoid survival curve obtained for the haploid with little scatter of points indicates that any physiological difference was negligible, at least for the haploid strain. The reason for the greater scatter of points on the diploid curve is difficult to establish. The haploid and diploid stock

cultures used in the experiments were considered to be in the stationary phase of the growth cycle when irradiated since they were at least two months old and there was no visible evidence of the accumulated growth becoming more dense.

The dose-survival curve for the haploid line reported in this thesis bears some similarity to the survival curve reported by Howard (1) in radiation studies with haploids. Each curve has an initial shoulder region followed by an exponential fall off. Howard reported survival data over two decades with a dose-range extending to 4 kilorads. However this compares with survival described in this thesis over three decades for a dose-range extending to 8 kilorads. The Do values were about the same, but the extrapolation number reported here was twice that found by Howard. Since the conditions of culturing, irradiating, and growing were slightly different between the two techniques, the duplication of results should not necessarily follow.

The phase of the cell cycle at the time the filaments were irradiated is not known for the investigations reported here for haploid and diploid Oe. cardiacum. It was thought that a comparison of the survival parameters reported here with those reported by Horsley and Pujara (44) for haploid Oe. cardiacum might be instructive in identifying the phase. A Do value of 836 rads reported in this thesis was also reported by Horsley and Pujara for both the late G_1 phase and the late S phase. On the basis of extrapolation numbers, the data reported by Horsley and Pujara implicate early G_2 as the phase in which the cells were irradiated. It is obvious that further studies are needed to identify the stage of the cell cycle in which irradiation occurred.

The information in this thesis has not added much to the understanding of the influence of ploidy on radiosensitivity. The results are essentially in agreement with other findings except for the unique variation of the survival parameters, n and Do. In this respect the experiments have failed to shed further light on this complex problem. On the other hand, the second aspect of the technique was very worthwhile to the research program involving Oe. cardiacum. The conclusion drawn was that the technique was not very good and hence this method of collecting cells for irradiation has been discontinued.

SUMMARY AND CONCLUSIONS

As indicated in the preface, one of the objectives of the experiments reported here was to measure and compare the γ-radiosensitivity of haploid and diploid Oedogonium cardiacum. The radiosensitivity has been measured and from the dose-survival curves it was found that, as the ploidy doubled, the Do value also doubled, but the extrapolation number decreased 4-fold. The conclusion that can best be drawn is that at all doses the diploid line was found to be more resistant than the haploid. The reasons for this are not understood but it probably depends in a complex way on genetic redundancy of the diploid cells and their ability to repair damage. Further experiments in this area might include studies of the repair capacity of both the haploid and diploid line of Oe. cardiacum using dose-fractionation techniques described for other cell lines by Davies (10) and Elkind and Sutton (46).

A second objective was to describe and evaluate a new radiation technique, modified from Howard (1) in studies with Oe. cardiacum. The Howard method permitted an analysis of radiosensitivity with a greater number of cells than that reported by Horsley and colleagues (23-25, 33). The greater number of cells enabled the haploid survival curve to be extended a decade lower than that reported by Howard who worked only with haploids. The diploid curve exhibited a scatter of points that was suggestive of some variability among the cultures. This may have been due to the use of physiologically different cultures, since an independent culture was required for each dose of radiation.

The new radiation technique, introduced by Howard (1), has been evaluated and compared with the procedure used routinely in the laboratory. The author reports that the Howard approach provided larger cultures of zoospores for measuring radiosensitivity and hence the results obtained should be more statistically meaningful, particularly at low levels of survival. Unfortunately, to obtain a single-dose survival curve, the Howard method necessitates irradiating several independent cultures which may visibly appear similar but physiologically be different. The smooth survival curve described for the haploid line seems to indicate that any physiological differences among the cultures were not significant. However, the same reassurance cannot be obtained from the diploid curve which exhibits a scatter of points that is suggestive of some variability among the diploid cultures.

The author concludes that no further experiments should be undertaken with the Howard technique because of the variability of the diploid data and of the uncertainty of the physiological status of the cultures.

During the past three or four years Horsley and his co-workers have developed a new procedure for growing large stock quantities of Oe. cardiacum in defined inorganic medium supplemented with trace elements and vitamin B_{12} that will produce large numbers of motile zoospores in a short period of time.

STUDIES ON THE SYNCHRONY OF *OEDOGONIUM CARDIACUM*

CHAPTER V

INTRODUCTION

Synchronous populations of cells are useful systems for investigating normal cellular activities and responses to environmental modifications of a single "typical" cell. In particular they are useful in the detection of small changes, such as the variations in DNA, RNA, and amino acid production with cell age, which otherwise would go undetected or be difficult to measure quantitatively. Many papers, including reviews (47-49), have been published regarding the theoretical concepts of synchrony and the practical approaches to synchronizing cell populations. In this chapter the aspects of synchrony which are pertinent to this thesis will be summarized.

A population of proliferating cells is synchronous if the cells proceed simultaneously around the cell cycle, passing at the same time through the various stages, for example, mitosis and DNA synthesis. First described by Howard and Pelc (50) using autoradiography of continuously labelled bean roots, this DNA synthetic period was called the "S" phase and the remainder of the interphase was divided into two phases, "G_1" and "G_2", to describe the gaps in time before and after the S phase. The cell cycle of Oe. cardiacum has been determined by Banerjee and Horsley (33) and is shown in Figure 5.

Measurements of the degree of synchrony of cell populations have involved the use of many biological end-points, for example, mitotic

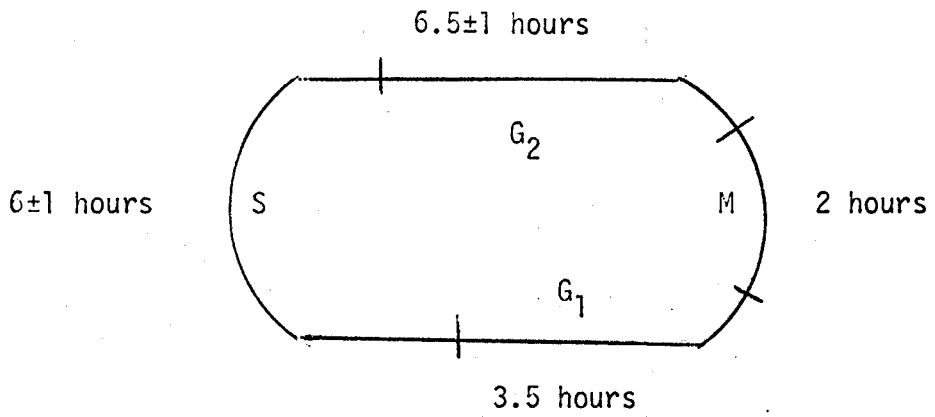

FIGURE 5

Phases of the cell cycle of Oe. cardiacum divided into the four phases: G_1, S, G_2 and M. The times shown are the lengths of the phases as determined by pulse-labelling with ^{14}C-TdR. From Banerjee and Horsley (33).

index (51, 52), cell size (52), cell number (52), and ^{3}H-TdR pulse labelling (52-54). Furthermore, even for any one end-point, different mathematical expressions have been proposed by Blumenthal and Zahler (55), Engelberg (56), and Zeuthen (57), for example, to express the degree of synchrony. Engelberg (56) stressed that any expression of the degree of synchrony was to some extent arbitrary and could only be judged by its usefulness in the context of the given application.

Reviews of cell synchrony (47, 48) indicate that several methods have been used to obtain synchronous cultures of a wide variety of cells. James (58) classified the methods as "induced synchrony" when produced by treatment of the cell culture, and "selection synchrony" when obtained by isolation of a specific stage. Induced synchrony has been accomplished by the use of temperature cycles and shocks, light cycles, nutritional changes, gas cycles, and inhibitory compounds. Selection synchrony has been done by filtration, shaking, sedimentation, sizing differences, and the ^{3}H-TdR "suicide" technique. The most satisfactory method of synchronization, from a physiological point of view, would be one which minimized the disturbance of the normal growth and development of the cells.

Since the selection method depends on the momentary existence at sometime in the cell cycle of a specific physical characteristic upon which an isolation technique can operate, it is obvious that the percentage yield from a logarithmically growing culture will be relatively small compared to the initial number of cells present.

As previously discussed in Chapter II A, synchronous cultures of Oe. cardiacum zoospores can be obtained if the zoospores liberated

by the filaments are collected within a short time interval. Synchronous populations of Oe. cardiacum zoospores have been used for a number of different investigations, for example, the time and rate of DNA synthesis through the cell cycle (33) and the determination of chromosome number (36). In addition, the loss of reproductive integrity following irradiation (40), dose-division delays in the cell cycle (39), and the morphological analysis of cells following lethal doses of irradiation in the cell cycle (59) have also been studied.

Synchronous populations of Oe. cardiacum zoospores are obtained by the induction of zoosporogenesis within the vegetative cells of the filaments by altering certain environmental factors such as the type of medium, the amount of light, and the level of free carbon dioxide in the medium. When the phototactic zoospores are liberated from the filaments, they can be collected by attracting them to glass microscope slides illuminated from below with fluorescent lights. This technique is fully outlined in the next chapter. Horsley and his colleagues (23, 33) reported that a collection period less than 90 minutes yielded a population of zoospores attaching to the glass slides that were approximately the same age. From autoradiographic studies, Banerjee and Horsley (33) reported that DNA synthesis began 3.5 hours after zoospore attachment, suggesting that the population was in the G_1 phase at the time of attachment.

However, it has been suggested (31, 40) that the degree of synchrony of populations of Oe. cardiacum cells declines as the cells progress through the cell cycle into the G_2 phase. This suggestion was

based on dose-survival curves (*) obtained when populations were irradiated in the G_2 period with UV (31) and X-rays (40). Since the curves exhibited inflexion points, the authors concluded that two sub-populations of differing radiosensitivity were actually present: a larger G_2 group and a smaller, lagging S group.

