

dicotyledonous plants react differently on 2,4-D treatment has not yet been answered but the slower absorption and translocation within the monocots and the rate of detoxification (formation of 2,4-D complex) may be the contributing factors.

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## INHIBITION OF GROWTH OF CHLORELLA PYRENOIDOSA BY BETA-EMITTING RADIOISOTOPES<sup>1, 2</sup>

J. W. PORTER AND H. J. KNAUSS

BIOLOGY SECTION, RADIOLOGICAL SCIENCES DEPARTMENT, GENERAL ELECTRIC Co.,  
RICHLAND, WASHINGTON

Many studies have been made during the past 50 years of the effects of ionizing radiations on plants, animals and microorganisms, but to our knowledge no extensive studies have been made on the effect of these radiations on *Chlorella*. Neither have attempts been made to grow this organism in the presence of radioactive elements, except in tracer experiments. Mutations of *Chlorella* have been produced, however, by Granick (3) with X-ray.

This paper reports (1) the methods used in growing algae in the presence of beta-emitting radioactive elements, and (2) the effect of several concentrations of tritium oxide, phosphorus-32, sulphur-35 and the equilibrium mixture Sr<sup>90</sup>-Y<sup>90</sup> on the growth of *Chlorella*. The results presented, which are largely descriptive, are preliminary to other extensive studies now being made on the effect of tritium oxide on cellular processes and biosynthetic reactions of *Chlorella*.

#### MATERIALS AND METHODS

The culture of *Chlorella*, #7516 of the American Type Culture Collection, was grown on agar slants and transfers were made at monthly intervals. Algae to serve as inocula in experiments were transferred from slants at approximately 6-week intervals to one

liter of sterile inorganic nutrient solution (Knop, modified). A modified, low-form, 3-liter culture flask mounted on an electrically driven rocker platform served as the container. The algae were grown under white fluorescent light of 100 to 300 fc, and aerated with a stream of 8 to 9% CO<sub>2</sub> in air. After 4 to 6 days' growth 900 ml of solution were drawn off 2 to 3 times a week and replaced with sterile nutrient solution. The algae withdrawn served as a source of inoculum for the experiments with the beta-emitting isotopes.

Algae were grown in the presence of each of the four isotopes in 20 × 150 mm tubes. The nutrient solution was that of Myers (4). Nutrient solutions containing tritium oxide were prepared as follows. Fifteen ml of nutrient solution, adjusted to pH 5.5, and distilled water were added to each tube. After autoclaving the solutions were cooled and tritium oxide was added. Cells to serve as inocula were removed from the continuous culture, centrifuged and then suspended in sterile nutrient solution. The suspension was adjusted to an optical density of 0.60 (660 mμ, Beckman Model DU Spectrophotometer) and then 1.0 ml of the suspension was added to each tube. The final volume in each tube was 18.0 ml.

Nutrient solutions containing the other beta-emitting isotopes were prepared somewhat differently. Six ml of nutrient solution were added to each tube. After sterilization 0.01 to 0.02 ml of 0.1% methyl red in alcohol was added. The radioactive isotope was added,

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and the solution was adjusted to pH 5.5 to 6.5, as judged by the indicator color, with KOH. If the pH of the solution dropped below 5.5 on aeration additional KOH was added. Sterile water and the inoculum were added. The final volume was usually 11.0 ml.

Carrier or acid, or both, were present in stock solutions of all radioisotopes, except tritium oxide. The pH of the solution of this isotope was approximately 7.0. The carrier and acid prevented excessive deposition of radioactivity on the walls of containers. Concentrations of phosphorus-32, expressed as  $\mu\text{C}/\text{ml}$  of final solution were those at the start of an experiment.<sup>3</sup> Concentrations of other isotopes did not change significantly during the experimental period.

During the growth of the algae, in a continuously exhausted hood, the light intensity (white fluorescent) incident on the tubes was approximately 400 fc, and the gas bubbled through the tubes was an 8 to 9%  $\text{CO}_2$  in air mixture. It flowed at the rate of approximately 100 ml per minute. The temperature of the nutrient solution was kept at 25 to 27°C.

