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May 15, 1975

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1975 - 76

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ANNUAL REPORT

FOR PERIOD ENDING APRIL 30, 1975

Table of Contents

✓ Radiation Physics Section.....A 1 - A 25

(see Section Index)

✓ Radiation Biochemistry Section.....B 1 - B 23

(See Section Index)

Cellular Radiobiology Section.....C 1 - C 14

(See Section Index)

Publications and Reports.....D 1 - D 3

Other Staff Activities.....D 4 - D 5

Meetings Attended, Papers Presented.....D 6 - D 7

00925

RADIATION PHYSICS SECTION INDEX

- A. Ionization of Liquids..... A 1
- B. Assessment of Mutagenesis by High LET Radiation with
Particular Reference to Plutonium Alpha Particles..... A 10
- C. Mitotic Delay and Killing of Resistant and Sensitive
Lines of Murine Leukemic Cells In Vitro..... A 18

A. Ionization of Liquids

1. AC Ionization Chamber

A new bridge circuit which measures chamber resistance and preamplifiers were constructed for our alternating current liquid ionization chamber. The oscillator frequency was 100 kHz and the ionization chamber capacity was 50 μF for a plate area of 0.73 cm^2 and separation of 1 mm. The potential applied to the chamber was 17.8 volts rms. For an electron beam charge of 8×10^{-9} coulombs delivered to the above chamber containing hexane, a straight line relationship was observed between the chamber resistance and the time extending out to 6 milliseconds. At six milliseconds, electrical noise became a limiting factor. The slope of the line was 6.62×10^{11} ohms/sec and the intercept was 2.65×10^7 ohms. From the fundamental equations for liquid ionization chambers, the following may be derived: Slope of chamber resistance as a function of time in ohms/sec = $dK/2eA\mu n_0$ and intercept in ohms = $d/2eA\mu n_0$ where d = collecting plate separation, K = recombination coefficient, μ = mobility, e = charge on the electron, A = area of collecting plates and n_0 = initial ion density, all in mks units. From the bridge data above and assuming $\mu \approx 10^{-7}$, we find $K/\mu = 15.5 \times 10^{-9}$ and $n_0 = 1.6 \times 10^{19}/\text{m}^3$. While these values agree quite well with data taken by the D.C. conductivity chamber, most important was the straight line relationship that shows that space charge is avoided in the AC chamber and may be neglected in the D.C. chamber out to at least 1 ms.

This work was reported at the 5th International Congress of Radiation Research in July, 1974 (C00-78-320).

2. Kinetics of the Reactions of Quasifree Electrons

Our study of the reactions of quasifree electrons with biologically relevant solutes and of the mobilities and reactions of the resulting solute anions was continued through application of the pulse-irradiation/ion current decay technique which we developed and described earlier (G. Bakale, E.C. Gregg, and R.D. McCreary, J. Chem. Phys. 57, 4246, 1972). Briefly, this technique consists of monitoring the electron and subsequent ion current decay following irradiation of the sample in a parallel plate ion chamber by a single pulse of 1 MeV electrons. The electron and/or ion currents are then analyzed to determine if linear, first- or second- order decay kinetics are followed. From the type of kinetics involved we can then deduce mobilities, attachment rates, and recombination coefficients.

Preliminary results on rates of quasifree electron attachment to several solutes of biological interest were reported at the 1974 International Congress of Radiation Research (C00-78-317). These results indicated that radiation sensitizers exhibited very high rates of reaction with quasifree electrons, whereas electron reaction rates with radiation protective additives and amino acids were markedly lower. Subsequent measurements of the reaction rates of several of these solutes have shown that this cursory conclusion was qualitatively correct, but our main effort since then has been toward refining our technique in order to understand quantitatively the electron and ion decay processes.

The first step toward improving the quality of our rate constant data was to decrease the impurity level in the solvents used so that

electron reactions with impurities were negligible compared to the electron reaction with the solute under study. To achieve this we developed a purification technique in which we routinely are able to reach impurity levels in Fisher Certified Grade n-hexane which are threefold lower than those we had reached earlier in Phillips Research Grade n-hexane (G. Bakale, E.C. Gregg, and R.D. McCreary, *op.cit*). The latter solvent is normally used in most studies of quasifree electrons; however, its high cost (four times that of the Fisher n-hexane) makes its use prohibitive for scavenger studies which consume large quantities of solvent.

The purification technique we developed involves an initial degassing by bubbling argon through the solvent and passing the solvent over a column of Molecular Sieve 4A followed by irradiating the solvent with ^{60}Co - γ radiation to a dose of $\sim 10^5$ rads. The solvent is then transferred to the ion chamber and again degassed by argon-bubbling. With this technique we routinely obtain electron half-lives of 1 - 1.5 μ seconds in Fisher n-hexane at 20°C. Since we add sufficient solute to decrease the electron's half-life to 40 - 400 nseconds, the reaction with adventitious impurities requires only a slight correction. This is demonstrated in Figure 1 in which the intercept--which corresponds to electron reactions with impurities--of the plot of the observed pseudo-first-order rate constants vs solute concentration is ~ 30 times less than the reaction rate at the greatest solute concentration. Fig. 1 also illustrates the marked difference in electron attachment rates between a radiation protective agent, Cleland's Reagent, and a typical

FIGURE 1

radiation sensitizer, dinitrobenzonitrile. The slope of such a plot is the bimolecular electron attachment rate constant, k_2 , and the values of k_2 of several solutes obtained from similar plots are listed in Table I.