One objective of the experiments reported in this thesis was the improvement of the degree of synchrony of Oe. cardiacum zoospore populations. A brief description follows of the two techniques used, but complete details are presented in the next chapter. The nutritional technique involved the substitution of an inorganic medium modified from Machlis (60) (**) for the Molisch inorganic medium used routinely in the growth of collected zoospores. Machlis reported that his medium, supplemented with trace elements (**), enhanced the growth of Oe. cardiacum cultures.

Another method of inducing synchrony, which was thought to be applicable to Oe. cardiacum cultures, was developed for Chinese hamster cells by Sinclair (61), and for HeLa cells by Pfeiffer and Tolmach (62), and by Young, Schochetman, and Karnovsky (63). They reported that cells in asynchronous populations, when exposed to hydroxyurea, were inhibited from going beyond the end of G_1 in the cell cycle. When the block was removed, the cells which had accumulated at the end of G_1 then proceeded synchronously into the S phase. Sinclair also reported the selective lethal action of hydroxyurea for S cells but such killing would not be

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(*) See Appendix C.

(**) See Appendix A.

important with Oe. cardiacum since it was not the intention to expose S cells to hydroxyurea. As mentioned previously, the zoospores are in the G_1 phase when harvested (33), and therefore it was anticipated that these cells, if treated with hydroxyurea at this time, would be blocked at the end of G_1. Further, upon removal of the hydroxyurea, the cells would be released synchronously into the S and G_2 phases.

The nutritional method is considered a general method of inducing synchrony, that is, the particular cellular processes affected are not known. On the other hand, the inhibitory approach has a specific site of action, that is, it inhibits DNA synthesis by interfering with the reduction of a DNA precursor.

The second objective of the experiments reported in this thesis was to increase the size of the synchronous zoospore populations collected. With the provision of larger populations the survival data would be more statistically meaningful. However, if the collection period were lengthened to collect more zoospores, the degree of synchrony would fall off. It was intended that if the collected zoospores were blocked at the end of G_1 with hydroxyurea, then the collection period could be extended as cells would accumulate at this point in the cell cycle and the degree of synchrony would not be diminished. Then removal of the block would presumably give rise to a large population of zoospores proceeding synchronously through the S and G_2 phases.

CHAPTER VI

MATERIALS AND METHODS

A. Stock Cultures of Oedogonium cardiacum

Female haploid cultures were prepared and maintained in a manner similar to that described in Chapter II B for the radiosensitivity investigations. The salient features of the method will now be described. The non-axenic cultures were grown in a 1:12 biphasic soil-water medium in 700-ml glass preserving jars. Each jar was boiled for one hour on two successive occasions about a week apart. The jars were covered with polyethylene sheeting which had been punctured and plugged with cotton. The medium was inoculated when there was no discernible turbidity of the supernatant.

Initially, the cultures were maintained at room temperature under a 12:12 hour light:dark regimen on an illuminated culture rack. After inoculation, the cultures were exposed to a light intensity of about 220 foot-candles. After growth was established the intensity was reduced to about 100 foot-candles, and finally, after 3 months, were transferred to a window rack exposed to natural north light.

B. Measurement of Synchrony

The degree of synchrony of cell populations may be quantitated with respect to a number of cellular events such as cell division, mitosis, DNA synthesis, and cell size. In the experiments reported here,

the event used was the initiation of cell division. A zoospore which showed signs of stretching of the cylinder of new wall material was considered to have divided.

The degree of synchrony is reflected by the growth curve which, in these investigations, shows the percentage of zoospores that have divided as a function of time from the mid-point of the collection period. Depending on the number of zoospores collected, samples were fixed every hour or half-hour during division until the percent division reached a plateau. The actual times of fixation are given later in the chapter. The particular mathematical expression of the degree of synchrony applied here was derived by Zeuthen (57) and has been used in previous studies with Oe. cardiacum by Parker (31). The expression, known as the percent phasing, is given as follows:

$$\text{Percent phasing} = \frac{T/2 - D\ 1/2}{T/2} \times 100$$

where T = generation time measured from the mid-point of the collection period to when 50% of the first burst of cells has divided.

D 1/2 = time taken for 50% of the first burst to divide measured from 25% to 75% on the rising portion of the growth curve.

This expression, applied consistently throughout the investigations, provided a record of the relative changes in synchrony of the populations studied.

In the Oedogonium system, the percent phasing, without supplementing data from radio-isotope labelling, is described as a division-

oriented, rather than a cycle-oriented, measure of synchrony. In the latter the emphasis lies on the simultaneity of the phases of the cell cycle as determined by the incorporation of a radioactive DNA precursor. In division-oriented synchrony, the emphasis is placed on the simultaneity of cell or nuclear divisions with little regard for interphase activities.

Since nuclear division normally precedes cell division in Oe. cardiacum the possibility arose that the degree of synchrony might be different if nuclear division were selected as the measured end-point. Therefore, an experiment was conducted to determine whether the percent phasing value would be affected by the choice of end-points. The growth curves, based on cell division and on nuclear division, in both Molisch and Machlis media are shown in Appendix D. From the table of percent phasing values, also given in Appendix D, it is apparent that this index was not affected by the end-point selected. Since cell division in Oe. cardiacum is easier to determine, it is used as the end-point in the following investigations.

C. (i) Synchronization -- Nutritional Technique

Healthy cultures of Oe. cardiacum were selected on the basis of abundant, dark green growth. The filaments were washed in distilled water and induced to sporulate by placing them in a glass vessel containing about 800 ml. of dilute soil extract. After a dark period of 60-70 hours at room temperature, the vessel was placed above a bank of fluorescent lamps with an intensity of 150-200 foot-candles. Until the zoospores were liberated, the filaments were exposed alternately to periods

of light and dark with temperatures fluctuating from 21-26° C. The initial appearance of zoospores was determined periodically by examining samples of the medium for swimming zoospores or by checking the glass microscope slides resting on the vessel floor for attached zoospores.

When it appeared that sufficient zoospores could be collected, the medium and filaments were transferred to a large perspex vessel blackened on all sides to permit the passage of light only through the bottom. About 50-60 glass microscope slides were placed on the vessel floor to collect the zoospores. When sufficient zoospores had been collected (about 800 cells per slide) the slides were removed from the medium, rinsed with a stream of distilled water to remove loosely attached zoospores and debris, and placed in a perspex vessel containing either of two inorganic growth media. Thus 25-30 slides were placed in 2-4 litres of either Molisch medium, which was the standard laboratory procedure, or Machlis medium, which was the experimental medium under investigation. The vessels were set in a water bath maintained at about 23° C and illuminated continuously from below at an intensity of 300 foot-candles.

Commencing at about the 10th hour from the midpoint of the collection period, slides were removed periodically from the two media and fixed in Carnoy's solution. Cell samples were fixed until the 22nd or 23rd hour beyond the collection mid-point. After fixing, the slides were covered with a glass cover slide and stored in 70% ethanol. The cells were then examined under the microscope to determine the growth of the zoospores during the period of fixation. The percentage of the

zoospores which had divided was plotted as a function of time from the mid-point of collection. From the growth curves, the percent phasing value was calculated for each of the two growth media, Molisch and Machlis.

(ii) Synchronization -- Inhibitory Technique

Before synchrony studies with hydroxyurea could be undertaken, it was necessary to investigate the tolerance of Oe. cardiacum to this drug. After healthy cultures were selected, zoosporogenesis was induced, and zoospores collected and grown in Machlis inorganic medium as described in Chapter VI C (i). The salient features of the method follow. The collected zoospores were divided into 7 groups, including a control prior to being placed in Machlis growth medium. Hydroxyurea (*) was added to the medium in a quantity measured to give the desired concentration. Each of the six experimental groups was treated with a certain concentration of hydroxyurea for a particular length of time. The six treatments were 400 µg/ml for 9, 19, and 29 hours and 800 µg/ml also for 9, 19, and 29 hours. Sinclair (53) reported concentrations of 76 µg/ml for Chinese hamster cells, but the concentrations selected here were increased to account for the fact that plant cells generally are more tolerant to environmental perturbations. After the treatment period, the cells were rinsed 3 times in Machlis medium to remove the hydroxyurea and then placed in fresh Machlis medium to grow.

Cell survival, as measured by the loss of proliferative capacity,

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(*) 1-hydroxyurea was obtained from the Pharmaceutical Department, Henderson General Hospital, Hamilton, and from National Biochemicals Corporation, U.S.A. The powder was dissolved in 10 ml distilled water and then added to the medium.

was determined by fixing the filaments in Carnoy's solution following a 5-day growth period and examining them under the microscope. As in the case of the radiation experiments reported earlier in the thesis, zoospores which grew into filaments with more than 12 cells were designated as survivors, and those with 12 cells or less as non-survivors. The hydroxyurea doses with which the cells were treated are given in µg. hours.

For the synchrony studies the method of induction of zoosporogenesis, and the collection and growth of zoospores in Machlis medium was similar to that described in Chapter VI C (i). The collection periods were short and varied from 100-165 minutes. Hydroxyurea was added to the growth medium for periods of 4-9 hours at concentrations of 200 or 400 µg/ml. The range of hydroxyurea exposures was 0.8×10^3 to 3.6×10^3 µg. hours, within the limits of survival as suggested in Chapter VII B.

Since collected zoospores are in the G_1 phase and this phase is 3.5 hours long (33), the collection periods could not exceed 3.5 hours otherwise the cells would progress into the S phase before treatment with hydroxyurea. Hydroxyurea is assumed to block the cells at the end of G_1.

Cells were released from the block by rinsing 3 times in Machlis medium and then placed in fresh Machlis medium for the remainder of the growth period. Slides were removed periodically for fixing in Carnoy's solution. The cells were then examined under the microscope to determine the percent of zoospores which had divided as a function of time from the mid-point of the collection period. From the graph, percent phasing values were calculated for the different hydroxyurea

concentrations.