Growth of the algae was followed over a three-day period by making daily light absorption readings<sup>4</sup> at 660  $\text{m}\mu$ . Controls, identical to experimental tubes except for radioactivity, generally showed logarithmic growth and a generation time of approximately 12 hours. Separate experiments showed a linear relationship between light absorption and cell mass (but not cell numbers) within the range in light absorption reported.

### RESULTS

The growth of algal cultures, in  $\log I_0/I$  values, was plotted against time. Figure 1 shows the changes in light absorption by algae growing in the presence of 5 to 40  $\text{mc}/\text{ml}$   $\text{H}_2^3\text{O}$  (1600 to 13,000 rep. (6) per day, calculated). Each point represents the average of the light absorption of duplicate tubes in a single experiment. Similar results have been obtained in 3 additional experiments with  $\text{H}_2^3\text{O}$ . It is evident from figure 1 that cell growth was not appreciably inhibited during the first day, but thereafter inhibition was a function of the dose of radiation. At the highest level of radiation growth was inhibited markedly at 72 hours.

Algae grown in the presence of other isotopes were exposed to the following dose rates of radiation (in rep. per day (calculated);  $\text{P}^{32}$ , 1000 to 5900;  $\text{Sr}^{90}\text{-Y}^{90}$ , 800 to 3200;  $\text{S}^{35}$ , 330 to 2700). Figure 2 shows an example of the changes in light absorption by algae growing in the presence of  $\text{P}^{32}$ . It will be noted that the weight of cells in the solution containing the high-

<sup>3</sup> All radioisotopes used in these studies were calibrated or recalibrated, by personnel of the Section's Analytical Group.

<sup>4</sup> Readings at 24 and 48 hours were made directly on the tubes, after removal of the aerators, in which the algae were growing. Readings at 72 hours were made on diluted samples if the log value was above 1.5. Loss of volume from the tubes during the growth period was minimized, except in those experiments with  $\text{H}_2^3\text{O}$ , by bubbling the air- $\text{CO}_2$  mixture through water before passage into the growing cultures.

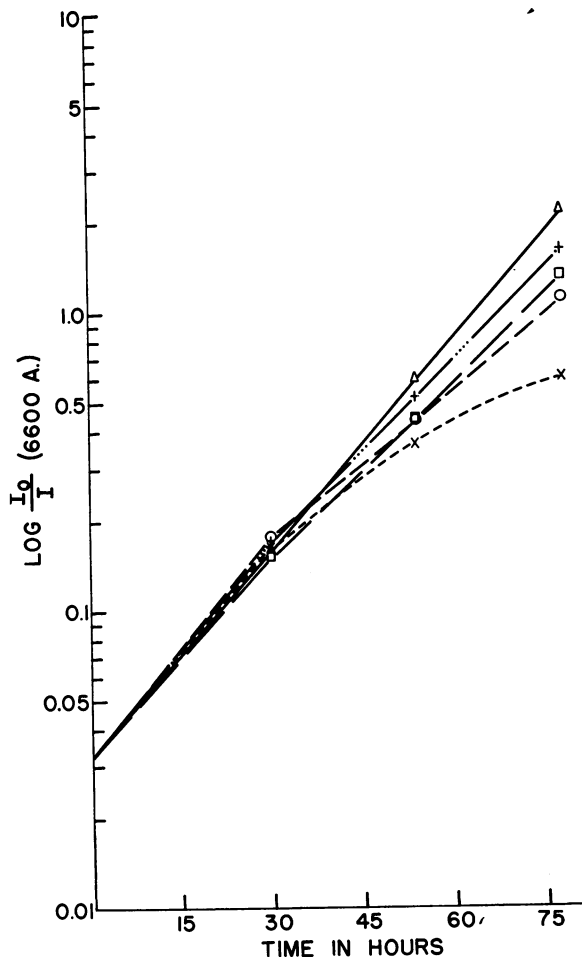


FIG. 1. The growth of *Chlorella* in the presence of tritium oxide.  $\Delta$ —Control,  $+$ —5  $\text{mc}/\text{ml}$ ,  $\square$ —10  $\text{mc}/\text{ml}$ ,  $\circ$ —20  $\text{mc}/\text{ml}$ ,  $\times$ —40  $\text{mc}/\text{ml}$ .

est level of  $\text{P}^{32}$  was less at 72 hours than that in the control solution. It will also be noted that growth in this experiment was more erratic than that shown in figure 1. Presumably this was due to the lower levels of radiation used and to our inability, because of the method used, to control the nutrient composition as precisely as in the experiments with tritium oxide.