From this table, one also sees that several of the radiation sensitizers have a rate constant of $\sim 2 \times 10^{12} \text{ M}^{-1} \text{ sec}^{-1}$ in n-hexane. We propose that this value of k_2 represents the limiting reaction rate of the quasifree electron in n-hexane at 20°C where the electron mobility is $0.07 \text{ cm}^2/\text{volt second}$ (R.M. Minday, L.D. Schmidt and H.T. Davis, J. Chem. Phys. 54, 3112, 1971). This limiting rate is consistent with the theory of diffusion-controlled reactions which can be stated in simplest terms by

$$k_2 = 4 \pi (r_s + r_e) (D_s + D_e) \quad (\text{I})$$

where k_2 is the bimolecular electron attachment rate constant, r is the effective radius of the reactants, D is the diffusion coefficient and the subscripts s and e denote the solute and electron, respectively. Since the electron is a much more mobile species than the solute (i.e., $D_e \gg D_s$), Equation I simplifies to

$$k_2 = 4 \pi (r_s + r_e) D_e \quad (\text{II})$$

The diffusion coefficient of the electron may be obtained from the electron mobility, μ_e , by applying the Nernst-Einstein equation:

$$D_e = \frac{kT}{e} \mu_e \quad (\text{III})$$

where k is the Boltzmann constant, T the absolute temperature and e the charge of the electron. At 20°C, where $\mu_e = 0.07 \text{ cm}^2/\text{volt second}$, Equation III yields $D_e = 1.8 \times 10^{-3} \text{ cm}^2/\text{sec}$. Substituting this

TABLE 1. Rate constants of electron attachment to solutes at 20°C in n-hexane except as noted*

Solute	Structure	$k_2 \times 10^{-12} (\text{M}^{-1} \text{sec}^{-1})$
4-hydroxy-2,2,6,6-tetramethyl piperid-1-yloxy		0.37
Cleland's Reagent (dithiothreitol)		0.43
p-quinone		1.3
menadione (2-methyl-1,4-naphthaquinone)		1.5
p-nitrotoluene		1.9
1,3-dinitrobenzo-5-nitrile		1.9
1,3-dinitrobenzene		2.2 5.1*
p-nitroacetophenone		2.3

* denotes c-hexane solvent

value of D_e in Equation II and converting the rate constant of 2×10^{12} liters/mole second into molecular units of 3.3×10^{-9} cm³/sec yields a reaction radii sum of $\sim 15 \text{ \AA}$. Since the radius of the solute is only $\sim 4 \text{ \AA}$, the remaining reaction radius must be attributed to the electron and suggests that the electron at the instant of attachment has an effective radius of $\sim 10 \text{ \AA}$. This in turn implies that the electron is in a trapped state formed by several n-hexane molecules when the reaction occurs.

Calculating similarly the reaction radius of the 1,3-dinitrobenzene / c-hexane system for which $k_2 = 5.1 \times 10^{12}$ liters/mole second or 8.5×10^{-9} cm³/second and $\mu_e = 0.21$ cm²/volt-second (A.O. Allen, T.E. Gangwer and R.A. Holroyd, J. Phys. Chem. 79, 25, 1975) yields $D_e = 5.25 \times 10^{-3}$ cm²/sec and $(r_e + r_s) = 13 \text{ \AA}$. Thus, it appears that the electron is reacting from a trapped state in both n- and c-hexane; however, further work is required to establish this unequivocally.

The influence of the solute's structure upon the attachment rate also can be seen from Table I. For example, comparing 1,3-dinitrobenzene with 1,3-dinitrobenzo-5-nitrile illustrates that the nitrile group has, if any, an inhibitory effect upon electron attachment. Similarly, one sees that the acetyl group of the p-nitroacetophenone makes the nitrobenzyl ring more reactive toward electron attachment than the methyl group of p-nitrotoluene. Also, the naphthyl ring structure of menadione appears to provide only a slight enhancement of electron reactivity compared to quinone. Through studies such as these we hope to gain sufficient knowledge of various functional groups' enhancing or inhibiting effects upon electron attachment which may

enable us to design and synthesize more efficient radiation sensitizers.

The temperature dependence of the electron attachment rate constant is shown in Figure 2 for 1,3-dinitrobenzo-5-nitrile, p-nitroacetophenone, p-nitrotoluene and Cleland's Reagent. The three former solutes are all considered to be good radiation sensitizers and show normal Arrhenius behavior with the slope of the plot yielding an activation energy of ~ 7.5 kcal/mole.

The temperature dependence of k_2 of these sensitizers is in marked contrast to that of Cleland's Reagent, which is a radiation protective agent. The electron attachment rate of Cleland's Reagent oscillates between a positive and a negative temperature coefficient which may be expressed as activation energies of 12.2 and -2.5 kcal/mole, respectively. Although negative activation energies of electron attachment rates have been observed in non-polar liquids by others (A.O. Allen and R.A. Holroyd, J. Phys. Chem. 78, 796, 1974) we are unaware of any observation of an Arrhenius plot which has four inflection points. This unusual dependence of k_2 upon temperature may be related to ring closure (via the -S-S- bonding) and opening but requires more detailed study before an electron attachment mechanism is proposed.

