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I am submitting herewith a thesis written by Henry J. Handler entitled "Oxygen and X-Ray Enhancement of Superoxide Dismutase in Tetrahymena pyriformis." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

Gary L. Whitson, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

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Graduate Studies and Research

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OXYGEN AND X-RAY ENHANCEMENT OF SUPEROXIDE DISMUTASE IN TETRAHYMENA PYRIFORMIS

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

Superoxide dismutase (SOD), an enzyme induced by O_2^- radicals, in aerobic organisms and its potential radioprotective effect were investigated in Tetrahymena pyriformis GL-C. Using standard assay conditions for SOD activity, SOD was found to be present in log phase, stationary phase and heat-shock synchronized cell cultures of Tetrahymena. The amount of SOD activity in log phase cultures, 1.42 units of SOD activity per mg-protein, was consistent with amounts reported for several other aerobic microorganisms. SOD activity in synchronized cultures was found to be elevated in respect to an increased amount of soluble protein which increases during the synchronization procedure. The activity of SOD dropped by one-half during the first division following the last heat shock.

SOD activity increased two-fold during growth of cells in 100% O_2 in both log phase and heat-shock synchronized cultures of Tetrahymena. X-irradiation of cells also resulted in increased amounts of SOD activity in cultures which were grown in medium in the presence of normal room air and in oxygenated cultures (100% O_2).

Enhancement of SOD activity prior to irradiation by high O_2 levels was examined in synchronized cultures in order to determine whether high levels of SOD would act in a radioprotective manner by reducing excess division delay caused by x-irradiation of synchronized Tetrahymena. Measurements of division index and changes in cell number were used to determine the amount and time of synchronized cell division in these

studies. However, results obtained indicated that enhanced amounts of intracellular SOD activity (a two-fold increase) did not act as an effective radioprotective agent in that excess division delay remained the same for both irradiated synchronized cell cultures and cell cultures incubated in high O_2 prior to x-irradiation.

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CHAPTER I

INTRODUCTION

The enzyme superoxide dismutase was first isolated and described as haemocuprein by Mann and Keilin (1938). Its name was derived from its description as a bluegreen protein containing copper isolated from ox-blood red cells. In the years that followed, similar metallo-proteins were isolated from a variety of sources such as bovine (Porter and Folch, 1957), human brain (Porter and Ainsworth, 1959), human red blood cells (Markowitiz et al., 1959), and even E. coli (Keele et al., 1970). The nomenclature of these proteins routinely reflects the tissue of origin and describes the various metals which complex with it. Determination of the actual role of this protein and its consequential naming superoxide dismutase (SOD) is attributed to McCord and Fridovich (1969). This enzyme was named SOD for its "dismutase" of superoxide anion radical (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2), (McCord and Fridovich, 1969). Since its initial discovery, the role of SOD in biological systems has been explored extensively with particular emphasis on its role in alleviating oxygen toxicity in a variety of cells.

The toxicity of cells to high levels of oxygen has been well documented (Gottleib, 1971; Haugaard, 1968). Cellular and molecular mechanisms in defense against O_2 toxicity have not been well defined until recent times. Through the pioneering studies of Fridovich and coworkers, it is now established that SOD plays a major role in the elimination of several harmful radicals formed by reactions utilizing

oxygen (Gregory and Fridovich, 1973b). It is also known that certain enzymatic reactions in biological systems involve several cellular enzymes including xanthine oxidase, aldehyde oxidase, tryptophan dioxygenase and peroxidases which can produce superoxide radicals (O_2^-) (Halliwell, 1978). These enzymes catalyse oxidation reactions in which a single electron is transferred from the substrate onto each O_2 molecule used to produce an oxygen-free radical known as super-oxide anion radical (O_2^-).

There are several lines of evidence that there are sources of O_2^- radicals in biological systems. For instance, autoxidation of the components of both mitochondrial and chloroplast electron transport chains have been shown to generate O_2^- (Loschen et al., 1973; Fridovich, 1975). O_2^- radicals can act in certain chemical reactions as either reducing agents or as oxidizing agents. For example, O_2^- radicals reduce cytochrome C (Halliwell, 1978), but oxidize ascorbic acid (Nischikimi, 1975). However, in comparison to other oxygen radicals, O_2^- is rather unreactive with cellular substrates. In the absence of anything else to react with, O_2^- has even been shown to react with itself (McCord and Fridovich, 1969). This "dismutation" of O_2^- produces H_2O_2 and O_2. H_2O_2 and O_2 can then apparently react to form hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen but how this reaction occurs is not clear (Cohen, 1977).

Hydroxyl radicals are among the most reactive species known to occur in organic reactions. They attack and damage almost every molecule found in living cells (Anbar and Neta, 1967). It is evident

that the formation of and presence of superoxide radicals are not good for the living cell, but the real danger seems to lie in the ability of O_2^- to form ·OH and singlet oxygen.

SOD provides a unique mechanism for the rapid removal of O_2^-, which thereby prevents formation of ·OH radicals. In addition, there is good evidence for a role of SOD *in vivo*. First of all, SOD seems ubiquitous to all aerobic organisms (Fridovich, 1975). Secondly, anaerobic organisms do not appear to have "normal" levels, and thirdly, SOD is an inducible enzyme. *E. coli* shows an increased resistance to hyperbaric O_2 which also corresponds to an increased level of SOD when grown under 100% O_2 (Gregory and Fridovich, 1973a). Similar results have been obtained with the unicellular green alga *Chlorella* (Pulich, 1974) and rats exposed to high O_2 tensions show an increased content of SOD in the lungs (Crapo and McCord, 1976). In assigning the unique role of SOD to the protection of biological systems from harmful radicals, especially O_2^-, an interesting area of recent research has been SOD's role in relation to ionizing radiation and SOD's use as a radioprotective agent.

There is ample evidence that ionizing radiation exerts damage to biological systems in at least two distinct ways. Energy from certain radiations can be directly imparted to a solid structure within a cell (e.g., hitting a chromosome with an x-ray) and this is called a direct action. However, damage can also be caused by the radiolysis of water, which makes up 95% of a cell. In this case the ionizing radiation produces radicals which then react with cell structures

causing the damage; this is considered to be an indirect effect (Arena, 1971).

There is good evidence that O_2^- is generated during radiolysis of aerated water (Anbar and Neta, 1967). Several attempts have been made, therefore, to characterize SOD as a radioprotective agent in a number of experimental systems. For instance, Petkau and Chelak (1974) showed an increase in survival by exogenous additions of SOD to the buffer solutions containing cells of the microorganism Achleoplasma laidlawii B both prior to and during x-irradiation. Since this first report, radioprotection by exogenous addition of SOD has been reported during administration of whole body doses of x-irradiation to mice (Petkau et al., 1975a; Petkau et al., 1976a), during irradiation of bone marrow stem cells in vivo (Petkau et al., 1975b), and erythrocytes of mice (Petkau et al., 1976b). The protective effect realized by addition of SOD to systems aforementioned seems to be due to the direct protection SOD offers to phospholipid membranes (Petkau and Chelak, 1976). SOD has also recently been used in connection with the radioprotective effect it produces to show that O_2^- may be the primary cause of the so-called "oxygen effect" (Goscin and Fridovich, 1973; Oberley et al., 1976).

All of the above-mentioned studies concerning the role of SOD in eliminating harmful radicals and its potential radioprotective nature has prompted this present investigation. Tetrahymena pyriformis was selected as a model test organism in this study for the following reasons: (1) it is easily grown and maintained in large sterile

cultures; (2) it is easily synchronized; (3) it has been studied extensively in relation to biochemical and irradiation studies; (4) synchronized cell cultures divide 75 min after the last heat shock (EHS) (when x-irradiation is given at the time of EHS an induced and predictable delay in division is caused); and (5) it is a primitive eukaryotic cell which has not been previously investigated in relation to SOD.

Tetrahymena has been the subject of extensive study in radiation biology primarily because of its radioresistance (Geise, 1967). LD_{50}'s (lethal dose to kill 50% of the cell population) have been reported in the range of 350,000 to 400,000 rads by both Elliott (1959) and Calkins (1964, 1967). Block or delays in cell division of synchronized cells by x-irradiation have been reported by Ducoff (1956). More recently it has been reported that the induced division delay has been found to be both dose and time-dependent by Hodge and Nachtwey (1972). Therefore, it is apparent that the radiation-induced division delay of synchronized Tetrahymena can be used as a suitable test system to measure radiation damage. The larger the dose, the greater the damage, hence the longer the delay. In this investigation, the delay in cell division was used to measure radioprotection by SOD. The purpose of this study was designed to answer the following questions:

1. Is there SOD activity in Tetrahymena?
2. Is SOD activity enhanced under conditions of high O_2 tension?
3. Does x-irradiation effect levels of SOD activity in log phase cultures?

4. Does levels of SOD activity fluctuate in synchronized cell populations during the first division following the last heat shock?

5. Is SOD activity in synchronized cell cultures affected by x-irradiation administered at the end of the last heat shock?

6. If SOD activity is enhancible, will increased levels of SOD activity act as a radioprotective agent in reducing division delay caused by x-irradiation?

CHAPTER II

MATERIALS AND METHODS

I. CULTURE METHODS

The experimental organism used in this study was the ciliated protozoan Tetrahymena pyriformis GL-C. This ciliate is an amicronucleated strain reaching a size of 40 by 60 μm when fully grown. Stock cultures were maintained in a medium containing 2.0% proteose peptone #3 (Difco), 0.2% liver extract (Nutritional Biochemical Corp.), and 0.5% dextrose (Merck). Cells were incubated at 28.5°C in glass screw cap culture tubes, 30 ml capacity, containing 10 ml aliquots of autoclaved medium (Whitson et al., 1968). Cultures were aseptically transferred under UV light by sterile pipette every 24 hours to maintain cells in logarithmic growth phase.

II. GROWTH STUDIES

Determination of generation time was performed in the following manner. An aliquot of cells (approximately 60,000) was inoculated into a 500 ml Erlenmeyer flask containing 200 ml of growth media and incubated at 28.5°C in a Blue M constant temperature shaking water bath. At various time intervals after inoculation, 1 ml of cells was removed by sterile pipette and diluted to a final volume of 10 ml with 0.3% NaCl containing 1.0% formaldehyde in glass-distilled water. This sample was then counted using a Coulter Cell Particle Counter Model B equipped

with a 100 μm aperature to determine the cell numbers. Cell numbers were displayed on a log scale versus time and generation times were calculated using the following equation of Buchanon and Fulmer (1928).

$$\text{Generation time} = \frac{T \times \log 2}{\log b/a}$$

Where T is the elapsed time between the final cell number "b" and the initial point "a" providing these points are on the straight line.

III. OXYGEN (O_2), CARBON DIOXIDE (CO_2), NITROGEN (N_2) STUDIES

Cultures for O_2 studies were grown under aseptic culture conditions with 100% O_2. O_2 was hospital grade, and sterilized by filtration with an in-line millipore filter (Type HA, 0.45 micron). One hundred percent O_2 was bubbled into culture medium at a pressure of 0.5 PSI for the length of the growth period up to treatment with x-irradiation. CO_2 and N_2 were sterilized using the same millipore filter set-up. In the case of CO_2 and N_2 studies, 20-hour old log phase cultures were subjected to 100% CO_2 or 100% N_2 for 4 hours prior to x-irradiation or sampling.