The growth capacity of algae following exposure to  $\text{H}_2^3\text{O}$ ,  $\text{P}^{32}$ ,  $\text{Sr}^{90}\text{-Y}^{90}$  or  $\text{S}^{35}$  was tested by subculture in non-radioactive nutrient solution. Algae grown for 3 days in radioactive nutrient solution were allowed to stand<sup>5</sup> an additional 3 days in the refrigerator in the

<sup>5</sup> In other experiments algae were centrifuged directly from the radioactive solution and then taken up in fresh nutrient solution. These cells and duplicates not centrifuged from the radioactive solution were stored for 3 days in the refrigerator. Then each lot of cells, free of radioactivity, except that incorporated in the cells, was grown in non-radioactive nutrient solution. Growth after 3 days subculture of those cells stored 3 days in the presence of radioactivity was approximately 75% of that of the cells centrifuged immediately after the growth period ended.

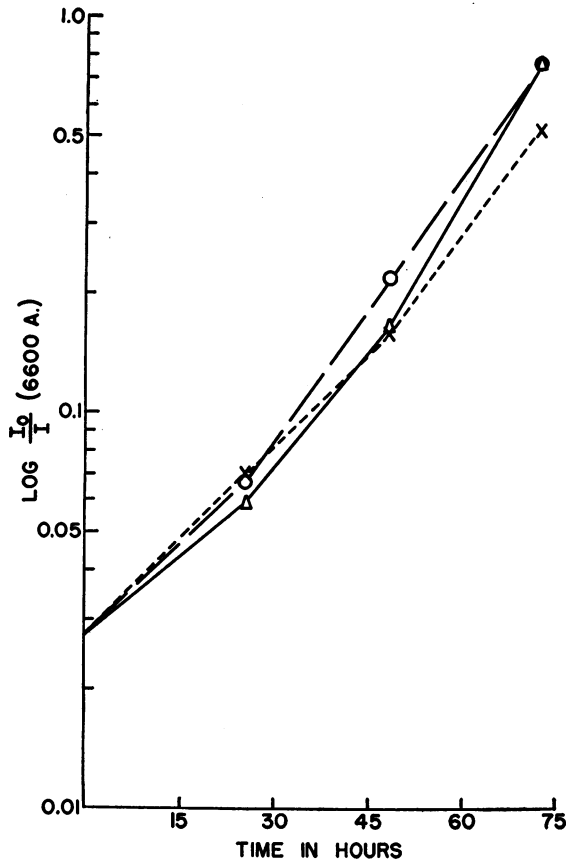


Fig. 2. The growth of *Chlorella* in the presence of  $P^{32}$ .  $\Delta$ —Control,  $\circ$ —77  $\mu\text{c/ml}$ ,  $\times$ —155  $\mu\text{c/ml}$ .

presence of the radioactive solution. The solution was then decanted from the cells, the cells resuspended in fresh nutrient solution and then centrifuged (2 minutes at approximately 10,000 rpm). The supernatant solution was discarded and the cells resuspended in fresh nutrient solution. All suspensions were adjusted to the same  $\log I_0/I$  value (660  $m\mu$ ) and then used as inocula for non-radioactive nutrient solutions. Growth of the cells was followed for 3 days.

Cells exposed to tritium oxide (fig. 1), when subcultured on inactive media gave the results shown in figure 3. Cells, exposed to  $P^{32}$  (fig. 2), when subcultured on inactive media gave the results shown in figure 4. The growth of experimental and control cells shown in these figures varied as a function of the concentration of isotope in which the cells were originally grown. Growth rates of those cultures exposed originally to the higher levels of radiation (20 and 40  $\text{mc/ml H}_2^3\text{O}$ ) were greatly different from that of control cultures throughout the 3-day period, but the growth rates of the other cultures paralleled that of the controls between the second and third day of subculture. Finally growth on subculture is nearly equal in per cent of mass of control cells, for those cells exposed to approximately equal doses of radiation from  $P^{32}$  and  $\text{H}_2^3\text{O}$  (3000 rep.; 116  $\mu\text{c P}^{32}/\text{ml}$ , 10  $\text{mc H}_2^3\text{O}/\text{ml}$ ).