Up to this point we have reported results related only to the quasifree electron decay rate; however, with our conductivity technique we also were able to simultaneously determine the yield of free ion-pairs produced by each pulse of radiation. We had pointed out earlier (G. Bakale, E.C. Gregg and R.D. McCreary, loc cit) that the initial electron current, i_0 , obtained by extrapolation of the exponentially decaying electron current to zero time was related to $G(e_f)$, the yield

FIGURE 2

of free electrons produced per 100 eV of energy absorbed by the sample, through the equation

$$G(e_f) = 100 i_o / DeAE_o \mu_e \quad (IV)$$

where D is the absorbed dose, A the electrode area, E_o the effective electric field and, again, e the electronic charge and μ_e the mobility of the electron.

We have begun measurements of free electron yields utilizing the above relation in order to determine what, if any, effect the presence of a sensitizer has upon the number of ion-pairs produced in the solvent. The effect of temperature upon the yield of free ion-pairs in a 1.9 μ M solution of p-nitrotoluene in n-hexane is shown in Fig. 3. The yield at 20°C is 0.40 which is only 10 percent greater than the yield in pure n-hexane, but the activation energy of 6.8 kcal/mole is considerably greater than the value of 4.3 kcal/mole for pure n-hexane (R.M. Minday, L.D. Schmidt, and H.T. Davis, J. Chem. Phys. 54, 3112, 1971). Activation energies of 6-8 kcal/mole were also found with other radiation sensitizers in n-hexane.

We had also observed a slight increase in the yield of free ion-pairs with the addition of a sensitizer. For example, at 20°C the $G(e_f)$ in pure n-hexane is 0.36 but this value increases to $\sim$ 0.50 in the presence of dinitrobenzonitrile at a concentration level of 1-2 μ moles/liter. An explanation of these enhanced free ion-pair yields and higher temperature coefficients in the presence of sensitizer requires further studies on the electron attachment process which are now progressing.

The final area of the ionization process in liquids which we studied

FIGURE 3

was the effect of solutes upon ion-ion recombination rates. As of this writing, we have observed that neither radiation sensitizers nor protective agents affected the ion-ion recombination rate.

B. Assessment of Mutagenesis by High LET Radiation with Particular Reference to Plutonium Alpha Particles

1. Overview

While it remains that we have done very little work with alpha particles to date, we have exercised a fair amount of effort in finding and characterizing a strain of L5178Y cells that can act as a parental line for subsequent production of radioresistant mutants. Most of this work has been funded by a NIH Grant on Production of Radiation Resistant Mutations of Mammalian Cells; however, it is very basic to and necessary for our proposed program in mutagenesis with High LET particles and which is not covered by NIH funding. While the material that follows is essentially a progress report to the NIH, it does explain our build-up of personnel, facilities and preliminary experiments so necessary to the initiation of work with alpha particles.

Over the past year we have been measuring and comparing the characteristics of "normal" L5178Y cells with a radioresistant mutant of same both obtained from V.D. Courtenay in England. While more detailed results and analyses of these two cell lines will be discussed later, we shall also report on the characteristics of several strains of L5178Y cells being considered as a standard parental line for our future research.

In August of 1974, we were very fortunate in obtaining the services of Dr. T. Yau who joined us as an Assistant Professor and whose major responsibility is in the operation of the tissue culture facility in growing and maintaining normal cells as well as producing radiation and chemically induced mutants of same. Besides developing some new

approaches to biochemical characterizations of those differences between normals and mutants, Dr. Yau is also concerning himself with developing a line of human cells in culture for similar studies.

Dr. Yau also obtained the services of Mr. S.C. Kim as a Research Assistant. Mr. Kim has had considerable experience with tissue culture and is proving to be a key person in our program.

Regarding equipment and facilities, we have decided against the use of tritium-loaded culture flasks in favor of continuous x-ray exposures for the production of mutations. This was dictated in part by the hazards of handling the tritium with a large number of culture flasks as well as the fact that we wish to use pulsed x-rays later in the program and the same x-ray equipment could be utilized for both modes of exposure. We have designed and built an x-ray machine that can deliver radiation at 5 r/hr simultaneously to 50 culture flasks for months if necessary. Further, a transmission air ion chamber was developed and is used to provide a feedback signal to regulate the x-ray intensity to better than 1%. For pulses, the ion chamber is to be used in an integrate mode to control the rads per pulse which pulses in turn can be programmed in any desirable time sequence.

2. Culture and Selection of "Normal" L5178Y Cells

a) Setting up of tissue culture facility

The initial efforts during this period were to order, set up, and test equipment necessary for the normal operation of a tissue culture laboratory. These include media preparation and quality control facilities, microscopy, laminar hood for aseptic techniques, CO₂ incubator for plating, and Coulter counting for cell enumeration and size distribution.

- b) Selection of an appropriate cell line for radiation-resistant mutation induction

The following sublines of L5178Y were obtained, maintained and tested in our laboratory:

LR₁ - This is the L5178Y subline used extensively by the Department of Radiation Biology and Biophysics at the University of Rochester. The line has been passaged indefinitely under in vitro conditions.

LR₂ - This line obtained from the Department of Pharmacology of the University of Rochester has been passaged in vitro - in vivo alternately.

AIV - A radiation resistant mutant from Dr. V.D. Courtenay.

LN - The "normal" strain of L5178Y used by Dr. V.D. Courtenay to induce radiation resistant mutants.