To check whether hydroxyurea was indeed inhibiting Oe. cardiacum cells, zoospores were exposed to protracted treatments of hydroxyurea after a two-hour collection period. The seven exposures tested were 0 µg/ml, 400 µg/ml for 9, 19, or 29 hours and 800 µg/ml for 9, 19, or 29 hours. After the hydroxyurea was removed, the subsequent growth to the two-cell stage was observed in Machlis medium. Although this investigation will not identify the actual location of the block in the cell cycle, it will reveal whether cell division is inhibited in the presence of hydroxyurea.

D. Large Collections of Zoospores with Hydroxyurea

In the investigations concerned with increasing the size of the synchronous zoospore population, the techniques used for the induction of zoosporogenesis, and the collection and growth of the zoospores in Machlis medium were similar to that described in Chapter VII C (i). It was intended to lengthen the collection period to obtain more zoospores, but this often results in a decline in the degree of synchrony of the population. Therefore hydroxyurea, at concentration of 300 µg and 400 µg/ml, was added to the collecting medium to block the cells from progressing to the S phase during collection periods of 3-3.5 hours. Hydroxyurea, at similar concentrations, was also added to the growth medium for 3.5-4.25 hours to permit zoospores collected at the end of the collection period to reach the block. The range of hydroxyurea exposures used was 2.3×10^3 - 2.6×10^3 µg.hours, which was within the limits outlined in Chapter VII B.

The techniques of removing the hydroxyurea block, the subsequent growing of the cells, and the compilation of data have been described previously in Chapter VII C (ii).

CHAPTER VII

RESULTS

A. Synchronization -- Nutritional Technique

The results of 4 separate experiments, undertaken to compare the degree of synchrony in Molisch and Machlis inorganic growth media, are summarized in Figure 6 (A, B, C, D). The x-axis indicates the time in hours after the mid-point of collection and the y-axis indicates the growth of the zoospores in terms of the percent of zoospores which have divided. Each experimental point represents the scoring of an average of about 800 cells, and the vertical bars represent the standard deviation which has been described previously.

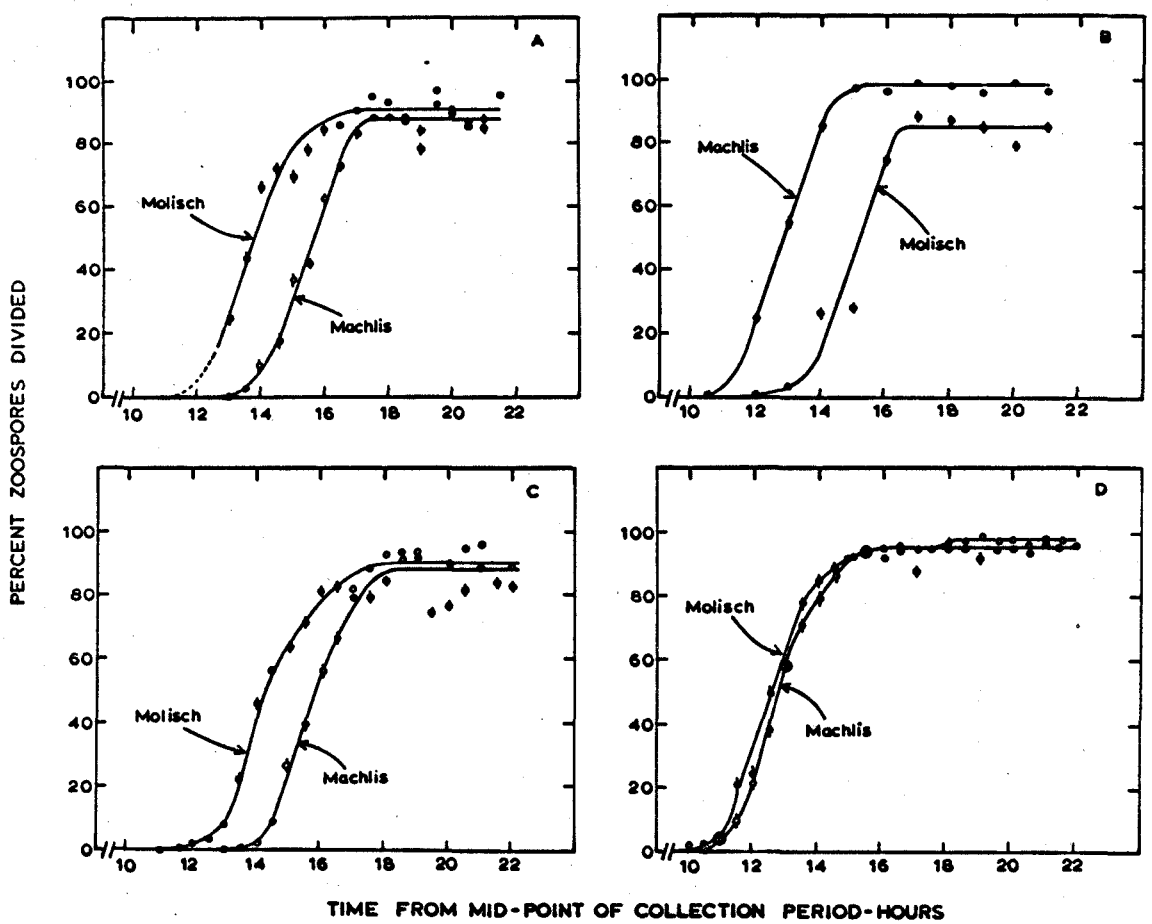
Each of the growth curves exhibits an initial lag period appearing 10-13 hours after the mid-point of the collection periods, which varied from 50-90 minutes. In Figures 6A and 6C, cell division occurred first (*) in Molisch medium, but in Figure 6B it occurred first in Machlis medium. In Figure 6D there is virtually no difference in the initiation of cell division for the two media. It appears that the variation in the initiation of cell division is not related to treatment but is a random phenomenon.

The lag period of growth curves is followed by a straight

(*) The time at which cell division was initiated was determined by extrapolating the linear rising portion of the growth curve back to the x-axis.

FIGURE 6

Growth curves from four experiments (A, B, C, D) in which Oe. cardiacum zoospores were grown in Molisch and Machlis inorganic media. The percentage of zoospores that have divided is plotted as a function of the time in hours after the mid-point of the collection period. Standard deviation has been indicated on the plots as vertical bars. The dotted line in Fig. 6A represents an estimated portion of the curve for which there were no data.


A
B
C
D
Molisch
Machlis
PERCENT ZOOSPORES DIVIDED
TIME FROM MID-POINT OF COLLECTION PERIOD-HOURS

line rising portion and then a plateau region, which appeared at about the 90% division level.

The degree of synchrony, expressed as percent phasing, and the generation times of the zoospore populations were determined from the growth curves and are given in Table 6. The average percent phasing as well as the average generation time, is the same for both Molisch and Machlis media.

B. Synchronization -- Inhibitory Technique

An experiment was carried out to determine the tolerance of Oe. cardiacum to hydroxyurea by treating the zoospores with various doses of hydroxyurea following a two-hour collection period. The dose-survival data are presented in Figure 7. The x-axis indicates the concentration of hydroxyurea in µg.hours and the y-axis indicates the percent survival after 5 days' growth in Machlis medium. The zero dose survival has been normalized to 100% and other values corrected accordingly, based on the plating efficiency in untreated controls of 85.3%. Unlike the survival criteria for the radiation studies, single cells were included as non-survivors in these experiments since their presence, as shown in Figure 7, appears related to the treatment. Each experimental point in the survival curve represents the scoring of an average of 860 cells and the error bars represent the standard deviation.

The dose-survival curve in Figure 7 indicates that survival falls off linearly with dose to a concentration of about 9×10^3 µg. hours and then levels off. For the investigations into synchrony with hydroxyurea which follow in this chapter, concentrations of hydroxyurea