IV. SYNCHRONIZATION OF CELLS

Cells were synchronized with a series of heat shocks using a modified method of Scherbaum and Zeuthen (1954). Synchronized cultures were started by adding log phase cells from one-day old stock cultures to 125 ml Erlenmeyer flasks containing 50 ml of medium. Cells were allowed to grow for 12 to 14 hours at 28.5°C to a density of approximately

20,000 cells/ml prior to the synchronizing treatment. At this time the cultures were subjected to six alternate heat shocks of 30 min duration at 33.9°C followed by recovery periods of 30 min duration at 28.5°C. The heat shocks and recovery periods were regulated by an automated synchronizing system consisting of two water baths and electric timers connected to solenoid valves regulating the flow of water to a heat transfer apparatus (third water bath) containing the flask of the cell cultures.

V. DIVISION INDEX

The division index of synchronized cell was determined at various times after the synchronization treatment by visual examination. Aliquots of cells were fixed with Bouin's fixative and visual examination was made with the use of a binocular Olympus microscope using a 20x objective. The division index was calculated as the number of cells in division (furrowing) divided by the total number of cells based on a sample size of at least 200 cells.

VI. RADIATION EXPOSURE

Cell samples (10^6/50 ml sample in growth medium) were irradiated in a 5 cm × 10 cm round glass dish (Fisher) using a General Electric Maxitron 300 x-ray machine. The parameters of irradiation were 250 KVP, 20 mA with 0.13 mm copper and 0.05 mm aluminum as added filtration. The target distance was 23.5 cm. Exposure rate was determined using a Radocon dosimeter (Victoreen of Cleveland, Ohio). The average corrected

dose rate was 1001 roentgen (R)/min with a dose corrected for temperature and pressure. Synchronized cell cultures were irradiated with the midpoint of radiation occurring at the time of the end of the last heat shock (EHS). Log phase cultures were irradiated after 24 hours of growth. Stationary phase cultures were irradiated after 48 hours of growth, which is the time when cell numbers reached maximum density.

VII. SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase samples from both control and experimental cells were obtained using a method described by McCord and Fridovich (1969). Initial starting material was 5 ml samples of culture medium containing cells which were centrifuged in an IEC clinical centrifuge at 1850 xg for 4 min. After centrifugation, the medium was decanted and then 5 ml of phosphate buffer (KH_2PO_4, 7.35 M; Na_2HPO_4, 7.04 M; $MgCl_2$, 0.32M; and NaCl, 3.42 M) pH 6.75 were added to the pellet of cells. The cells were then resuspended by gentle mixing and centrifuged again at 1850 xg for 4 min. At the end of the second centrifugation, the buffer was decanted and 1 ml of ice cold glass-distilled H_2O was added to the pellet. Cells were once again resuspended and homogenized using a teflon-glass Potter-Elvelhjem homogenizer. To the homogenate, 1 ml of ethanol-chloroform (5V/3V) was added and then mixed vigorously with a vortex mixer. This mixture was centrifuged for 4 min at 1850 xg and the upper phase of the supernate was retained for assay of SOD activity. Fractions containing varying volumes of supernatant were assayed for

SOD using a procedure of Marklund and Marklund (1974) based on the autoxidation of pyrogallol, the higher the amount of SOD activity the less the autoxidation (lower rate) of pyrogallol. SOD samples were assayed in 0.2 mM pyrogallol in air equilibrated 50 mM Tris-cacodylic acid buffer, pH 8.20 containing 0.1 mM diethylenetriaminepentaacetic acid. The rate of autoxidation was taken from the increase in A_{420} (absorbance) seen per min incubation using a Beckman DBG spectrophotometer equipped with a 12" strip chart recorder. Bovine SOD (Worthington Biochemical) with 1670 units SOD activity per mg protein was used as a standard.

VIII. PROTEIN DETERMINATIONS

Total soluble protein obtained from cell samples at different times was determined using a procedure of Bradford (1976). Samples were collected as in section VII, with a slight modification. After the second centrifugation cells were homogenized in 1 ml phosphate buffer pH 6.75 and immediately centrifuged for 4 min at 1850 xg. The pellet was discarded and the supernatant was assayed for total soluble protein. Ten to 100 µg samples were assayed in 5 ml of 0.01% (W/V) Coomassie Brilliant Blue G-250 (Eastman), 4.7% (W/V) ethanol, and 8.5% (W/V) phosphoric acid and the A_{595} was measured using a DBG spectrophotometer. Bovine Serum Albumin FR V Powder (ICN Pharmaceuticals) was used as a standard in this procedure.

CHAPTER III

RESULTS

I. SOD ACTIVITY IN LOG PHASE CULTURES OF TETRAHYMENA

Average Generation Times for Log Phase Cultures

In order to establish parameters for experimental protocols, information was first obtained concerning normal cell cycles of Tetrahymena. Generation times were estimated at 28.5°C for cell cultures grown under two different growth conditions; one using normal atmospheric levels of 0_2 approximately 20%, and the other using the administration of 100% 0_2. These generation times are displayed in Table I. The results shown are estimates based on three separate growth curves for each growth condition. The generation times expressed were averages calculated from semilogarithmic regression plots performed for each set of experiments. Typical growth curves are shown in Figure 1. The average generation time for cell cultures grown under normal atmospheric room air was 3.58 hours. This is in contrast to cell cultures which were grown under 100% 0_2 conditions, which show a slightly longer average generation time—3.71 hours. Statistical analysis (ANOVA) determined by comparison of the different logarithmic slopes of the growth curves shown in Figure 1 indicates that the difference in average generation times for normal air and 0_2 bubbled cultures are not significantly different.

TABLE I

GENERATION TIMES

Condition	Generation Time (hours) ± S. E.[a]
Normal Room Air (20% O_2)	3.58 ± 0.05
100% O_2	3.71 ± 0.02

[a]S. E. is standard error of the mean.

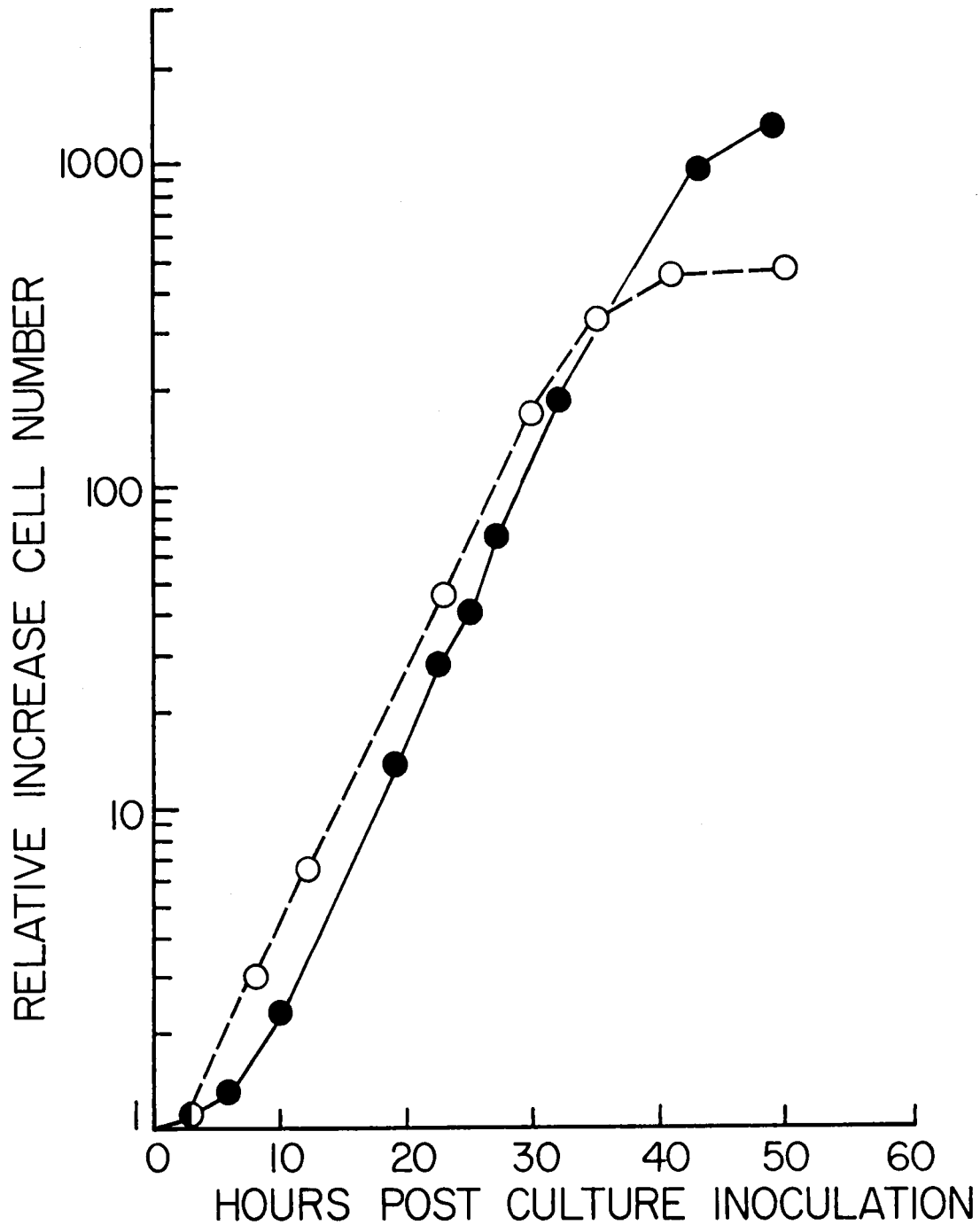


Figure 1. Typical growth curves of Tetrahymena pyriformis GL-C. (Normal room air 20% O_2, ●——●, 100% O_2 ○——○.)

SOD Activity of Tetrahymena under Normal (20% O_2) Growth Conditions

The next set of experiments was performed in order to determine whether there is SOD in Tetrahymena. All of the following experiments were done a minimum of three times. Using standard procedures for the identification of SOD, this enzyme was found to be present in log phase cultures and stationary phase cultures under normal growth conditions. Results are summarized in Table II. SOD activity in normal log phase is approximately 0.94 units per 10^6 cells. When the activity of SOD is expressed in relation to total soluble protein, there is approximately 1.42 units of SOD activity per mg-protein. There is an approximate decrease of 10% in SOD activity in stationary phase cultures compared to log phase cultures.