- c) Results of measurements

The above four sublines of L5178Y were maintained in Fischer's Medium supplemented with 10% Heat-Inactivated Horse Serum. Mean generation time for LR₁, LR₂, AIV, and LN are 10-12 hours, 12-16 hours, 16-20 hours, and 12-14 hours, respectively. Cell volume modal distribution in increasing order is as follows: LR₁, LN, LR₂, and AIV. Cells frozen in liquid nitrogen at the rate of 1 - 2° C per minute with cell density above 2×10^6 cells per ml. revive after thawing without any difficulty. Plating efficiency in soft agar for LR₁, LR₂, AIV, and LN are 35%, 25%, 16% and 45%, respectively. With the possible exception of AIV and LN, all these sublines produce ascites tumors in DBA/2 mice within 8 days when 1×10^5 cells in 0.1 ml saline were injected by the intraperitoneal route.

A series of dose-response survival studies were made with these sublines. Log phase cultures were irradiated at a dose rate of 100 rads per minute 40 cm. from the source at 37°C with a Picker Vanguard x-ray unit. The following parameters were derived from the survival curves for single exposures:

	LN	LR ₁	LR ₂	AIV
n (Extrapolation #)	7	3.5	3.5	9
D _q (Shoulder)	40r	40r	40r	120r
D ₀ (Slope)	80r	140r	140r	190r
LD ₅₀	100r	135r	135r	250r

3. Biophysical Characterizations

As mentioned, two lines of strain L5178Y mouse lymphoblasts were obtained from Dr. V.D. Vourtenay and compared. The relative resistance of the AIV line to X-radiation (250 kV) was confirmed using both colony formation and growth curve extrapolation as assays: for example, in our hands, survival after 400 rads was less than 1% for the sensitive LN cells compared with 20% - 30% for the AIV cells. Besides being more resistant to radiation in terms of reproductive capacity, AIV cells were found to be more resistant also in terms of radiation-induced mitotic delay.

These two cell lines were found to differ in other properties besides their responses to radiation. The population doubling time (T_D) and the modal cell volume both are larger for the radioresistant cells:

for example, T_D for log phase AIV cells was 16 to 18 hr compared with 11 to 13 hr for the radiosensitive strain. Observations of cells which had been fixed and stained for purposes of measuring chromosome number indicate that interphase nuclei of AIV cells are also larger than those of the more radiosensitive cells. Preliminary observations, however, indicate the average chromosome number per cell (40) is not different for the two lines.

Finally, it was found that the two cell lines differ with regard to their sensitivity to antimetabolites which are known inhibitors of DNA synthesis. AIV cells are resistant (by colony-formation assay) to excessive thymidine compared with the radiosensitive cells and also slightly more resistant to methotrexate. Hydroxyurea, however, appeared to kill AIV cells at a slightly lower concentration than was needed to kill the radiosensitive line.

The results so far indicate that either the AIV cells differ from the "parent" radiosensitive cells by multiple mutations or, if they differ by a point-mutation, the mutation is pleiomorphic.

4. Biochemical Characterizations

The LN line and the radiation resistant mutant (AIV) derived from these cells were tested for their abilities to exhibit inhibition of oxygen utilization upon addition of glucose, cyanide, and amytal. The magnitude of such inhibition serves as an index for characterizing the biochemical functioning of the oxidative machinery. Specifically, these measurements would indicate alterations of the respiratory-linked energy-yielding reactions resulting from radiation-induced mutations. Changes in glucose-inhibited respiration would indicate alterations in a spe-

cific metabolic control between the two major energy-yielding reactions within the cell, namely, mitochondrial versus glycolytic.

The addition of glucose to respiring LN cells resulted in a 32-37% inhibition of the oxidative rate; with the radioresistant AIV cells there was either a stimulation or no effect. Repeat experiments demonstrated a 70% inhibition with LN cells and 10% stimulation with AIV cells.

The absence of the Crabtree effect suggests that the resistant cells might be lacking in respiratory capacity, possibly as a result of a decrease in mitochondrial content or enzyme activity within the mitochondria. For this reason it was of interest to test the effect of the known respiratory inhibitor, potassium cyanide (KCN), on oxygen consumption. KCN is known to react with cytochrome oxidase of the mitochondria resulting in complete inhibition of electron transport via the cytochrome chain to oxygen. Also, a certain amount of cyanide-insensitive respiration or oxygen consumption occurs in the cytoplasm and nucleus so that the relative rate of oxygen consumption after KCN treatment would give some indication of extra-mitochondrial oxidation.

The inhibition of the rate of O_2 consumption by KCN was 50-60% with the LN cell and 6% or less with the AIV cell, i.e. both cells had appreciable cyanide-insensitive respiration, with the greater amount occurring with the resistant mutant. If glucose was present in the buffered saline used, the effect of cyanide was reduced with the LN cell to values between 0-17% inhibition. There was no effect of glucose on cyanide-sensitive respiration with the normal cell.

As an additional check the cells were tested for the effects of Antimycin-a addition. Antimycin-a is believed to be a respiratory inhibitor reacting on the electron transport chain within the mitochondrion. Like cyanide it will inhibit cellular respiration. Antimycin-a addition resulted in 15% inhibition of respiration with the LN cell and 0% inhibition with the AIV cell. These results agree with the cyanide data in that they indicate a greater amount of non-mitochondrial oxidation was occurring within the radioresistant AIV cell.