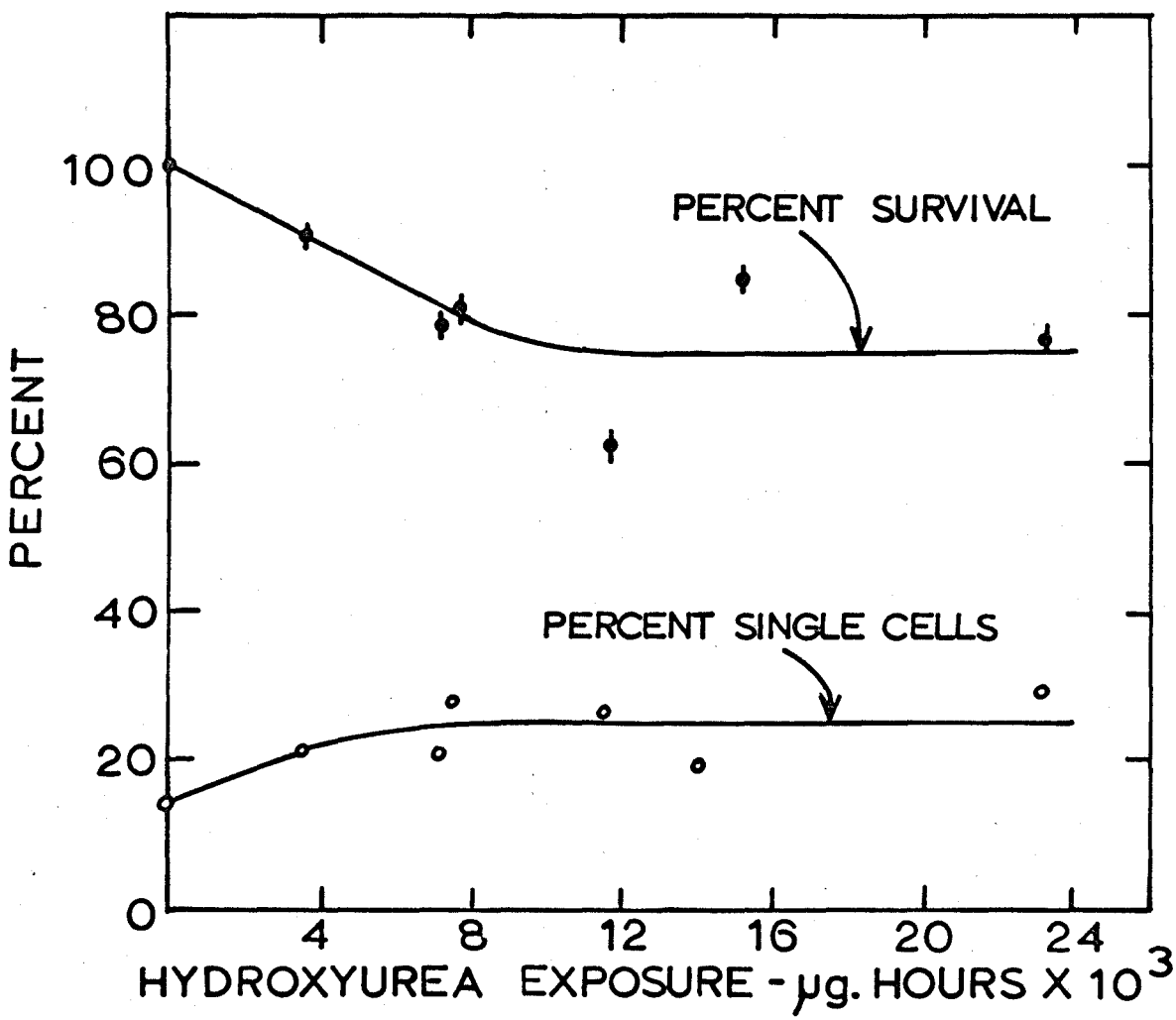
TABLE 6

Percent phasing values and generation times for four experiments in which Oe. cardiacum zoospores were grown in Molisch and Machlis media.

	Percent Phasing		Generation Time (hours)	
Figure	Molisch Medium	Machlis Medium	Molisch Medium	Machlis Medium
6A	82.3	80.7	13.6	15.4
6B	77.7	78.2	15.0	12.8
6C	75.8	80.8	14.1	15.7
6D	76.0	76.3	12.4	12.7
Average	78.0	79.0	13.8	14.2

FIGURE 7

Survival of Oe. cardiacum at various exposures of hydroxyurea. Standard deviation has been indicated by vertical bars. The percent single cells as a function of hydroxyurea exposure is also shown.


100
80
60
40
20
0
PERCENT
PERCENT SURVIVAL
PERCENT SINGLE CELLS
4
8
12
16
20
24
HYDROXYUREA EXPOSURE - µg. HOURS X 10³

which would yield less than 90% survival, as indicated in Figure 7, were not used. Therefore, only hydroxyurea concentrations not exceeding 3.6×10^3 µg.hours could be used. The lethal damage induced by hydroxyurea manifested itself primarily in the form of single cells.

The results of three separate experiments in which the synchrony of Oe. cardiacum zoospores was measured following an hydroxyurea treatment are summarized in Figures 8 (A, B) and 9. The x-axis indicates the time in hours after the mid-point of collection and the y-axis indicates the growth of the zoospores measured as the percent of zoospores which have divided. In Figure 8A, the cells were exposed to a 4-hour hydroxyurea treatment at a concentration of 0, 200, and 400 µg/ml, and in Figure 8B the cells were exposed to a 9-hour treatment at a concentration of 0, 400, and 800 µg/ml. In Figure 9, the hydroxyurea concentration was fixed at 400 µg/ml with treatment times of 0, 4, and 7 hours. The lengths of the collection periods were as follows: Figure 8A, 100 minutes, Figure 8B, 120 minutes and Figure 9, 150 minutes. Each experimental point in the two figures represents the scoring of an average of 730 cells and the error bars represent the standard deviation.

Each of the 3 growth curve exhibits an initial lag period followed by a linear rising portion and then a plateau region. In Figure 8B portions of the curves were estimated since the availability of slides limited the intervals at which cells could be fixed. The initiation of cell division in the untreated control cells occurred at the 13th hour after the mid-point of collection, but in the treated populations it was delayed 2.6-5.0 hours. The plateau regions of the experimental curves were not significantly different from the controls

FIGURE 8

Growth of Oe. cardiacum zoospores following an exposure to hydroxyurea after the collection period: A, a 4-hour exposure at concentrations of 0, 200, and 400 µg/ml; B, a 9-hour exposure at 0, 400, and 800 µg/ml. The percentage of zoospores that have divided is plotted as a function of the time in hours after the mid-point of the collection period. Standard deviation has been indicated as vertical bars. The dotted lines in Fig. 8B represent estimated portions of the curve for which no data was available.

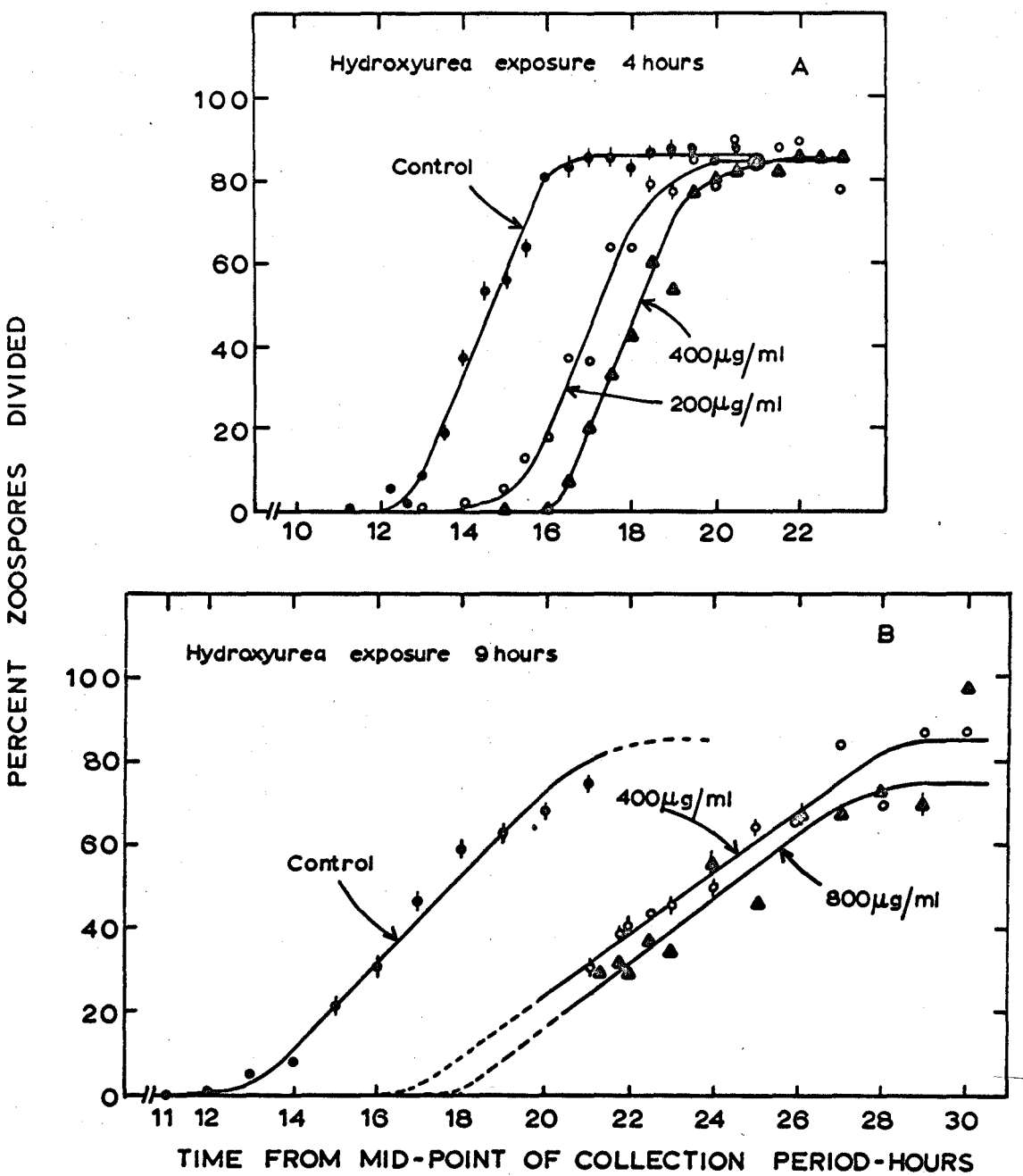
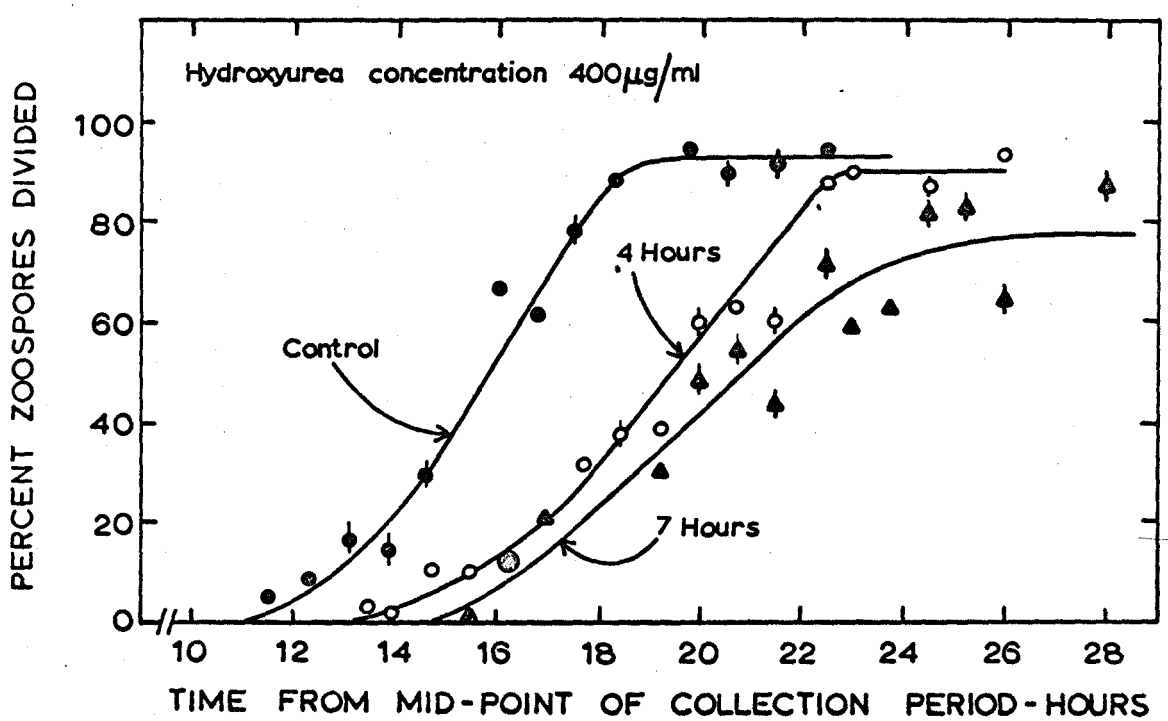