SOD Activity of Tetrahymena under Growth Conditions of 100% O_2

SOD activity was measured in Tetrahymena grown with 100% O_2 bubbled through the medium to determine if SOD activity is enhanced by high O_2 tension. Using previously described assay procedures, increased levels of SOD activity were found in cell cultures grown utilizing 100% O_2. The increased SOD activity was apparently enhanced by molecular O_2. Results are summarized in Table II. Oxygenated log phase cultures contain approximately 2.09 units of SOD activity per 10^6 cells. When SOD activity is expressed in relation to protein, there is approximately 3.5 units of SOD activity per mg-protein. These levels represent a two-fold increase in activity in oxygenated cultures versus cultures grown

TABLE II

UNITS OF SOD ACTIVITY FOR LOG AND STATIONARY PHASE CULTURES

Growth Conditions	U-SOD-A/10^6 cells[a]	mg-protein/10^6 cells[b]	U-SOD-A/mg-protein
24 hr log phase Room Air 20% O_2	0.94 ± 0.04	0.66 ± 0.02	1.42 ± 0.03
48 hr stationary Room Air 20% O_2	0.59 ± 0.05	0.47 ± 0.05	1.26 ± 0.01
24 hr log phase 100% O_2	2.09 ± 0.02	0.60 ± 0.02	3.50 ± 0.10
48 hr stationary 100% O_2	1.57 ± 0.03	0.50 ± 0.03	3.14 ± 0.09
20 hr log phase 4 hr 100% CO_2	0.63 ± 0.04	0.57 ± 0.03	1.10 ± 0.05
20 hr log phase 4 hr 100% N_2	0.62 ± 0.01	0.59 ± 0.04	1.05 ± 0.07

[a]U-SOD-A is units of superoxide dismutase activity.

[b]mg-protein is milligrams of total soluble protein.

under normal room air. In the case of oxygenated stationary phase cultures, there is also a 10% decrease in comparison to log phase cultures grown under the same conditions. There is a two-fold increase of SOD activity in oxygenated stationary phase cultures in comparison to stationary cultures which were not oxygenated.

Further evidence of the enhancibility of SOD was found when log phase cultures were grown for 20 hours under normal conditions and then subjected to environments which simulate anaerobic growth conditions. Cultures were bubbled with either 100% CO_2 or 100% N_2 for four hours. Log phase cultures showed a 30% decrease in SOD activity due to a lack of molecular O_2.

The Effects of X-Irradiation on SOD Activity in Log Phase Cultures

Since it was established that SOD was indeed present in Tetrahymena and that its activity could be enhanced or elevated by high O_2 tension, SOD activity was determined for log phase cultures after x-irradiation to determine if x-rays had any effect on SOD activity. A typical experiment indicating the effects of two different x-ray doses, one of 5,000 R and one of 10,000 R, on SOD activity are shown in Table III, which also describes the two different growth conditions previously characterized.

From the data obtained in the experiments outlined in Table III, there is a clear indication of a significant increase in SOD activity within 15 min following x-ray treatment. These elevated levels gradually fell off to near normal log phase culture levels after one

TABLE III

LEVELS OF SOD IN LOG PHASE CULTURES POST X-IRRADIATION TREATMENT
WITH 5,000 R and 10,000 R

Time (min) Post Treatment	U-SOD-A/10^6 cells	mg-protein/10^6 cells	U-SOD-A/mg-protein
24 hr log phase; 5,000 R			
15	3.00 ± 0.32	0.55 ± 0.07	5.45 ± 0.07
30	1.47 ± 0.04	0.57 ± 0.07	2.60 ± 0.24
45	1.40 ± 0.25	0.60 ± 0.03	2.33 ± 0.03
60	0.76 ± 0.08	0.66 ± 0.09	1.15 ± 0.05
24 hr log phase; 10,000 R			
15	2.10 ± 0.29	0.52 ± 0.05	4.00 ± 0.20
30	1.20 ± 0.10	0.68 ± 0.06	1.80 ± 0.14
45	1.30 ± 0.15	0.61 ± 0.04	2.10 ± 0.11
60	1.30 ± 0.18	0.68 ± 0.04	1.90 ± 0.16
24 hr log phase, 100% O_2; 5,000 R			
15	3.70 ± 0.29	0.92 ± 0.05	4.00 ± 0.10
30	2.60 ± 0.19	0.72 ± 0.06	3.61 ± 0.05
45	2.39 ± 0.04	0.83 ± 0.03	2.88 ± 0.04
60	1.80 ± 0.12	0.81 ± 0.07	2.22 ± 0.02
24 hr log phase, 100% O_2; 10,000 R			
15	2.85 ± 0.02	1.05 ± 0.01	2.71 ± 0.21
30	2.52 ± 0.03	1.00 ± 0.06	2.50 ± 0.12
45	1.90 ± 0.18	1.04 ± 0.12	1.83 ± 0.03
60	1.66 ± 0.05	1.22 ± 0.01	1.36 ± 0.04

hour following x-irradiation. These data also indicate that an x-ray dose of 10,000 R does not result in as great an increase in SOD activity as compared with that found for cells exposed to 5,000 R. In the case of cultures grown utilizing 100% 0_2, where SOD activity was increased, the x-irradiation does not result in as large an elevation of SOD activity in comparison to cultures grown under normal room air (20% 0_2). Nevertheless, these data clearly indicate that x-irradiation somehow causes elevated SOD activity. Considering the similarities of x-ray enhancement of SOD activity and increased SOD activity stimulation by molecular 0_2, the formation of radicals in both of the aforementioned insults, enhancement may be envoked by similar mechanisms.

With time, the SOD activity changes in irradiated cultures under different conditions of aeration and changes are shown in Figures 2 through 5. All of the results as shown indicate that x-rays stimulate increased SOD activity rapidly and that elevated enhanced levels fall to near normal levels in a short time following irradiation.

II. SOD ACTIVITY IN HEAT SHOCK SYNCHRONIZED TETRAHYMENA

Characterization of Division and X-Ray Induced Division Delay in Synchronized Tetrahymena

Once establishing that enhancement of SOD activity in log phase cultures of Tetrahymena could be obtained by both 100% 0_2 and x-irradiation, experiments were designed to determine if SOD activity fluctuated during the first division cycle in synchronized cultures of Tetrahymena. Data were gathered to characterize the first division cycle and the pattern of x-ray induced division delay (Figure 6). Division index and

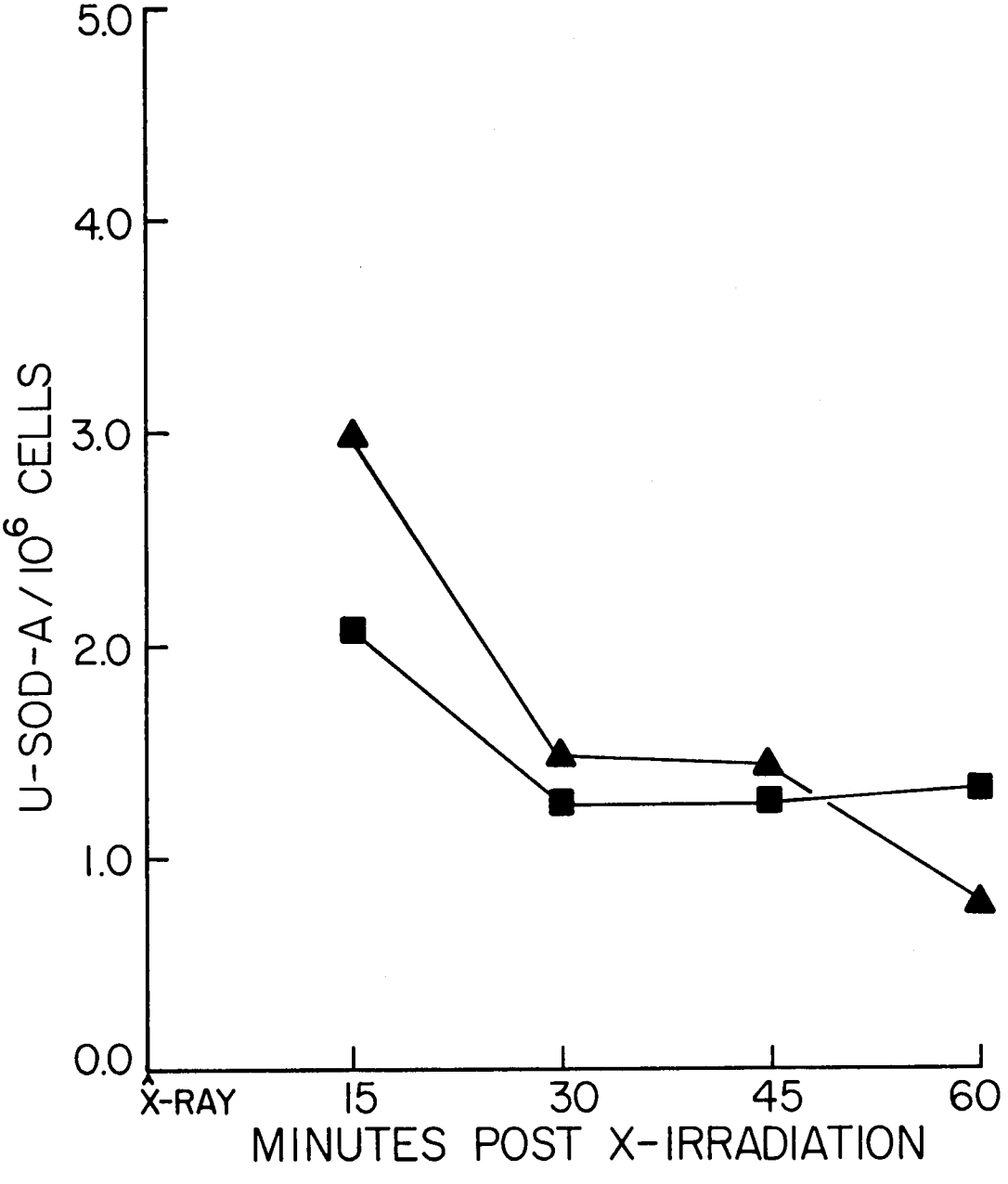


Figure 2. Units of SOD activity/10^6 cells of log phase Tetrahymena grown under normal room air (20% O_2) after x-irradiation. (5,000 R ▲—▲, 10,000 R ■—■.)