The results obtained so far seem to indicate that the radiosensitive LN cell has a relatively greater mitochondrial capacity for oxidation compared to the resistant AIV cell. This was demonstrated in the differences obtained upon glucose, KCN, and Antimycin-a additions. These results also demonstrated that the AIV cells has a greater capacity for non-mitochondrial oxidations. However, both L5178Y cells appear to have increased non-mitochondrial oxidative capacity when compared to the CHO, EAT cells, and lymphocytic R₁ and P388 tumor cells.

Of major significance is the lack of a Crabtree effect in the radioresistant cell. The Crabtree effect is one of the two most significant control factors in cellular metabolism (the Pasteur effect is the other). The alteration of the parent cell to a radiation-resistant mutant lacking this metabolic control suggest biochemical alterations in the energy producing system of the cell.

5. Summary

We believe we are currently at the point where we are ready for full scale operation of the program. The LN cell line appears to be

best choice for the "parent strain" from which radiation resistant mutants will be derived. Our preliminary studies with the LN and the derived (by Courtenay) radioresistant line AIV suggest that meaningful biochemical and biophysical differences between related cell lines that differ in their overall radiation response may indeed be established.

C. Mitotic Delay and Killing of Resistant And Sensitive Lines of Murine Leukemic Cells In Vitro

1. Introduction

This research was supported by both the NIH Grant--on Production of Radioresistant Mutants of Mammalian Cells--and the present ERDA Contract No. AT(11-1)2486. Since maintenance of steady-state cultures under conditions of continuous environmental radiation--wherein half the cells die each interdivision cycle--involve questions concerning mitotic delay when quantitative RBE as well as mutation rate, this investigation has been felt to be quite necessary to establish basic principles for our investigating mutagenesis of High LET α -particles.

There are extensive experimental results from which it may be inferred that damage to nuclear DNA is a major cause of radiation-induced reproductive death of mammalian cells, but the exact nature of the damage is still not known (c.f. Okada 1970a). Even less is known about the damage caused by ionizing radiation which results in mitotic delay, although a major target molecule(s) apparently is inside or near the nucleus of Chinese hamster ovary cells (Munro 1966). There are conflicting reports, however, dealing with the question of the possible involvement of DNA in mitotic inhibition (Schneider and Johns 1966; Humphrey and Dewey 1965). For several reasons, it is generally believed that mitotic delay and reproductive death are relatively independent in that the targets are different for the two phenomena: (1) dose response curves for the two phenomena differ in shape; (2) LET-RBE relationships for mitotic delay are different from those for cell death; and

(3) the curves describing the variations in radiosensitivity for delay and killing, as a function of the stage in the cell cycle, are out of phase for some cell lines (Okada 1970b). Based on the results of studies concerned with the cyclic response of mammalian cells to ionizing radiation, it has been suggested that the two phenomena may be associated inversely; "that is, an S cell may survive well because it has a long delay before division during which damage can be repaired prior to expression." (Sinclair 1968).

Support for the idea that longer delay may, in general, result in more repair and, therefore, greater survival, apparently was obtained from experiments with vegetative amoeboid cells of Dictyostelium discoideum (Cleveland and Deering 1972). Cleveland and Deering found that the survivors of irradiated cells of radiosensitive strains suffered unusually long division delays compared to the total cells in the population. Furthermore, the number of these survivors, for a given dose, increased significantly when division delay was experimentally prolonged by "holding" irradiated cells for different time intervals in buffer solution without a food supply. When radiosensitive and radioresistant strains are compared in terms of division delay of the total cells in the population, the dose-response curves are indistinguishable. Thus differential radiosensitivity of the different strains of amoeboid cells apparently is not reflected generally in both end points. Therefore, mitotic delay and killing appear to be relatively independent phenomena in these cells.

Recently, however, it was suggested that the extreme radiosensitivity of murine leukemic L5178Y S/S cells, in terms of killing, may be

related to a high frequency of abnormal divisions which are a result of unusually long x-radiation induced mitotic delay in these mammalian cells (Ehmann *et al.* 1974). In the present study, we have compared two L5178Y cell lines graciously provided by Dr. V.D. Courtenay. One was a radioresistant line (AIV) which was obtained after about three years of continuous irradiation from increasing amounts of tritiated water in the growth medium and the other was a more radiosensitive line (LS) from which the AIV's were derived (Courtenay 1969).

2. Methods and Materials

Both L5178Y cell lines were maintained in suspension culture in Fischer's medium supplemented with horse serum (10%, heat-inactivated), penicillin (100 units/ml) and streptomycin (100 µg/ml). The LS cells at 37.5°C have an average population doubling time of 12 hr whereas that for the AIV line is 17 hr. Asynchronous cells at $1-3 \times 10^5$ cells/ml were used in the present experiments.

Survival after irradiation was determined by counting colonies formed by cells plated in soft agar (0.2% - 0.3% agar in growth medium with 20% horse serum) in Falcon plastic petri dishes (60 x 15 mm). Cells were added, in small volumes, to the agar solutions after appropriate dilutions with fresh medium and 10 ml of these suspensions were pipetted into each petri dish. At least three dishes were used per concentration of cells plated and usually three different concentrations (obtained by serial dilution) of cells in soft agar were plated per dose of radiation. Cell number was determined electronically with a Coulter-orifice pulse-height analyzer system (Gregg and Steidley 1965). Plating efficiency was determined similarly, using unirradiated control

cells. In our hands, the plating efficiency of AIV cells (20% - 40%) was lower than that of LS cells (60% - 80%). Colonies were counted after 10-14 days of incubation (37.5°C) in a humidified CO₂ incubator.