Hydroxyurea exposure 4 hours
A
Control
400µg/ml
200µg/ml
100
80
60
40
20
0
10
12
14
16
18
20
22
Hydroxyurea exposure 9 hours
B
Control
400µg/ml
800µg/ml
11
12
14
16
18
20
22
24
26
28
30
PERCENT ZOOSPORES DIVIDED
TIME FROM MID-POINT OF COLLECTION PERIOD-HOURS

FIGURE 9

Growth of Oe. cardiacum zoospores following an exposure to hydroxyurea at a concentration of 400 µg/ml for 0, 4, and 7 hours after the collection period. The percentage of zoospores that have divided is plotted as a function of the time in hours after the mid-point of the collection period. The vertical bars represent standard deviation.


Hydroxyurea concentration 400µg/ml
Control
4 Hours
7 Hours
PERCENT ZOOSPORES DIVIDED
0
20
40
60
80
100
10
12
14
16
18
20
22
24
26
28
TIME FROM MID-POINT OF COLLECTION PERIOD - HOURS

which levelled off at a dividing fraction of about 0.8.

The percent phasing values and the generation times given in Table 7 were determined from the growth curves in Figures 8 and 9. It is apparent that the degree of synchrony was not improved by the hydroxyurea treatments, and also, that the generation time was lengthened as the hydroxyurea exposure increased.

An experiment was conducted in which the growth of Oe. cardiacum zoospores was measured following protracted treatments with hydroxyurea to determine whether the hydroxyurea was indeed functioning as a block and inhibiting cell division. The results are shown in Figure 10 (A, B). The x-axis indicates the time in hours after the mid-point of a two-hour collection and the y-axis indicates the growth of zoospores expressed as the percent of zoospores which have divided. In Figure 10A the concentration of hydroxyurea was 400 µg/ml and the length of exposure was 0, 9, 19, and 29 hours. In Figure 10B the length of exposure was the same but the concentration of hydroxyurea was doubled to 800 µg/ml. Each experimental point represents the scoring of an average of 900 cells and the vertical bars represent the standard deviation.

Each of the growth curves exhibits an initial lag period followed by a linear rising portion and then a plateau region. Portions of the curves were estimated, as shown by dotted lines, since the availability of slides restricted the number which could be fixed for each curve. The control curves in Figures 10A and 10B are identical since they represent the growth of the same population of untreated cells and indicate that the initiation of cell division occurred at the

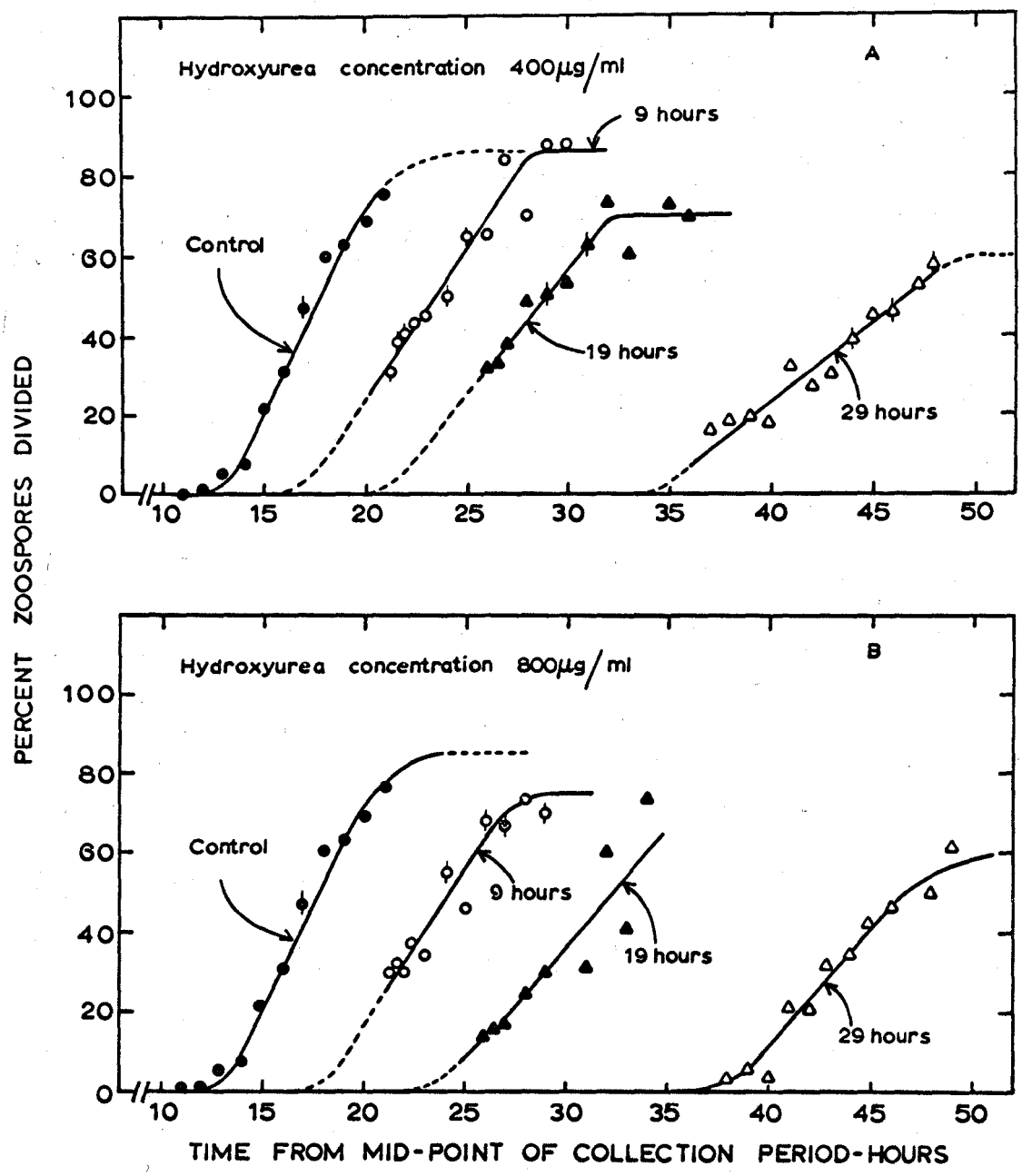
TABLE 7

Percent phasing values from three experiments in which cultures of Oe. cardiacum zoospores were treated with hydroxyurea after the collection period.

Figure	Hydroxyurea Concentration (µg/ml)	Hydroxyurea Treatment Time (Hours)	Percent Phasing	Generation Time (Hours)
8A	0	0	72.2	14.4
	200	4	72.9	16.9
	400	4	75.6	17.8
8B	0	0	42.4	17.1
	400	9	43.8	22.5
	800	9	33.3	22.9
9	0	0	60.3	15.6
	400	4	59.4	19.1
	400	7	34.3	19.7

FIGURE 10

Growth of Oe. cardiacum zoospores following a protracted exposure to hydroxyurea after the collection period: A, a concentration of 400 µg/ml for 0, 9, 19, and 29 hours, and B, a concentration of 800 µg/ml for 0, 9, 19, and 29 hours. The percentage of zoospores that have divided is plotted as a function of the time in hours after the mid-point of the collection period. The vertical bars represent standard deviation. The dotted lines represent portions of the curves for which no data were available.


Hydroxyurea concentration 400µg/ml
A
9 hours
Control
19 hours
29 hours
Hydroxyurea concentration 800µg/ml
B
Control
9 hours
19 hours
29 hours
PERCENT ZOOSPORES DIVIDED
TIME FROM MID-POINT OF COLLECTION PERIOD-HOURS

13th hour. Further, the process of cell division was completed in 8 hours and the percent division levelled off at 88%. In the treated population, the initiation of cell division was delayed by 3.6-24.8 hours and the length of the division process was extended an additional 6.9-14.6 hours, depending on the extent of the hydroxyurea treatment. The plateau regions of the curves were not significantly affected by the 9-hour hydroxyurea treatments, but they were lower for the 19-hour and 29-hour treatments. Survival data, determined after the fifth day of growth, is summarized in Table 8.