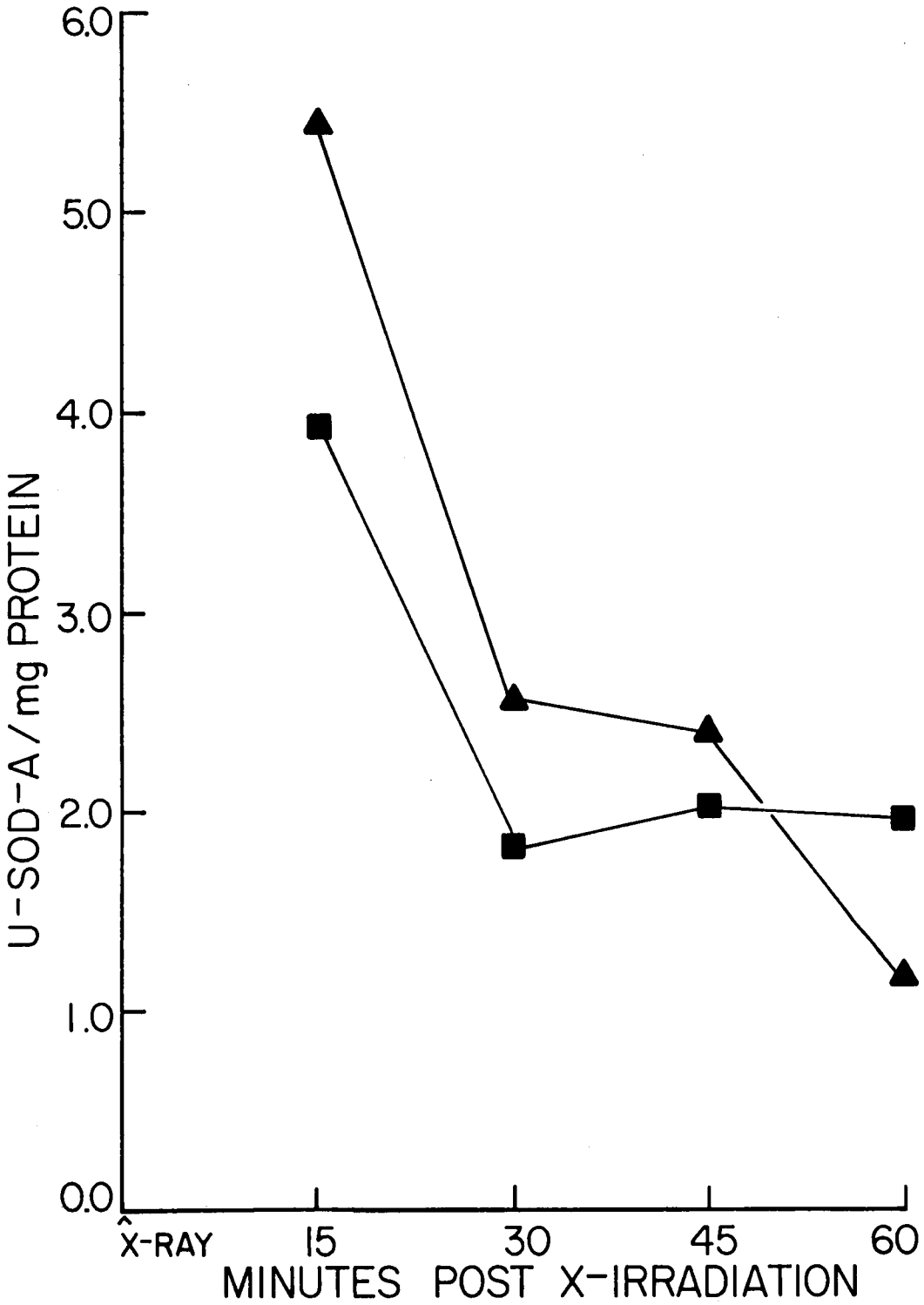


Figure 3. Units of SOD activity/mg-protein of log phase Tetrahymena grown under normal room air (20% 0_2) after x-irradiation. (5,000 R ▲——▲, 10,000 R ■——■.)

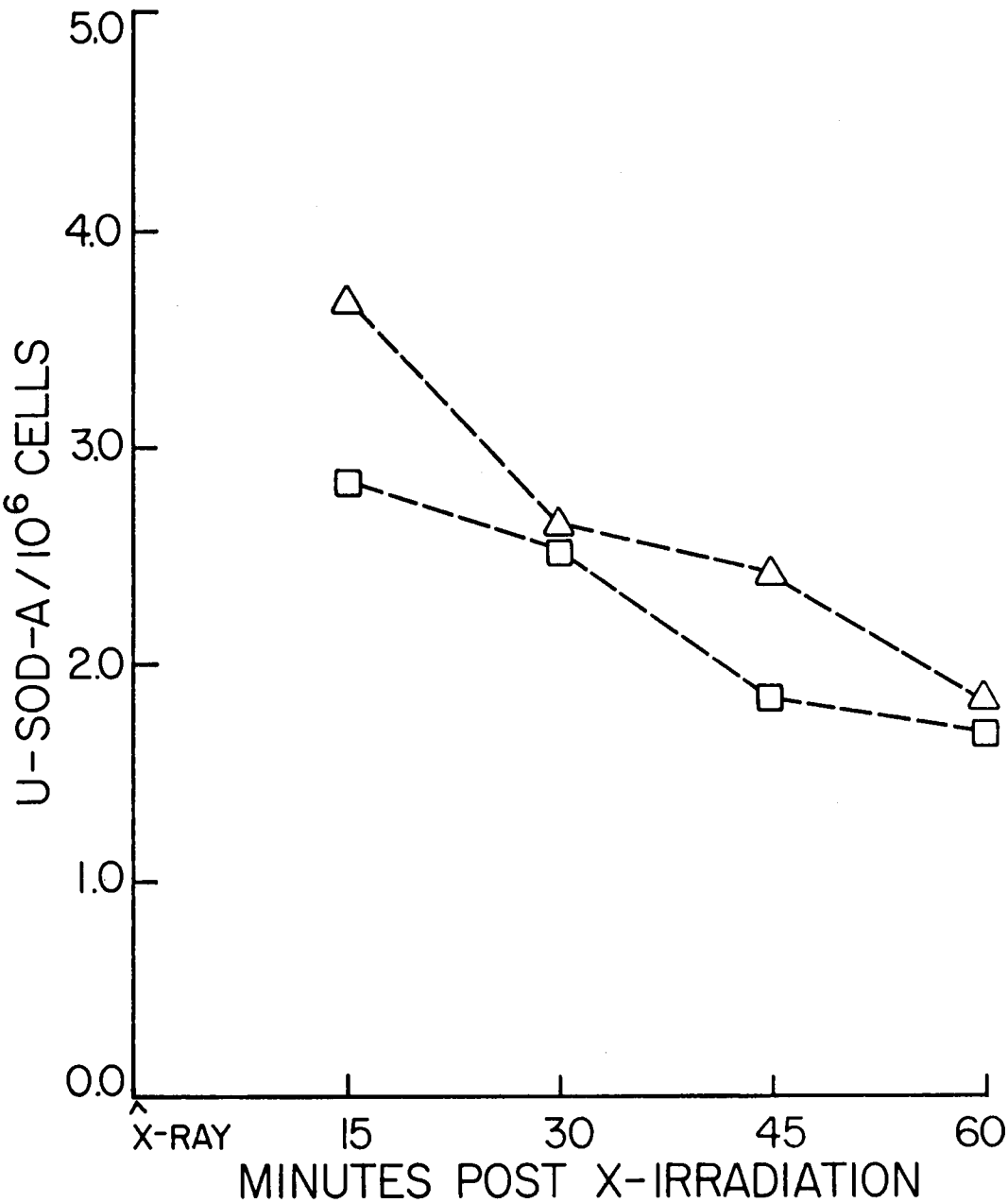


Figure 4. Units of SOD activity/10^6 cells of log phase Tetrahymena grown under 100% O_2 after x-irradiation. (5,000 R △—△, 10,000 R □—□.)

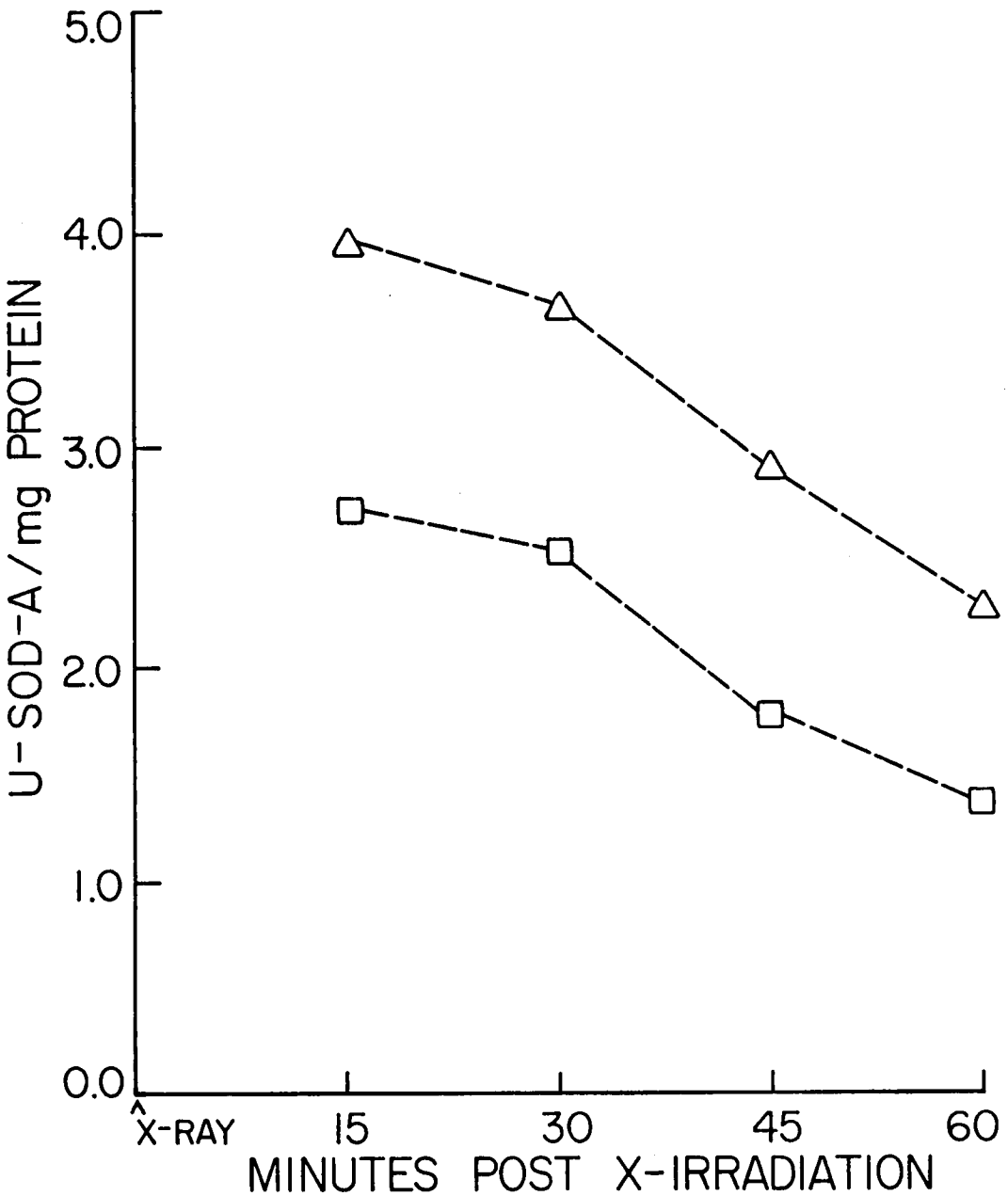


Figure 5. Units of SOD activity/mg-protein of log phase Tetrahymena grown under 100% 0_2 after x-irradiation. (5,000 R△--△, 10,000 R □--□.)