Mitotic delay was estimated from curves of percentage of colchicine (10 µg/ml) blocked metaphase (% C-metaphase) cells versus time. These curves were extrapolated back to the time axis and the difference in those times between irradiated samples and the appropriate unirradiated controls was used as a measure of mitotic delay. The frequency of metaphase cells was estimated from microscope slides prepared with cells from 5-8 ml aliquots of cell suspensions taken at different times from samples (originally 50 ml) kept in the CO₂ incubator. Before the cells were put on the slides, they were swelled in hypotonic sucrose and fixed with freshly prepared alcohol-glacial acetic acid (3:1) solution. The cells were stained with Giemsa and Jenner. A minimum of 500 cells was observed per determination of % C-metaphase cells.

Hypoxic conditions were obtained by gently bubbling for 30 min N₂ (95%) and CO₂ (5%) into 50 ml suspensions of cells in complete growth medium contained in rubber stoppered Brockway bottles. The rubber tubing from the exit port was then clamped with a hemostat, and the entry ports were similarly clamped. In this way some positive pressure could be maintained inside the bottle during exposure to radiation. Control cells were similarly gassed but not irradiated. In one experiment with AIV cells and in one with LS cells, cell suspensions were gassed also with air (95%) and CO₂ (5%) simultaneously with hypoxic cells.

X-radiation, from a 250 kV machine operating at 15 ma filament current, half value layer equal to 1.5 mm copper, was delivered at a

dose rate of either 80 rads/min or 50 rads/min. Samples were gassed and irradiated at room temperature.

3. Results and Discussion

Figure 4 depicts the percentage survival (on log scale) of AIV and LS cells determined for several different doses of x-radiation administered under aerated conditions (but the cells were not gassed). Similar differences in dose-survival curves for these cells have been reported previously (Fox and Fox 1973; Fox 1974).

Figure 5 summarizes our results obtained in terms of radiation-induced mitotic delay. The open and closed symbols respectively indicate data obtained under aerated and hypoxic conditions. It is clear that AIV cells are relatively resistant to ionizing radiation also in terms of mitotic delay per unit dose. It has been recognized that delay per unit dose is approximately proportional to the population doubling time for several different types of mammalian cells (Dewey et al. 1965). In other words, if mitotic (or division) delay is normalized with respect to population doubling time, the dose-response curves are essentially indistinguishable for these cells. Also, when the population doubling time of a given cell line is made to increase by growing the cells at a suboptimal temperature, division delay of the total cells in the population per unit dose increases in the same proportion as the doubling time (Rosenberg et al., 1967). Thus, normalized mitotic delay may be a more meaningful measure of radiosensitivity than absolute delays per unit dose. The slope (hrs delay per rad per hrs doubling time) is approximately equal to $1.1 \times 10^{-3} \text{ rad}^{-1}$ for the several different mammalian cell lines reviewed by Dewey et al.

FIGURE 4

FIGURE 5

Using 12 hr and 17 hr as the doubling times respectively for LS and AIV cells, the corresponding slopes calculated from the data obtained under aerated conditions (Fig. 5) are $4.2 \times 10^{-3} \text{ rad}^{-1}$ (LS cells) and $0.34 \times 10^{-3} \text{ rad}^{-1}$ (AIV cells). Thus, LS cells are about 12 ($4.3/0.34$) times more sensitive than AIV cells in terms of radiation induced mitotic delay. It may be noted that LS cells are 4 ($4.2/1.1$) times more sensitive and AIV cells are one third ($.34/1.1$) as sensitive as the cell lines reviewed by Dewey et al. A similar calculation from the data of Ehmann et al., shows that the L5178Y S/S cells are about ten times more sensitive than those lines reviewed by Dewey et al. It may be recalled that L5178Y S/S cells also are "extremely" sensitive in terms of radiation-induced reproductive death. These results obtained with different lines of L5178Y cells apparently are incompatible with the generalization stating that mitotic delay and killing are universally related but, instead, suggest a positive correlation between radiation-sensitivity in terms of delay and sensitivity in terms of killing when the two end points are determined with asynchronous populations of cells.

Regarding radiosensitivity in general, it is theoretically possible for cells to differ because of differences related to the oxygen effect. For example, relatively resistant cells may have higher levels of (or strategically located) superoxide dismutase, or the O_2 tension may be lower in the radiation target volume (nucleus) of resistant cells because they have more, or strategically located, mitochondria. Under these conditions, one might expect that relatively resistant cells would not manifest as great an oxygen effect as would the more sensitive

cells. So far, however, no evidence supporting the above possibility has been reported. On the contrary, the results of a recent study with highly radioresistant rat sarcoma (RT-2) cells showed that there is an oxygen enhancement ratio (OER at 1% survival) equal to about 3 with these cells (Nash et al. 1974). Similarly we found that irradiation of both AIV and LS cells under hypoxic conditions, compared to aerated (air plus CO₂) conditions, resulted in less killing [e.g. 1200 rads, AIV: 0.14% S (air), 15% S (N₂); 120 rads, LS: 17% S (air), 54% S (N₂)] although detailed dose-survival curves have not been determined for hypoxic AIV and LS cells. With regard to mitotic delay, the sensitivities (as measured by the slopes in Fig. 2) of both AIV and LS cells were reduced by a factor of about 2.3 when irradiated under hypoxic conditions. Since a significant oxygen effect is observed, mitotic delay may be a useful end point for investigating the so-called oxygen-mimetic radiosensitizers which are known to enhance killing of cells irradiated under anoxic conditions. Drugs which indeed act through a mechanism common to that of oxygen would be expected to sensitize anoxic cells also in terms of mitotic delay, a phenomenon which occurs shortly after irradiation.