The percent phasing values were not calculated since, at most exposures of hydroxyurea, the minimum dividing fraction of 0.75 was not attained. However, the degree of synchrony is reflected by the slope of the growth curve, and on this basis, it is apparent from Figure 10 that the degree of synchrony decreased with increasing exposure to hydroxyurea, at both 400 and 800 µg/ml. Calculations from Figure 10 indicate that both the generation time and the division delay increased with increasing exposure to hydroxyurea, at both 400 and 800 µg/ml.

C. Large Collections of Zoospores with Hydroxyurea

The results of two separate experiments in which the growth of Oe. cardiacum zoospores (that is, percent zoospores divided) was measured after an exposure to hydroxyurea are summarized in Figures 11 and 12. Hydroxyurea was administered during both the collection period, which was lengthened to increase the number of zoospores collected, and a post-collection period, which was long enough to enable all cells to reach the block at the end of G_1. The x-axis indicates the time in

TABLE 8

Survival of Oe. cardiacum cells determined 5 days after various treatments with hydroxyurea following the collection period.

Hydroxyurea Treatment Time (hours)	Percent Survival	
	400 µg HU/ml	800 µg HU/ml
0	85.3	85.3
9	78.1	67.7
19	69.1	73.7
29	53.7	65.8

FIGURE 11

Growth of Oe. cardiacum zoospores following an exposure to hydroxyurea at a concentration of 300 µg/ml for 7.75 hours. This exposure time includes the collection period of 3.5 hours and a post-collection period of 4.25 hours. The percentage of zoospores that have divided is plotted as a function of the time in hours after the mid-point of the collection period. The vertical bars represent the standard deviation.

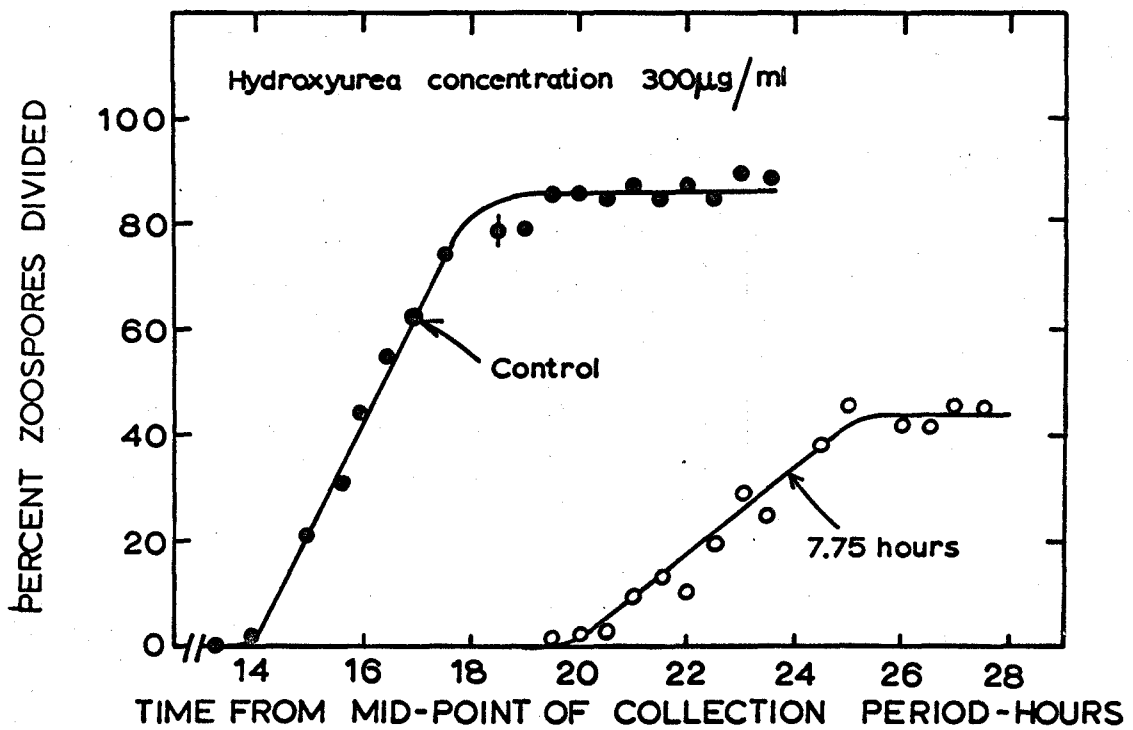
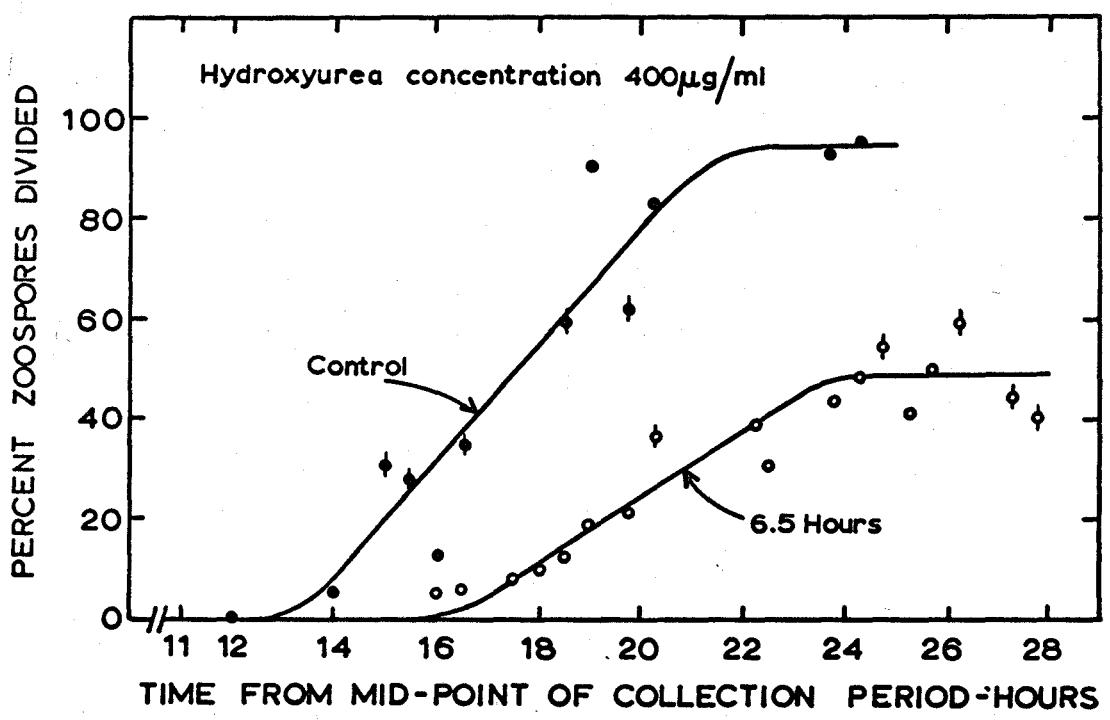

Hydroxyurea concentration 300µg/ml
PERCENT ZOOSPORES DIVIDED
100
80
60
40
20
0
Control
7.75 hours
14
16
18
20
22
24
26
28
TIME FROM MID-POINT OF COLLECTION PERIOD-HOURS

FIGURE 12

Growth of Oe. cardiacum zoospores following an exposure to hydroxyurea at a concentration of 400 µg/ml for 6.5 hours. This exposure time includes the collection period of 3.0 hours and a post-collection period of 3.5 hours. The percentage of zoospores that have divided is plotted as a function of the time in hours after the mid-point of the collection period. The vertical bars represent the standard deviation.


Hydroxyurea concentration 400µg/ml
PERCENT ZOOSPORES DIVIDED
100
80
60
40
20
0
Control
6.5 Hours
11 12 14 16 18 20 22 24 26 28
TIME FROM MID-POINT OF COLLECTION PERIOD-HOURS

hours after the mid-point of collection and the y-axis indicates the growth of the zoospores in terms of the percentage of zoospores which have divided. In Figure 11 the concentration of hydroxyurea was 300 µg/ml and the length of treatment was 7.75 hours (including the 3.5-hour collection period). In Figure 12 the concentration was 400 µg/ml and the treatment time 6.5 hours (including the 3.0-hour collection period). Each experimental point in the two figures represents the scoring of a minimum of 1,000 cells. The vertical bars represent the standard deviation.

Each of the growth curves exhibits an initial lag period followed by a linear rising portion and then a plateau region. The control curves show the lag period beginning at the 13th-14th hour after the mid-point of the collection period. The linear rising portion levelled off at the 18th-21st hour to a dividing fraction of 0.90. With cells treated at 300 µg/ml of hydroxyurea (Fig. 11) the initiation of cell division was delayed 6 hours and the division time increased by 1.2 hours to 5.3 hours. With cells treated at 400 µg/ml (Fig. 12) the initiation of cell division was delayed 3 hours and the division time decreased by 0.5 hours to 7.3 hours. The plateau regions in both experimental curves represented a dividing fraction of 0.5.

The percent phasing values could not be calculated for the experimental curves in Figures 11 and 12 since the minimum dividing fraction of 0.75 was not attained. The slopes of the curves, however, reveal that the degree of synchrony in treated populations was less than that in the untreated controls.

CHAPTER VIII

DISCUSSION

As stated previously in the introduction, one of the objectives of the experiments reported in this thesis was to improve the degree of synchrony of Oedogonium cardiacum zoospore cultures. Synchrony was measured by calculating the percent phasing with the initiation of cell division as the biological end-point. One of the two approaches undertaken was a study of the growth of Oe. cardiacum zoospores in Molisch and Machlis inorganic media and a comparison of percent phasing values. Since these values were similar for cultures grown in both media, it follows then that the substitution of Machlis medium for the routine Molisch medium did not significantly affect the degree of synchrony. Further, the Machlis medium did not appear to influence other growth parameters, such as the generation time and the fraction of the population undergoing division.