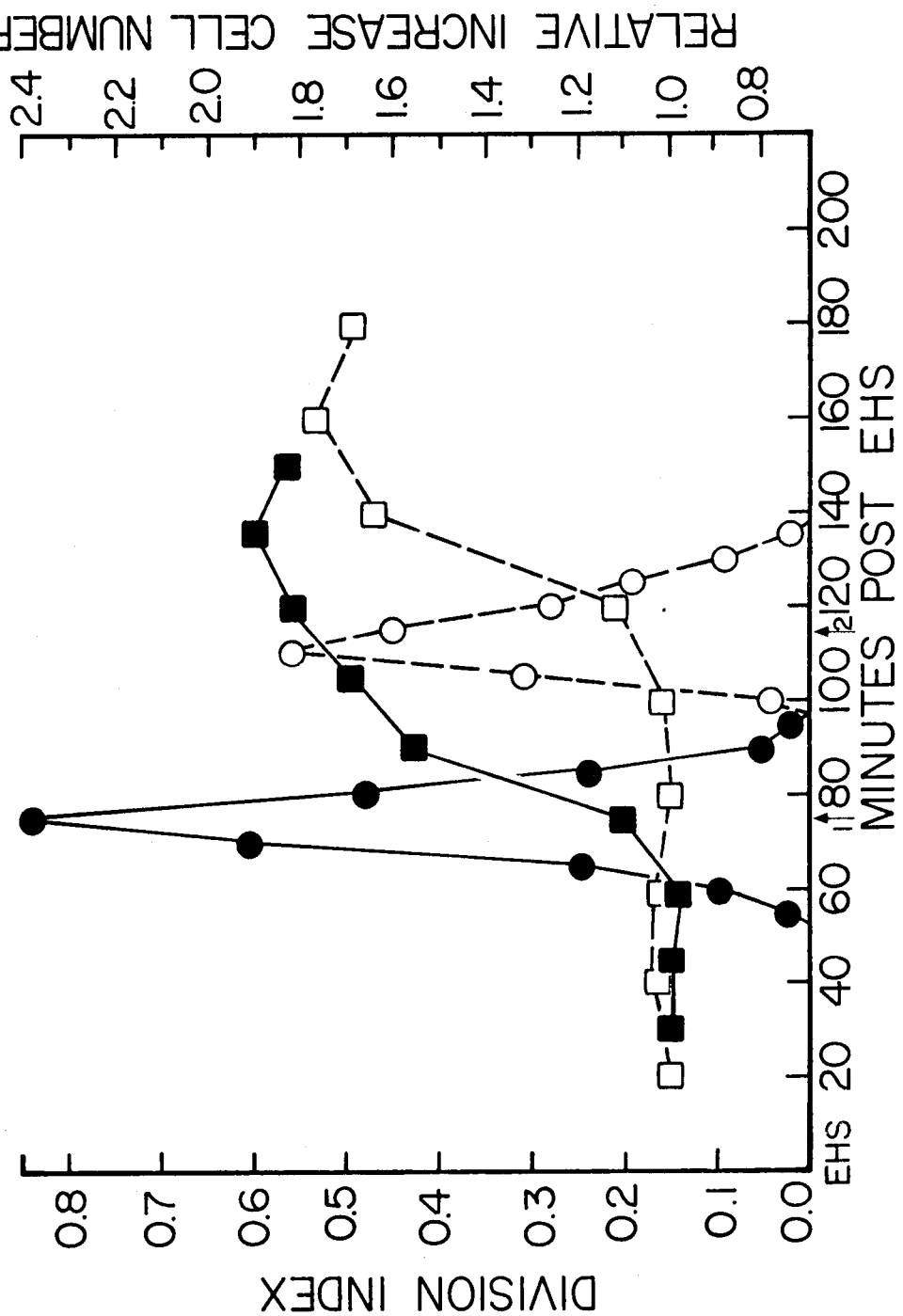


Figure 6. X-ray induced division delay in synchronized Tetrahymena. Division index, control ●—●, 5,000 R ○—○. Relative increase cell number, control ■—■, 5,000 R □—□. Arrows indicate peak of cell division, (1) = control, (2) = 5,000 R.

relative increase in cell number were used to measure the first division cycle in control and x-ray treated cultures of heat-shock synchronized Tetrahymena. Results are summarized in Tables IV and V. As is readily seen, the data clearly indicate that control cells initiate cell division at about EHS (end of last heat shock) + 60 min and reach a peak of about 84% synchrony 15 min later. At this time (EHS + 75 min) there is a corresponding increase in cell number which continues through EHS + 110 min with an eventual approximate doubling of cell number after division is complete. In the case of x-irradiated culture cells exposed to 5,000 R at EHS, there is a division delay of 35 min. In these experiments division begins at approximately EHS + 95 min with a peak in division index of only 55% synchrony. In x-irradiated cultures, cell numbers begin to increase at the peak of cell division and continue to increase until division ends with a 75% increase in cell number levels. Results for normal growth conditions are represented in Figure 6 with data for 100% oxygen utilization graphed in Figure 7 for comparison.

SOD Activity in Synchronized Cultures of Tetrahymena

The activity of SOD in heat-shock synchronized cultures was determined at scheduled times after EHS through cell division to determine whether SOD activity fluctuates during the synchronized division cycle. Synchronized cultures were also subjected to x-irradiation at EHS to induce a predictable and reproducible division delay; the SOD activity was measured during the period of excess delay. Results for typical experiments are summarized in Table VI. In synchronized cultures, there is an approximate two-fold increase of SOD activity (Figure 8) over that obtained for log phase cultures which corresponds

TABLE IV

DIVISION INDEX OF SYNCHRONIZED CULTURES POST END OF LAST HEAT SHOCK (EHS)

Time (min) Post EHS	Division Index 20% O_2 Control	Division Index 100% O_2
55	1.9 ± 0.7	
60	9.5 ± 0.8	11.0 ± 2.9
65	24.0 ± 1.8	26.0 ± 3.2
70	61.0 ± 3.1	65.0 ± 8.2
75	84.0 ± 3.2	82.0 ± 4.5
80	48.0 ± 2.8	44.0 ± 7.5
85	23.9 ± 0.8	23.0 ± 3.2
90	5.0 ± 2.5	6.0 ± 1.0
95	1.4 ± 0.5	
Cultures given 5,000 R at EHS		
100	4.0 ± 1.9	2.9 ± 0.3
105	31.0 ± 5.2	8.0 ± 3.7
110	56.0 ± 5.9	50.0 ± 8.2
115	45.0 ± 3.2	45.0 ± 3.1
120	28.0 ± 3.4	39.0 ± 6.1
125	15.0 ± 4.8	34.0 ± 3.1
130	9.0 ± 1.3	27.0 ± 3.1
135	2.1 ± 0.6	12.0 ± 1.6
140		4.0 ± 0.7

TABLE V

RELATIVE INCREASE IN CELL NUMBER OF SYNCHRONIZED CULTURES POST EHS

Time (min) Post EHS	Control	5,000 R at EHS
20		1.00 ± 0.00
30	1.00 ± 0.00	
40		1.03 ± 0.07
45	1.00 ± 0.00	
60	0.98 ± 0.04	1.02 ± 0.05
75	1.10 ± 0.04	
80		0.99 ± 0.04
90	1.60 ± 0.10	
100		1.01 ± 0.06
105	1.70 ± 0.07	
120	1.82 ± 0.06	1.21 ± 0.09
135	1.90 ± 0.02	
140		1.64 ± 0.04
150	1.83 ± 0.01	
160		1.77 ± 0.04
180		1.68 ± 0.06
Cultures grown 100% O_2		
20		1.00 ± 0.00
30	1.00 ± 0.00	
40		0.98 ± 0.05
45	0.99 ± 0.02	
60	0.96 ± 0.01	1.03 ± 0.04
75	0.96 ± 0.02	
80		1.00 ± 0.07
90	1.41 ± 0.04	
100		1.07 ± 0.08
105	1.83 ± 0.02	
120	1.89 ± 0.03	1.21 ± 0.06
135	1.91 ± 0.01	
140		1.66 ± 0.08
150	1.87 ± 0.03	
160		1.81 ± 0.01
180		1.78 ± 0.01

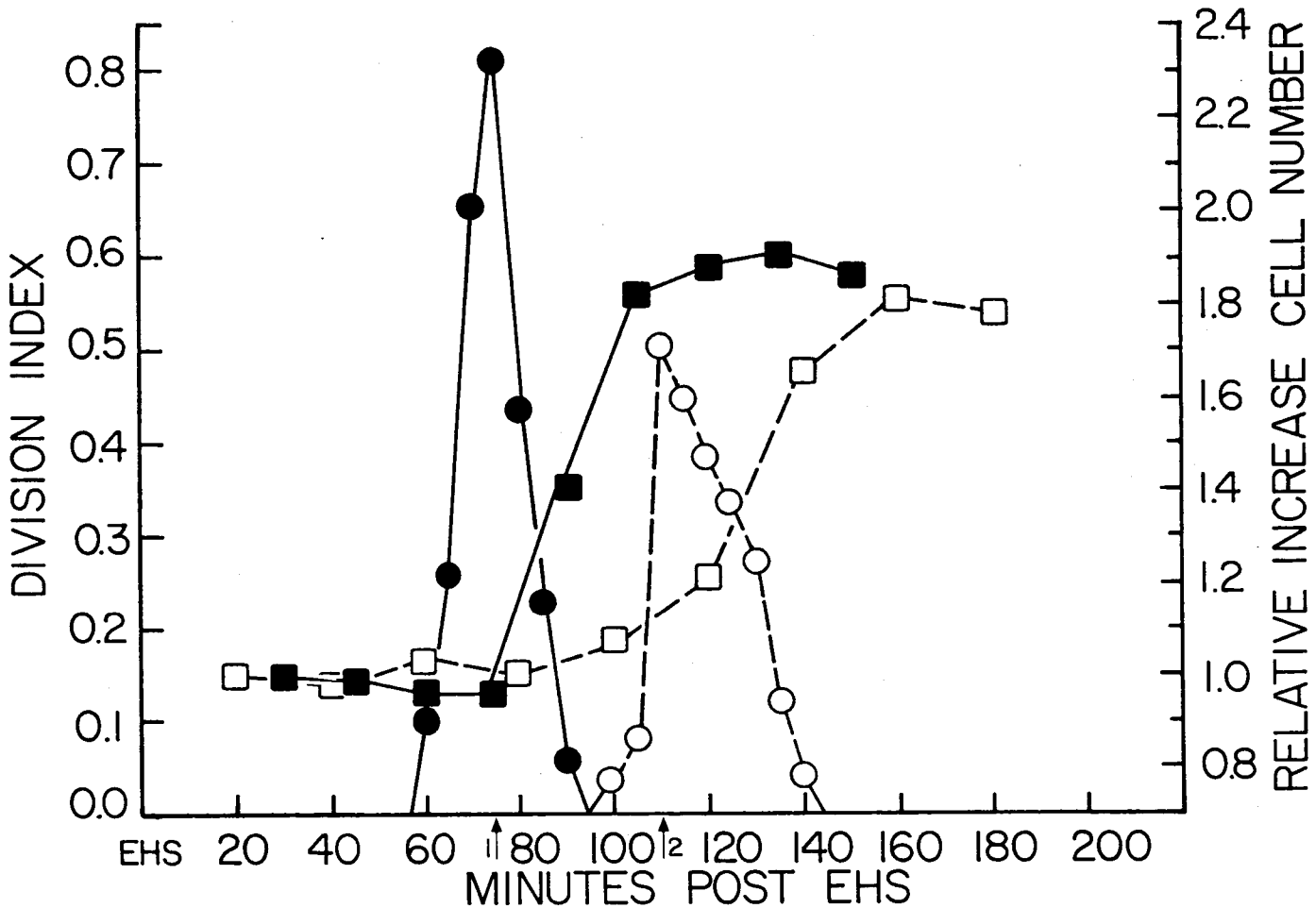


Figure 7. X-ray induced division delay in synchronized Tetrahymena, grown under 100% O_2. Division index, control ●—●, 5,000 R ○—○. Relative increase in cell number, control ■—■ 5,000 R □—□. Arrows indicate peak of cell division, (1) = control, (2) = 5,000 R.