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Dosimetric and Radiobiologic Considerations
of Inhaled Plutonium *

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I. Basic Particle Dosimetry

Whenever a charged particle traverses a medium, it leaves in its wake a number of ions, directly broken molecular bonds, and ultimately free radicals if it is an aqueous medium. In turn, these free radicals also break molecular bonds resulting in the largest portion of biologic effects attributed to such "indirect" effects. The path of the particle and the distribution of the ions and resultant radicals are determined by the charge, mass, and velocity of the original particle. Electrons, being of relatively small mass and unity charge, produce some 6 ion pairs per micron of path (an energy loss of 210 ev/ μ) when traveling with a velocity near that of light (say for a particle energy of 1 Mev and higher). However, as the electron gradually loses energy, both the path becomes very tortuous due to atomic and nuclear deflections and the rate of energy loss increases because of the decreased velocity and increased time spent in the vicinity of atoms along the path. This loss increases to about 66 ion pairs/ μ at an energy of 10 Kv which is near the end of its range. However, by this time, the electron has migrated many hundreds of microns from a colinear projection of its original direction. Thus, for a circular beam of electrons incident on a given medium, one visualizes a cylindrical volume filled with a reasonably uniform density of

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ions and/or free radicals. Consider a $0.01\mu\text{a}/\text{cm}^2$ beam of 5 Mev electrons incident on a water medium for one second such that 0.01μ coulomb of charge is delivered. Thus some 6×10^{10} electrons are delivered depositing a total energy of 5×10^5 ergs. Now the range of these electrons is 2.4 cm. leading to a volume of 2.4 ml. or an irradiated mass of 2.4 grams. Since the ions are distributed uniformly due to scatter, this is an energy deposition of 2×10^5 ergs/gm or 2000 rads. Observe that the average energy loss is 5 Mev/2.4 cm or 5.6 ip/ μ which implies that the end of the range contributes little to the total loss. One last figure of importance is that the total beam produces 9×10^{15} ion pairs which lead to a spacing of about 0.06μ between ion pairs. This in turn may be used to judge biologic effects relative to critical biologic structure. These considerations for electrons also hold for x-rays whose biologic actions are due to electrons produced by photon absorption.

Now, alpha particles being some 7200 times heavier than electrons are much slower for the same initial energy. Thus, such heavy ions at non-relativistic energies lose energy quite rapidly and produce a very dense column of ionization with very little deflection from their original path. The range is also relatively short because of this high rate of loss along the path. In addition, due to statistical variations, there is about a 2% spread in the overall range of individual particles (straggling). A 5.3 Mev alpha particle which

has a range of 41μ in water also has an average loss of 129 Kv or 3500 ion pairs per micron. However, due to slowing down (with little deflection) the ion density increases by about a factor of 2 for 5 Mev particles at the end of their range. This is known as the Bragg effect and is much more predominant for alpha particles than for electrons. Now, the alpha particle track is cylindrical with 90% of the ions within a diameter of 0.01μ . The remaining 10% are due to recoil electrons with sufficient energy to produce their own ionizations (delta rays) and such ions are present out to about 0.2μ . As with the electron beam discussed previously, consider a 5 Mev alpha particle beam of 6×10^{10} particles/cm². Since we have the same number of particles of the same energy we should deposit the same total energy of 5×10^5 ergs. However, because the range is only 40μ , we have a smaller volume which results in a dose of 1.25×10^6 rads. To produce the same 2000 rads as with electrons we should need only 10^8 particles/cm² which is a spacing of 1μ on the average between incident particles. However, the ion pairs are only about 3 Angstroms apart along the path of the particle.

These calculations of the dose in rads assume the ionization to be uniformly distributed throughout the volume as with electrons and x-rays. That this restriction does not apply to alpha particles on the semi-microscopic basis is rather obvious as discussed above. To further illustrate this, we can consider the energy of one alpha particle to be deposited only in the volume of its path. This produces 8×10^{-6} ergs deposited in 4×10^{-15} cm³ or a local dose of 2×10^7 rads.

II. Radiobiologic Effects

The above dosimetric considerations illustrate the inapplicability of the concept of dose in rads to biologic effects where those volumes sensitive to radiation (i.e. nuclei) are small and far apart in the millieu being radiated. Now, it is well known that nuclei of mammalian cells are at least a thousand times more sensitive to radiation than the cytoplasm (Monro, 1970). Further, heavily irradiating the cytoplasm seems to produce a prompt death rather than a loss of reproductive integrity as found with radiating nuclei. Regarding x-rays, a dose of 200 rads to a typical mammalian cell (Chinese hamster fibroblasts) will prevent division (a genetic death) half the time. Since typical masses of cell nuclei are 2×10^{-10} g, this corresponds to a delivery of 4×10^{-6} ergs to the nucleus which in turn is 2.5×10^6 ev or about 71,000 ion pairs. It is important to remember that this energy is distributed reasonably uniformly throughout the nuclear volume and is not all used in destroying critical molecules. Further, the local molecular damage produced by forming one ion pair in the vicinity of a key molecule may be slight enough to allow subsequent repair or rejoining.