Growth rates of cells on subculture were a function of dose of radiation for cells grown in the presence of

$\text{Sr}^{90}\text{-Y}^{90}$  and  $\text{S}^{35}$ . Mass of cells, in per cent of controls, was also nearly the same at 3 days of subculture for cells exposed to approximately 3000 rep. per day from  $\text{Sr}^{90}\text{-Y}^{90}$  or  $\text{S}^{35}$  as for cells exposed to the same level of radiation from  $P^{32}$  or  $\text{H}_2^3\text{O}$ .

Studies were made of the average size, and numbers of cells per ml per  $\log I_0/I$  value of 0.60 at 660  $m\mu$ , of cells grown 3 days in the presence of tritium oxide. An increase in the average size of the cells following beta irradiation was characteristic. Typical results are given in table I.

TABLE I

THE EFFECT OF 40  $\text{MC/ML}$  OF TRITIUM OXIDE ON THE DIAMETER, VOLUME AND NUMBER OF *CHLORELLA* CELLS

Cells *	Average diameter $\mu$	Average volume (calculated) $\mu^3$	Number of cells per unit of volume †
Control . . . . .	3.7	26	$29 \times 10^6$
40 $\text{mc/ml}$ . . . .	5.7	97	$8 \times 10^6$

\* Grown for three days in the presence or absence of  $\text{H}_2^3\text{O}$ .

† Number of cells per ml per  $\log I_0/I$  (6600 A) of 0.60.

#### DISCUSSION

Action of the ionizing radiations reduced the growth rate of the cells in proportion to the dose of radiation received. The reduction in growth rate was not evident, at the levels we used, until after the first day, but then the effect became progressively greater, and it persisted on subculture in non-radioactive media. Action of the ionizing radiations also resulted in an increase in cell size.

The results reported do not allow a deduction of the specific site or mode of action of the ionizing radiations on algae. A reduction in content of one or more enzymes below a growth-limiting level would, of course, reduce the growth rate of the cells, and the cells would not return to the normal rate until the enzyme content of the cell had returned to the growth-limiting level. This might take several generations. Likewise interference with cell division, or the gradual dropping out of lethal mutants over several generations would reduce the growth rate of the cells. Toxic products formed by living cells, or resulting from cell death, would also slow the growth rate of cells.

The increase in size of the algae during irradiation might result from an interference with cell division or from a change in the metabolism of the algae following changes in enzyme content. An increase in the content per cell of enzymes for starch synthesis and a decrease in content of other enzymes could result in an increased size of the cell if cell division is independent of the quantity of stored carbohydrate per cell. Likewise an enlarged size could result from damage to either the cell division mechanism or the enzymes synthesizing factors for cell division if the cell division process is relatively independent of the growth process.

It seems to us superfluous to speculate further on the sites of action of the beta-emitting isotopes in

Chlorella. Instead it is anticipated that further experimentation, now in progress, will yield data on biochemical and physiological sites in Chlorella which are most sensitive to damage by beta-emitting isotopes. We believe also that comparisons between the results we have obtained and those obtained by others with ultraviolet light (1, 2 and 5) would be meaningless. It is quite probable that the mechanisms of action, and the sites of damage, for these radiations are quite different. In general ultraviolet light brings about activation of those molecules which can absorb the light quanta, principally nucleic acids, whereas beta particles bring about ionization of molecules near the path of the particle. The latter action may be direct or mediated through products resulting from the ionization of water.

#### SUMMARY

Algae have been grown in the presence of several levels of  $H^3_2O$ ,  $P^{32}$ ,  $Sr^{90}$ - $Y^{90}$  and  $S^{35}$  and then subcultured in non-radioactive inorganic nutrient solution. Growth rates of cells were decreased in proportion to the dose of radiation received. The reduced rate of growth continued in subculture as a function of the dose received. An increase in the average cell size also occurred during growth of the algae in the radioactive solutions.