TABLE VI

LEVELS OF SOD IN SYNCHRONIZED CULTURES POST EHS

Time (min) Post EHS	U-SOD-A/10^6 cells	mg-protein/10^6 cells	U-SOD-A/mg-protein
Control			
30	2.30 ± 0.05	1.73 ± 0.05	1.33 ± 0.01
45	2.60 ± 0.11	2.10 ± 0.14	1.24 ± 0.03
60	2.10 ± 0.27	1.90 ± 0.14	1.11 ± 0.06
75	2.30 ± 0.24	1.70 ± 0.15	1.35 ± 0.03
90	1.50 ± 0.20	1.30 ± 0.13	1.15 ± 0.04
105	1.20 ± 0.15	1.24 ± 0.05	0.97 ± 0.09
120	1.15 ± 0.05	1.06 ± 0.03	1.08 ± 0.02
135	1.10 ± 0.24	1.05 ± 0.01	1.05 ± 0.02
150	1.05 ± 0.15	1.20 ± 0.11	0.88 ± 0.04
5,000 R at EHS			
20	2.70 ± 0.12	1.80 ± 0.10	1.50 ± 0.02
40	2.40 ± 0.10	1.92 ± 0.05	1.25 ± 0.02
60	2.20 ± 0.11	1.80 ± 0.13	1.22 ± 0.02
80	1.90 ± 0.11	1.70 ± 0.10	1.12 ± 0.01
100	1.64 ± 0.01	1.80 ± 0.13	0.91 ± 0.06
120	1.60 ± 0.10	1.60 ± 0.13	1.00 ± 0.02
140	1.36 ± 0.03	1.24 ± 0.04	1.10 ± 0.04
160	1.05 ± 0.09	1.10 ± 0.71	0.95 ± 0.01
180	0.78 ± 0.02	1.24 ± 0.04	0.63 ± 0.01

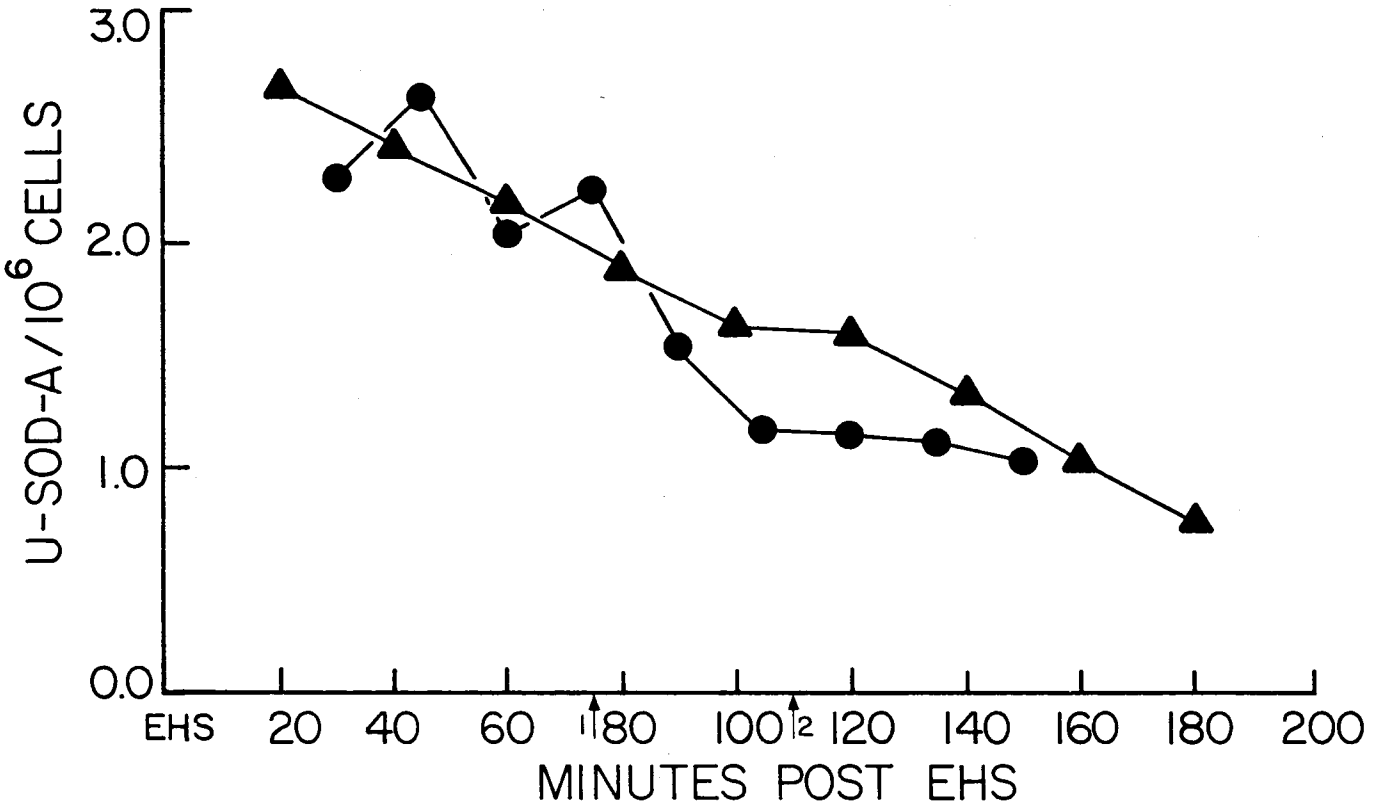


Figure 8. Units of SOD activity/10^6 cells of synchronized Tetrahymena. (Control ●——●, 5,000 R, given at EHS, ▲——▲.) Arrows indicate peak of cell division, (1) = control, (2) = 5,000 R.

to a concurrent intracellular increase (nearly two-fold) in protein. After completion of the first division, SOD activity returns to values obtained for normal log phase cultures.

When synchronized cultures were x-irradiated at EHS and sampled at EHS + 20 min, there was a slight increase in SOD activity. This level of activity then drops to control (nonirradiated synchronized cultures) levels by the next sampling point at EHS + 40 min. X-irradiated cultures after cell division also demonstrate that SOD activity returns to levels found for normal log phase cultures. To demonstrate the pattern of change in SOD after EHS, the data in Table VI are graphed in Figures 8 and 9.

SOD Activity in Synchronized Tetrahymena Grown and Synchronized in 100% O_2

The activity of SOD was determined at various times after EHS in synchronized cultures grown in 100% O_2 in order to determine whether SOD activity fluctuates in response to high O_2. SOD activity was also measured in cells from cultures grown in 100% O_2 and x-irradiated at EHS. SOD activity levels elevate in cultures grown and synchronized with 100% O_2, in both control (nonirradiated cultures) and x-irradiated cultures. This elevated SOD activity was found to be approximately 50% greater as compared to cells grown and synchronized in normal room air. Elevated SOD activity, as in the case of nonoxygenated cultures, was observed to parallel an increase in intracellular protein in synchronized cultures. Following the first synchronized cell division, there is a return of SOD activity to that found for normal log phase cultures.

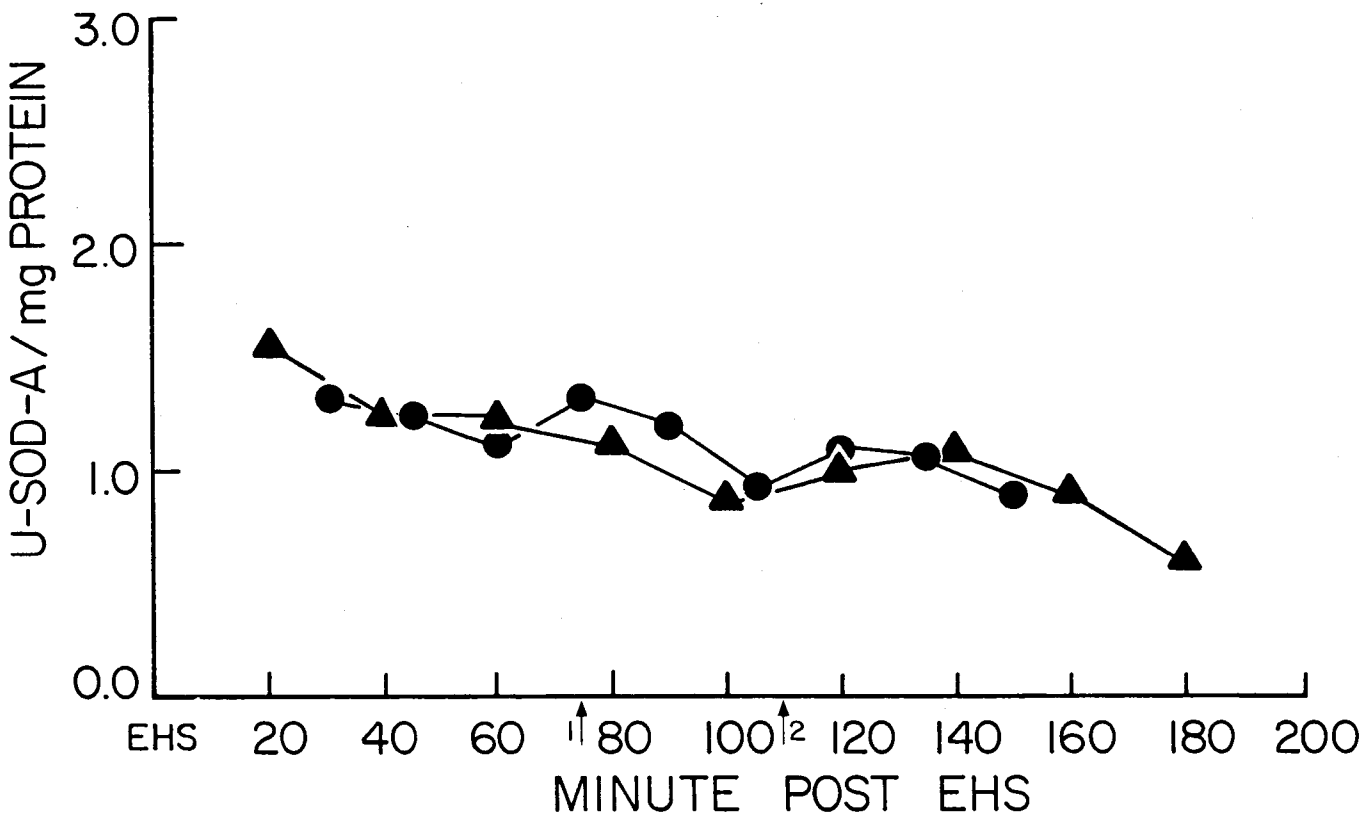


Figure 9. Units of SOD activity/mg-protein of synchronized Tetrahymena. (Control ●——●, 5,000 R ▲——▲.) Arrows indicate peak of cell division, (1) = control, (2) = 5,000 R.