The essential difference in the composition of the two media (*) studied is that the Machlis medium is supplemented with trace elements and vitamin B_{12}. Machlis (60) found that the supplements enhanced the growth of Oe. cardiacum filaments over a five-day period. However, in the synchrony studies reported here, the growth of zoospores is followed only to the completion of the first cell division, about 18 hours after

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(*) See Appendix B.

the collection period. In this short time period, any growth differences due to the nutritional change from Molisch to Machlis did not appear to manifest themselves.

The second approach to improving synchrony involved the use of an inhibitor, hydroxyurea. Initial experiments were carried out to determine the dose-survival response of Oe. cardiacum to various doses of hydroxyurea. It was essential that zoospores exposed to hydroxyurea-synchronizing treatments be able to grow normally when the block was removed. From the dose-survival data it is apparent that there is a killing effect and, although it does not continue to fall off with dose, can significantly reduce the size of the viable population. An hydroxyurea exposure of 3.6×10^3 µg.hours was selected as the maximum dose with which cells would be treated in the subsequent synchronization attempts. This was expected to maintain viability to within 90% of the untreated controls.

Survival was measured by the loss of reproductive capacity after a five-day growth period. Since the lethal damage induced by hydroxyurea manifested itself primarily in the form of single cells, one could speculate that the mitotic mechanism had been disrupted and the cells inhibited from dividing. The killing mechanism, as suggested by Sinclair (61), probably acts when the cells reach a critical stage about the time the untreated cells should have divided. Their RNA and protein levels (and presumably various other facets of the cells' progress in age) reach the stage when division should occur and only DNA synthesis is not complete. This unbalanced condition becomes critical and thus causes cell death.

From the studies of synchronization with hydroxyurea it is apparent that no significant increase in the percent phasing values was obtained for treated populations beyond that for the untreated controls. Therefore, it follows that hydroxyurea did not improve the degree of synchrony of Oe. cardiacum zoospores. However, hydroxyurea did cause a delay in the initiation of cell division and hence in the generation time of the populations. In each of the three experiments reported, the length of the division delay and the generation time was dependent upon the extent of the hydroxyurea treatment. The plateau regions of the experimental curves were not significantly different from the controls, which suggests that cell killing by hydroxyurea is not important at the particular concentration used.

Since the synchrony was not improved with the hydroxyurea treatments, then possibly some of the cells had slipped through the block, presumed to be at the end of G_1, and reached the period of cell division somewhat delayed beyond the normal time. It was apparent that hydroxyurea delayed cell division, but the problem arose whether hydroxyurea did in fact act as an inhibitor and could inhibit cell division in the Oedogonium cardiacum system.

In an attempt to determine whether hydroxyurea could inhibit, as well as delay, cell division, populations of zoospores were collected and then exposed to protracted treatments of hydroxyurea. Normally, populations of Oe. cardiacum zoospores are expected to divide approximately 18 hours after the mid-point of the collection period (33). However, from the data presented in Figure 10 it is apparent that cell division does not occur during hydroxyurea treatments of 9, 19, and 29 hours

at concentrations of 400 and 800 µg/ml. It is concluded that hydroxyurea inhibited cell division, but the particular site of inhibition in the cell cycle cannot be identified from the reported data. In mammalian cell lines the block has been reported to occur at the end of the G_1 phase (61-63). The inhibiting mechanism of hydroxyurea has been investigated by Young and Hodas (64) and identified as the interference with the reduction of thymidine ribonucleotide, a step in the synthesis of DNA.

The data from the experiments discussed above, which are presented in Figure 10, indicate further that the lengthening of the hydroxyurea exposure time increased the division delay. The length of the delay, however, was not equal to the length of the hydroxyurea exposure. For example, a 9-hour hydroxyurea exposure of 400 µg/ml did not induce a delay of 9 hours, but rather 3.9 hours. It appears that if the block is holding the cells at the end of G_1, then the progression around the remainder of the cell cycle when the block is removed is more rapid than the normal rate. Sinclair (61) reported that hydroxyurea slightly reduced the length of the S phase in Chinese hamster cells.

Figure 10 also shows that as the length of treatment increased, the fraction of the population undergoing division decreased. This suggests a more pronounced killing effect at the higher doses of hydroxyurea and is borne out by the survival data determined after the fifth day of growth. Further, the slopes of the growth curves suggest that the degree of synchrony declines as the hydroxyurea exposure time increased.

The attempts to harvest larger, synchronous cultures of Oe. cardiacum zoospores were carried out by lengthening the collection

to harvest more zoospores. Hydroxyurea was administered to the cells during both the collection period and a short post-collection period to synchronize the culture. Data from three experiments indicated that the lengthening of the collection period decreased the degree of synchrony, as shown by the percent phasing values, which ranged from 55.3-71.3. The optimum range of percent phasing values for Oe. cardiacum is above 75.0. The synchrony decreased because the age difference among the harvested zoospores was enhanced by the extension of the collection time. Thus it was necessary to treat the cells with hydroxyurea in an effort to improve the degree of synchrony. However, as the slopes of the growth curves indicate, the hydroxyurea treatments lowered, rather than improved, the degree of synchrony.

Further, the hydroxyurea treatments significantly lowered the fraction of zoospores dividing. This is in contrast with the results reported previously in this thesis for hydroxyurea treatments which were administered only during a period subsequent to the collection, rather than both during and following the collection period. The results reported previously do not show a significant lowering of the fraction dividing (0.8) for the same dose ranges. If it is postulated that the zoospores are particularly sensitive to hydroxyurea during the collection period when they are swimming or attaching to the microscope slides, then one could speculate that the hydroxyurea treatment during the collection period caused the fraction of zoospores dividing to be lowered, as shown in Figures 11 and 12.

From the investigations described in this thesis, it is apparent that no improvement in the degree of synchrony of Oe. cardiacum

zoospores or of the size of the synchronous zoospore collections was accomplished.

SUMMARY AND CONCLUSIONS

As indicated in the introduction, one of the aims of these investigations was to measure the synchrony of Oedogonium cardiacum zoospore cultures under different conditions. Firstly, the synchrony of zoospore cultures was measured growing in Molisch and in Machlis inorganic media following the collection period. A comparison of the percent phasing values indicates the degree of synchrony was the same for both growth media. Secondly, the synchrony was measured after treating zoospore cultures with an inhibitor, hydroxyurea. However, this approach did not improve synchrony either, and, in experiments with high doses of hydroxyurea, caused the synchrony to decline.

The second aim of these investigations, to enlarge the size of synchronous zoospore cultures using hydroxyurea, did not meet with success. Since the technique necessitated the exposure of the cells to hydroxyurea during the collection period as well as the initial portion of the growth period, the total dose of hydroxyurea was too severe on the subsequent growth of the cells. Therefore, in these studies, the degree of synchrony and the size of synchronous zoospore cultures were not improved beyond that obtained routinely in the laboratory with established techniques.

Future synchrony studies might include the use of radioactive isotopes to detect the specific location of the hydroxyurea block in the cell cycle and to measure the synchrony using the initiation of

DNA synthesis as the biological end-point. Investigations could be undertaken to determine the effect on synchrony of different conditions under which zoosporogenesis is induced.

APPENDIX

APPENDIX A

MODIFIED MOLISCH MEDIUM -- from Pringsheim (65)

$(NH_4)_2$ HPO_4	6.06×10^{-3} M
K_2HPO_4	2.30×10^{-3}
$Mg\ SO_4$	1.67×10^{-3}
$Ca(NO_3)_2\ 4H_2O$	1.72×10^{-3}

MODIFIED MACHLIS MEDIUM -- after Machlis (60)

KH_2PO_4	1.48×10^{-3} M
K_2HPO_4	0.52×10^{-3}
$CaCl_2$	0.50×10^{-3}
$Mg\ SO_4\ 7H_2O$	1.00×10^{-3}
KNO_3	20.00×10^{-3}
trace element solution	1 ml
Vitamin B_{12}	100 µg/litre

TRACE ELEMENT SOLUTION

H_3BO_3	1.00 gm
$CuSO_4\ 7H_2O$	0.15
EDTA (Na)	5.00
$ZnSO_4\ 7H_2O$	2.20
$CaCl_2$	0.50
$Mn\ Cl_2\ 4H_2O$	0.50

$FeSO_4 \quad 7H_2O$	0.50 gm
$CoCl_2 \quad 6H_2O$	0.15
$(NH_4)_6 \, Mo_7O_{24} \quad 4H_2O$	0.10

Dissolve the above salts in 75 ml distilled water. KOH pellets were added to keep the pH close to neutrality. The resulting solution was then diluted to 100 ml with distilled water.

SOIL EXTRACT -- after Horsley and Fucikovsky (24)

Mix 50 cc of cleaned garden soil with 600 ml distilled water in flasks plugged with cotton. Boil the mixture for 1 hour on two separate occasions about a week apart. When the supernatant clears, decant, and filter 3 times with fibreglass paper. Store in the refrigerator.

APPENDIX B

TABLE 9

Percentage of single haploid Oe. cardiacum cells, irradiated at various doses with X-rays, which failed to divide after a 10-day growth period.

Dose (rads)	Percent Single Cells
0	0.1
842	0
1229	0
1660	1.6
2045	0
2525	3.8
2871	0
3331	2.4
4207	1.6
5042	3.3
5365	1.1
5816	3.1
6706	5.5
7382	1.4

TABLE 10

Percentage of single diploid Oe. cardiacum cells, irradiated at various doses with γ-rays, which failed to divide after a 10-day growth period.