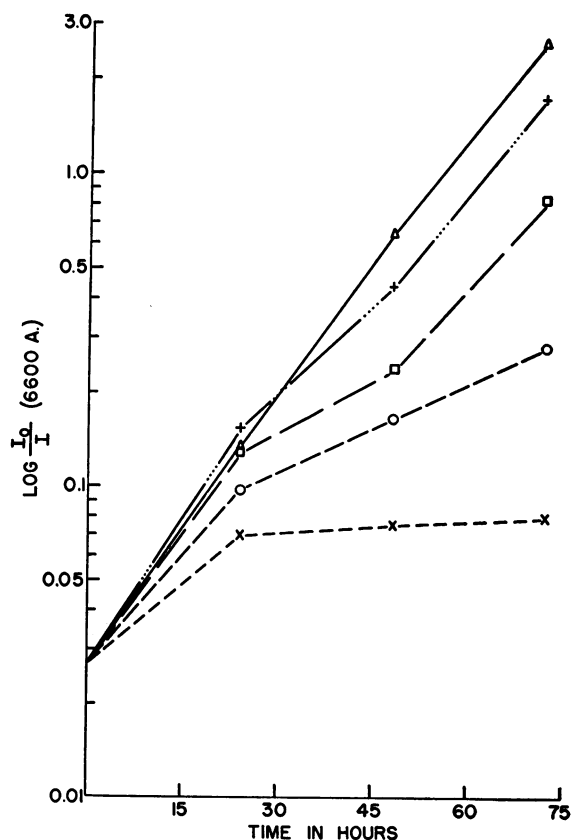


Fig. 3. The growth of Chlorella after exposure to tritium oxide. Inoculum grown in the presence of no  $H^3_2O$ — $\Delta$ ; 5 mc/ml—+; 10 mc/ml— $\square$ ; 20 mc/ml— $\circ$ ; and 40 mc/ml— $\times$ .

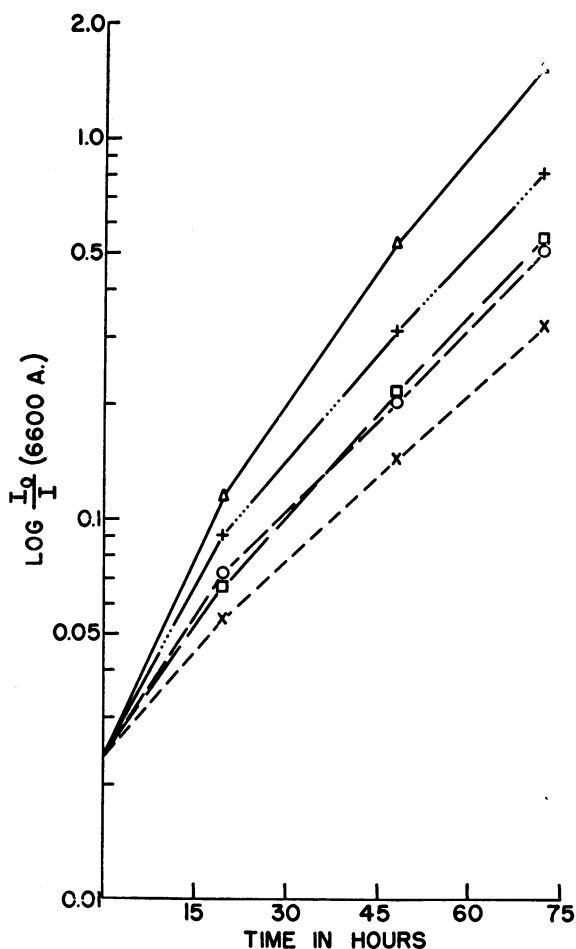


Fig. 4. The growth of Chlorella after exposure to phosphorus-32. Inoculum grown in the presence of no  $P^{32}$ — $\Delta$ ; 39  $\mu\text{c/ml}$ —+; 77  $\mu\text{c/ml}$ — $\square$ ; 116  $\mu\text{c/ml}$ — $\circ$ ; 155  $\mu\text{c/ml}$ — $\times$ .

The results reported do not allow a deduction of the specific site of action of the ionizing radiations on the algae.

Contributions of Dr. D. Weinberger and Mrs. M. S. Watson to this work are gratefully acknowledged.

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