These results are summarized in Table VII and graphed in Figures 10 and 11.

X-irradiated cultures which were grown and synchronized with 100% 0_2 show a large increase in SOD activity at EHS + 20 min due to x-irradiation. This increased amount of SOD activity quickly drops to levels observed for nonirradiated cultures grown under the same growth conditions and the same synchronization procedures. Once again, the SOD activity drops after delayed cell division to near levels in log phase cultures.

The Radioprotective Effect of Increased SOD Activity in Synchronized Tetrahymena Grown and Synchronized in 100% 0_2

It has been previously established that molecular 0_2 (100%) can cause increased SOD activity in both log phase and heat shock synchronized cultures. Excess division delay has also been characterized in heat shock synchronized cultures which have been x-irradiated at EHS. Experiments were next performed to determine whether increased SOD activity caused by 100% 0_2 could impart a radioprotective effect by reducing or removing the excess division delay. Division index and relative increase in cell number were used as parameters to characterize division and excess division delay in cell cultures which were grown and synchronized in 100% 0_2. The results are summarized in Tables IV and V, pages 26 and 27, and graphed in Figures 6 and 7, pages 24 and 28.

Cells in oxygenated control cultures initiate cell division at approximately EHS + 60 min and reach a peak of 82% synchrony 15 min later. At this time (EHS + 75 min) there is a corresponding increase in

TABLE VII

LEVELS OF SOD IN SYNCHRONIZED CULTURES, GROWN 100% O_2, POST EHS

Time (min) Post EHS	U-SOD-A/10^6 cells	mg-protein/10^6 cells	U-SOD-A/mg-protein
Control			
30	3.10 ± 0.10	2.00 ± 0.11	1.55 ± 0.04
45	3.10 ± 0.18	1.84 ± 0.08	1.68 ± 0.02
60	3.20 ± 0.15	1.90 ± 0.10	1.70 ± 0.10
75	2.50 ± 0.20	1.58 ± 0.02	1.60 ± 0.10
90	2.10 ± 0.12	1.05 ± 0.07	2.00 ± 0.02
105	1.80 ± 0.10	1.01 ± 0.05	1.78 ± 0.01
120	1.40 ± 0.16	1.10 ± 0.12	1.27 ± 0.01
135	1.40 ± 0.16	1.00 ± 0.03	1.40 ± 0.12
150	1.40 ± 0.17	1.07 ± 0.06	1.31 ± 0.08
5,000 R at EHS			
20	4.00 ± 0.26	2.28 ± 0.04	1.75 ± 0.08
40	3.50 ± 0.23	2.30 ± 0.10	1.52 ± 0.03
60	3.11 ± 0.03	2.42 ± 0.09	1.29 ± 0.04
80	3.20 ± 0.18	2.39 ± 0.07	1.34 ± 0.04
100	2.65 ± 0.04	2.27 ± 0.09	1.17 ± 0.05
120	2.50 ± 0.25	2.20 ± 0.13	1.14 ± 0.08
140	1.60 ± 0.22	1.10 ± 0.10	1.45 ± 0.06
160	1.03 ± 0.06	1.25 ± 0.09	0.82 ± 0.02
180	1.00 ± 0.13	1.26 ± 0.06	0.79 ± 0.07

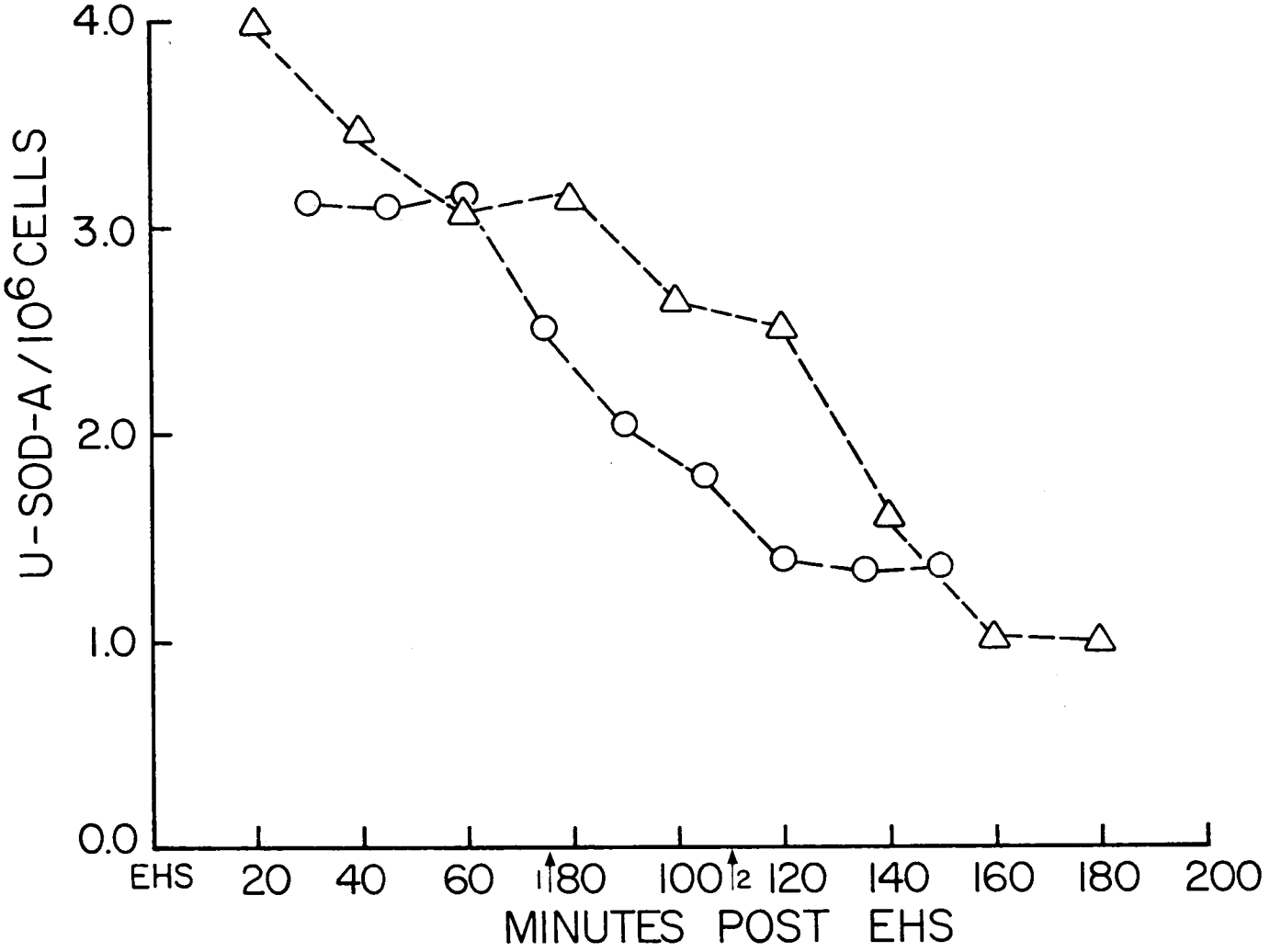


Figure 10. Units of SOD activity/10^6 cells of synchronized Tetrahymena grown under 100% O_2. (Control ○—○, 5,000 R △—△.) Arrows indicate peak of cell division, (1) = control, (2) = 5,000 R.

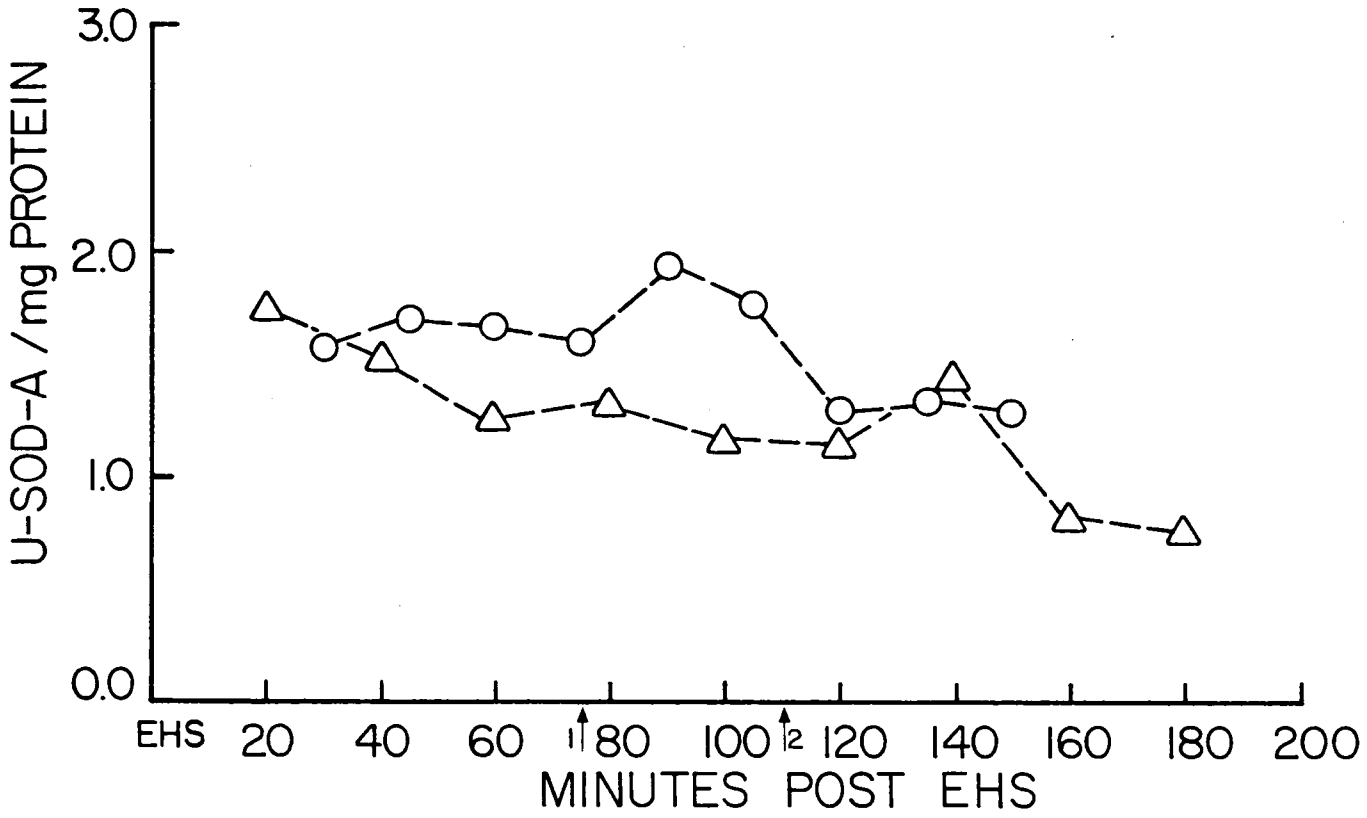


Figure 11. Units of SOD activity/mg-protein of synchronized Tetrahymena grown under 100% O_2. (Control○—○, 5,000 R△—△.) Arrows indicate peak of cell division, (1) = control, (2) = 5,000 R.

cell number which continues through approximately EHS + 110 min; changes in cell numbers indicate that there is approximately a doubling of cell number during the synchronized division cycle.