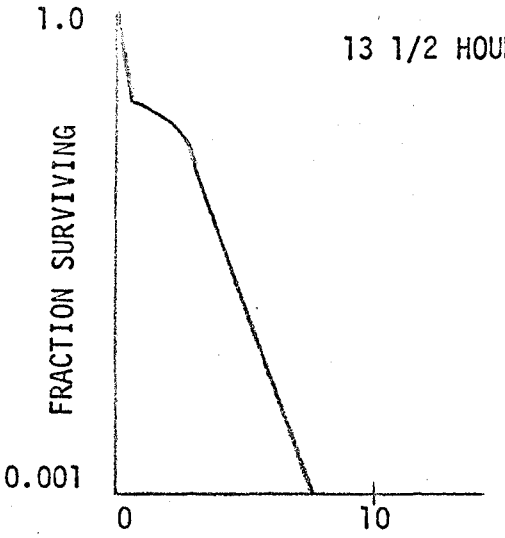
Dose (rads)	Percent Single Cells
0	0
858	0
1716	0.2
2125	1.4
2574	0
2970	0.1
3820	0.2
4670	1.0
6520	5.1
6440	2.0
6864	1.8
7290	0.2
8080	0.7

APPENDIX C

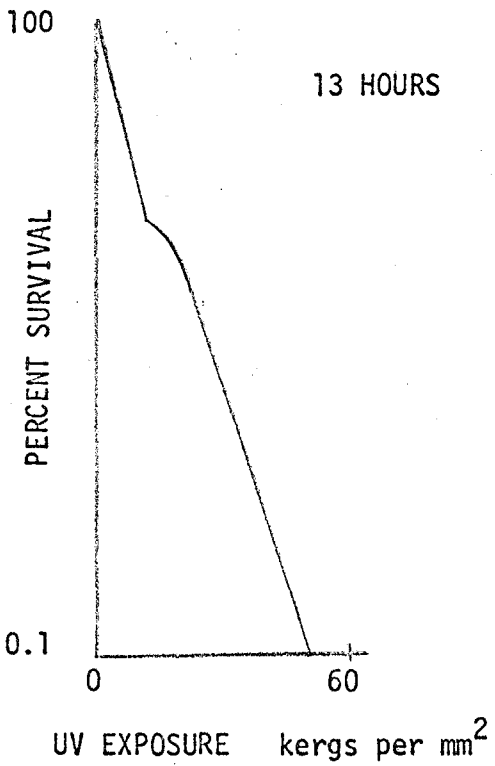
FIGURE 13

Dose-survival curves of Oe. cardiacum irradiated during the G_2 period with X-rays and with UV. Each curve exhibits an inflexion point suggestive of two subpopulations with different radiosensitivities. Figure 13A is taken from Horsley, Fucikovsky, and Banerjee (40); Figure 13B from Parker (31).

A


1.0
13 1/2 HOURS
FRACTION SURVIVING
0.001
0
10
COBALT 60 DOSE IN KILORADS

B


100
13 HOURS
PERCENT SURVIVAL
0.1
0
60
UV EXPOSURE kergs per mm2

APPENDIX D

A comparison of the degree of synchrony, based on two different biological end-points, was undertaken to determine the effect of the end-point selected on the percent phasing value. The growth curves are presented in Figure 14 (A, B). The percent phasing values are given in Table 11.

FIGURE 14

Growth of Oe. cardiacum zoospores in Molisch and Machlis inorganic media. The percentage of zoospores that have divided is plotted as a function of time in hours after the mid-point of the collection period. The biological end-points used to determine the percentage of zoospores which had divided were cell division and mitotic division. Standard deviation has been indicated on the plots as vertical bars.

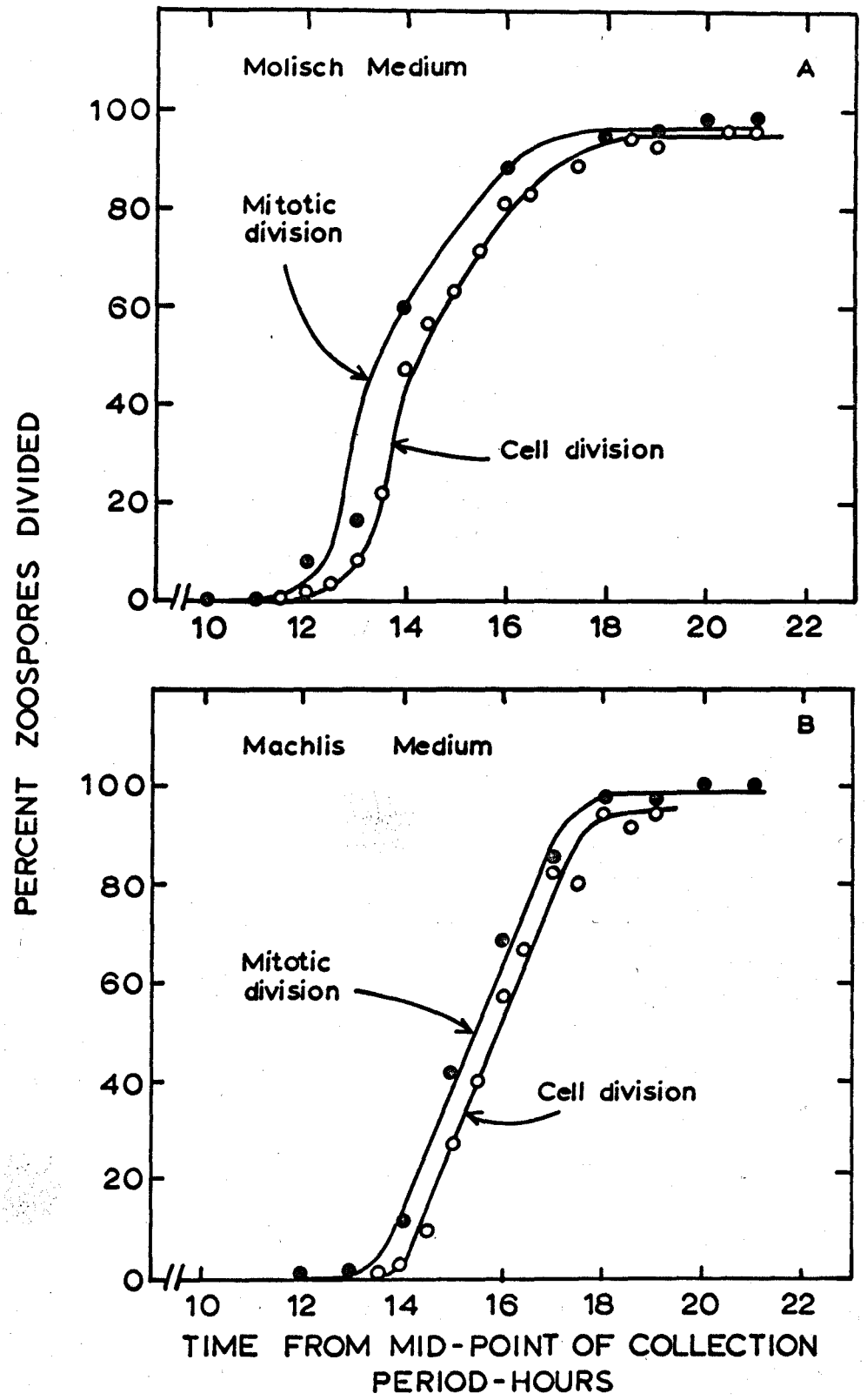

Molisch Medium
A
100
80
60
40
20
0
Mitotic division
Cell division
10
12
14
16
18
20
22
Machlis Medium
B
PERCENT ZOOSPORES DIVIDED
TIME FROM MID-POINT OF COLLECTION PERIOD-HOURS

TABLE 11

A comparison of percent phasing values using two different endpoints, nuclear division and cell division, in Machlis and Molisch media.

Growth Medium	Percent Phasing	
	Nuclear division	Cell division
Machlis	0.75	0.75
Molisch	0.69	0.70

APPENDIX E

FEULGEN STAINING TECHNIQUE (66)

FIXING: Remove slides from growth vessel and rinse with distilled water. Fix in Carnoy's solution (saturated with ferric ammonium sulphate as a mordant) for 15 minutes. Place in 95% E+OH for 5 minutes and then dip in dilute collodion (*) for 2 minutes. This prevents the loss of cells from the slide during staining. Place slides in ethanol chloroform (*) for 5 minutes to harden the collodion.

Pass slides through the following series:

STAINING: 70% E+OH -- 5 min.

50% E+OH -- 5 min.

60° C IN HCl -- 13 to 17 min.

Feulgen's reagent -- 1 hour

SO_2 water -- 13 min.

running tap water -- 15 min.

DEHYDRATION: 50% E+OH -- 3 min.

70% E+OH -- 3 min.

80% E+OH -- 3 min.

95% E+OH -- 3 min.

(*) 10 ml collodion, 10 ml ether, 80 ml absolute ether.

(**) 9 parts 80% E+OH : 1 part chloroform.

100% EtOH -- 3 min.

100% EtOH + ether (9:1) -- 2 + 5 min.

until collodion is removed.

100% EtOH -- 5 min.

100% EtOH and xylol (1:1) -- 15 min.

xylol -- 15 min.

xylol (fresh) -- 15 min.

Mount with diluted permount (*).

CARNOY'S SOLUTION:

Mix 1 part glacial acetic acid with 3 parts 95% EtOH. Add ferric ammonium sulphate and stir occasionally until saturated. Filter.

FEULGEN'S REAGENT:

Dissolve 2 gm basic fuchsin in 400 ml of boiling distilled water. Cool to 50° C and add 40 ml IN HCl. Cool to 25° C and add 2 gm sodium metabisulphite. Store overnight in the refrigerator. Next day shake the solution with 4 gm activated charcoal. Filter and store tightly capped in the refrigerator.

SO_2 WATER:

Mix 30 ml of 10% sodium bisulphite solution and 25 ml of IN HCl. Dilute with distilled water to a total volume of 500 ml.

(*) 1 part permount : 2 parts xylol.

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