In the case of x-irradiated cultures, there is a division delay of approximately 35 min. Thus division begins at approximately EHS + 95 min with a peak of only 50% synchrony. Cell number increases during division to approximately 65% of that found for nonirradiated cell cultures with final cells reaching a 75% increase. These parameters of division index and relative increase in cell number do not show any major differences between cultures which were oxygenated versus cultures grown with normal room air.

From these data it does not appear that any increase in SOD activity prior to irradiation protects cells by reversing the excess division delay imparted by x-irradiation.

CHAPTER IV

DISCUSSION

One of the major findings of this research was the discovery that there is SOD in Tetrahymena. SOD activity was studied in relation to its enhancement by high O_2 tension and whether this enhancement plays a role in producing a radioprotective effect in heat-shock synchronized cells of Tetrahymena.

Preliminary experiments concerning generation times of Tetrahymena were performed in order to determine whether high O_2 levels had a deleterious effect on cell growth. Results showed no statistical difference between the average generation times of cultures grown utilizing normal room air (20% O_2) versus the average generation times of cultures which were grown in media supplemented with 100% O_2. Growth under high O_2 tension was studied because it has been previously reported that high O_2 can be toxic to growing cells ranging from bacteria to mammalian cells (Haugaard, 1968; Gottleib, 1971). Results obtained in this investigation, however, indicate that growth rate is not significantly altered in Tetrahymena by high levels of O_2.

It is generally known that Tetrahymena is a ubiquitous organism which can exist in a variety of habitats involving both anaerobic and aerobic environments. Under aeration conditions, Tetrahymena seems to be an organism which can tolerate drastic changes of O_2 levels.

The possibility that tolerance of Tetrahymena to higher than normal levels of O_2 may be linked to the enhancement of SOD, was one of the main

reasons for this study. SOD activity was found to be present in log phase cultures, stationary phase cultures and even in heat-shock synchronized cultures of Tetrahymena. Its presence in Tetrahymena adds to the list of organisms which indicates that SOD is ubiquitous to all aerobic organisms (Fridovich, 1975). In the case of log phase cell cultures, which were grown in normal atmospheric air (20% 0_2), the amount of SOD activity was approximately 1.42 units per mg-protein. The amount of SOD activity was found to be consistent with amounts of SOD activity reported for various other microorganisms (McCord et al., 1971). Aerobic microorganisms contain SOD activity ranging from 1.4 units per mg-protein in Salmenella typhimurium to 7.0 units in Micrococcus radiodurans (a known radioresistant organism) (McCord et al., 1971). Studies on primitive eukaryotic cells such as yeast (Saccharomyces cerevisiae) indicate that the SOD activity in log phase cultures is approximately 2.5 units of SOD activity per mg-protein (Gregory et al., 1974).

SOD has been implicated to play a major role in the abating of the harmful or toxic effects of high levels of oxygen (Fridovich, 1975). It is now accepted that the role of SOD is to eliminate harmful radicals, especially 0_2^- radicals, which are highly reactive and harmful to molecular and cellular processes (Halliwell, 1978).

In this investigation, Tetrahymena were grown under conditions of elevated 0_2 tension which probably results in an intracellular increase in the amount of 0_2^- radicals. The amount of SOD activity present in Tetrahymena cultures grown under conditions utilizing 100% 0_2 showed a

two-fold increase in SOD activity as compared to that found for cells grown in normal room air. The increased SOD activity in cultures grown in 100% 0_2 may be sufficient to explain the high tolerance of Tetrahymena to increased 0_2 tension. Enhancement or elevation of SOD enzyme activity by molecular 0_2 has been reported to occur in a variety of organisms (Fridovich, 1975); the belief that high levels of atmospheric 0_2 directly stimulates increased amounts of SOD activity comes from studies in which Fridovich (1975) has shown that 0_2^- radicals alone can increase SOD activity. 0_2^- radicals become much more prevalent in an organism when 0_2 is present in high amounts.

0_2^- radicals in addition to enhancing SOD activity are also substrates for the SOD enzyme. This is an example of substrate stimulation to increase enzyme activity. With increased amounts of 0_2^- radicals present in its environment, organisms would necessarily require the need for increased amounts of SOD enzyme activity in order to eliminate or alleviate the harmful or toxic effects produced by 0_2^- radicals. In fact, the deleterious nature of 0_2^- radicals would demand almost immediate removal of these 0_2^- radicals in order for an organism to survive (Halliwell, 1978).

To further substantiate the role of 0_2^- radicals in the enhancement of SOD, experiments were performed to test whether the removal or lowering of high oxygen levels would result in a subsequential drop in SOD activity. Indeed, a drop in SOD activity occurred in Tetrahymena when cell cultures were subjected to simulated anaerobic conditions. There was an approximate 30% decrease in SOD activity in cell cultures

subjected to anaerobic conditions (see Table II, page 16). This finding is consistent with evidence gathered on Escherichia coli (Hassan and Fridovich, 1977) and Saccharomyces cerevisiae (Gregory et al., 1974), which showed that there was a decrease in SOD activity when cell cultures were subjected to anaerobic conditions.

Once the role of O_2^- radicals was established for the enhancement of SOD, there were subsequent studies which showed that O_2^- radicals may play a role in the mechanism in which ionizing radiation events damage living organisms (Fridovich, 1972). For instance, there is evidence that O_2^- radicals are generated during radiolysis of aerated water (Anbar and Neta, 1967). O_2^- radicals most recently have been implicated to play a large part in the so-called "oxygen effect" (Goscin and Fridovich, 1973; Oberley et al., 1976). The "oxygen effect" is the phenomenon in which an increased amount of damage is imparted to cells by ionizing radiation when a high level of O_2 is present during that irradiation. Because of the possibility of an "oxygen effect" experiments were then undertaken to determine whether x-irradiation had an effect on SOD activity in Tetrahymena. If O_2^- radicals could account for some of the x-irradiation damage to cells of Tetrahymena, one would then expect to see fluctuations in SOD activity due to elevation in O_2^- radicals. The results of x-irradiation experiments did show a marked increase in SOD activity following x-irradiation in log phase cell cultures and in heat-shock synchronized cell cultures of Tetrahymena. One would then think that this apparent change occurring from x-irradiation would necessarily involve an increase in O_2^- radicals, due

to radiolysis of water and the presence of high oxygen; an increase in O_2^- radicals would then lead to an increase in SOD activity to eliminate O_2^- radicals which otherwise would react with cell macromolecules and form even more harmful radicals such as ·OH radicals (Cohen, 1977).

Thus, SOD must play a role in the protection of living organisms from radicals formed by ionizing radiations. A very prominent example has already been shown in Micrococcus radiodurans, which is a microorganism known to have above normal levels of SOD activity as compared to other microorganisms (Fridovich, 1975). This organism derives its name because of its great resistance to damage imparted by ionizing radiation. The enhancement of SOD by x-irradiation could therefore be a major feature of the defense of living organisms against the damaging effects of radicals formed by ionizing radiation; in essence, SOD is a radioprotective agent whose role is to eliminate harmful radicals caused by radiation.

The role of SOD as a radioprotection agent has recently been examined using exogenous addition of SOD during x-irradiation treatment in a number of organisms—Acheleoplasma laidlawii B (Petkau and Chelak, 1974), mice (Petkau et al., 1975a), and more specifically bone marrow stem cells of mice (Petkau et al., 1975b). All of these studies showed that exogenous additions of SOD to either buffer solutions of microorganisms or injected into whole animals does act as a radioprotective agent reducing mortality rates.

With the belief that induction or increases in levels of SOD activity would act as an effective radioprotective agent in Tetrahymena,

experiments were undertaken to determine whether conditions previously described herein for enhancing SOD activity would effectively abate or alleviate radiation damage. In particular, it was shown that SOD activity could be enhanced by high levels of 0_2 in Tetrahymena. Thus, when Tetrahymena was grown and synchronized with 100% 0_2, there was a noticeable increase in the amount of SOD activity after EHS. There was also an excess delay induced by x-irradiation in the synchronized cell division of Tetrahymena. Therefore, it was decided to determine if conditions which would result in an increase of intracellular amounts of SOD activity prior to x-irradiation would effectively act in a radioprotective manner by shortening the excess division delay in the synchronized cultures. However, conditions which elevated SOD activity did not reduce excess division delay.

These negative findings could be interpreted in a variety of ways, all of which are speculative. For one thing, it has never been clearly established what mechanisms are mainly responsible for the radiation damage resulting in the excess delay. If the damage results in formation of free radicals which exceed the capacity of the enzyme (SOD), then even a two-fold increase in SOD activity would not necessarily be sufficient to remove enough radicals to shorten the delay. Another possibility exists in respect to the defense mechanisms to sublethal damage in organisms, for the protection against 0_2 toxicity are not the same as the defense mechanisms developed for ionizing radiation and the different systems do not overlap. It is obvious from this study that increases in intracellular levels of SOD activity do not function

effectively as a radioprotection agent in relation to cell division. Exogenous addition of SOD and its possible roles in radioprotection in Tetrahymena remains for further investigation.

CHAPTER V

SUMMARY

1. SOD activity was found to be present in Tetrahymena pyriformis GL-C.

2. The SOD activity in log phase cultures of Tetrahymena was found to be elevated by a two-fold increase, when Tetrahymena were subjected to high levels of (100%) oxygen.

3. The amount of SOD activity, as compared with normal log phase cultures, decreased by approximately 30% when cell cultures were subjected to anaerobic conditions.

4. Log phase cultures of Tetrahymena which were subjected to x-irradiation treatment showed an increase in SOD activity at 15 min post x-ray treatment. The elevated amounts of SOD activity returned to that of normal log phase levels within one hour post x-ray treatment.

5. SOD activity in heat-shock synchronized cultures of Tetrahymena at EHS was found to be higher than that found in log phase cultures. The increased amount of SOD activity corresponded to an increase in the intracellular increase of soluble protein of synchronized cultures. The SOD activity dropped by one-half during the first division following EHS.

6. When x-irradiation was given to heat-shock synchronized cell cultures of Tetrahymena, there was an excess delay in synchronized cell division. The x-irradiation treatment also resulted in an increase in the intracellular amount of SOD activity in synchronized cultures. The increased amount of SOD activity at EHS + 20 min dropped slightly at

about EHS + 40 min and then again by one-half following the synchronized division.

7. SOD activity was enhanced by about two-fold when cultures of Tetrahymena were grown and synchronized in an atmosphere of 100% oxygen. The pre-enhanced amount of SOD activity produced by oxygenation prior to irradiation did not decrease the amount of excess division delay caused by x-irradiation given at EHS.

8. It was concluded from this study that an increase in intracellular amounts of SOD activity did not act as an effective radioprotective agent.

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