On the other hand, when an alpha particle penetrates the nucleus, the damage in the path of the particle is very high and repair quite unlikely. Further, there is no oxygen effect as with x-rays due to the preponderance of ions which scavenge the oxygen. Thus, small doses of energy from alpha particles as averaged over the whole irradiated volume will produce the same effects as larger doses from electrons. A relative biologic effectiveness (RBE) ranging from 2 to 5 has been found for loss of reproductive integrity in mammalian cells by 5.3 Mev alpha particles which implies that 50 rads

due to an alpha particle averaged over the whole medium will do the same damage as 200 rads of x-rays. Since any one 5 Mev alpha particle is depositing about 5×10^5 rads per micron of path, which amounts to about 2×10^4 rads when averaged over a typical nucleus, even one particle will produce "over kill". This has been shown experimentally where only a 2mm penetration of one alpha particle into a nucleus was necessary to kill the cell (Monro, 1970). The above data on "killing" can be explained only on the basis that 50 rads corresponds to 7μ between alpha particles which on a probability basis will hit a 7μ diameter nucleus just a little over half the time. This, of course, is about the size of a nucleus in the above cell.

Thus, it is reasonably obvious that instead of using dose in rads as delivered by alpha particles, we should more properly consider the probability of a cell nucleus being struck by such a particle.

III. Lung Model and Cells at Risk

Consider the lung which is of immediate concern in the "hot particle" problem. Firstly, we shall assume that the pieces of radioactive material are of small enough size (say 1μ) to become trapped in the alveoli and radiate alpha particles in all directions. Since each alpha particle will traverse 40μ of solid tissue, such will obviously travel much further in the less dense lung tissue. In fact, the range varies inversely with the density (ρ). Not so obvious is the fact that even though the total mass traversed along one path remains a constant, the number of cells within the increased volume determined by the range will be greater. The irradiated volume (v) varies inversely

as the cube of the density while the total mass irradiated varies as ρv or $1/\rho^2$. Since the density of the actual cell does not vary, then the number of cells at risk must vary as $1/\rho^2$. Now, the density of lung tissue changes during breathing so a number of different values are reported in the literature (ICRP, 1968). We shall consider the average lung (men and women) to weigh 1000 grams, have a residual volume of 3.0 liters, and inspire about 1 liter under light exercise. This produces a minimum density of 0.2 at reasonable inspiration and a maximum density of 0.25 at expiration. Now, rather than average the density at this point, it will be more correct to average the cells at risk later in the calculations even though the effect is small. Microscopic examination of lung tissue shows that about 1/3 of the tissue traversed by an alpha particle from a source located in an alveolus consists of epithelial cells, which are most suspect of becoming carcinogenic. The rest of the tissue mass consists of blood, plasma and connective tissue. Now, in spite of very irregular and spread-out cells, we are more concerned with the volume of the cells and the projected cross sectional area of the nuclei. We shall assume these to be $1500\mu^3$ and $10\mu^2$ respectively for typical epithelial cells. The average cell size if forced into a cubical shape is 11μ on a side or a diameter of 14μ if spherical. From the previous discussion, we see:

$$\text{Alpha Particle Range (cm)} = R = 4 \times 10^{-3}/\rho$$

$$\text{Lung volume} = V(\text{cm}^3) = 4500 + 500 \sin wt$$

$$\text{Lung Density (gms/cm}^3\text{)} = \rho = 1/(4.5 + 0.5 \sin wt)$$

$$\text{Mass of Tissue within } R = M(\text{gms}) = 4\pi R^3 \rho / 3 = \frac{8 \times 10^{-7}}{3\rho^2}$$

$$\text{No. of epithelial cells within } R = N = M/(3)(1500) \times 10^{-12} = \frac{60}{\bar{\rho}^2}$$

Now, to find the average number of cells at risk due to breathing we must average N .

$$\bar{N} = \frac{W}{2\pi} \int_0^{2\pi/w} N dt$$

$$\bar{N} = \frac{60W}{2\pi} \int_0^{2\pi/w} (4.5 + 0.5 \sin wt)^2 dt$$

evaluating, we find

$$\bar{N} = \frac{(1.01)60}{(\bar{\rho})^2}$$

where $\bar{\rho}$ is the linear average of the excursion due to breathing.

Thus weighting the influence of breathing on the lung density only produces a 1% correction which is quite negligible.

Using the originally assumed value for cell size, we find:

$$\bar{N} = 1240 \text{ cells}$$

$$\bar{R} = 4 \times 10^{-3} / \bar{\rho} = 182\mu$$

and an average volume of lung tissue per cell of $20,000\mu^3$ or one cell in a box 27μ on a side.

IV. Probability Considerations

While the above calculations imply that 6 cells can be in series along a 180μ path, it is obvious that no more than three or four such cells can be penetrated since these would completely absorb the energy of one alpha particle. Further, since the total effective tissue path is still 40μ of which only $1/3$ is cells, it follows that on the average only one cell will actually be in the path of an alpha particle, however, the number hit can

vary between zero and three. Since the nucleus is the critical target and it presents an area of $10\mu^2$ to any incident particle, it would follow that the probability of hitting a nucleus would be $10/(27)^2 = 0.014$ since the nuclei have the same spacing as the cells. Thus, the probability of not hitting a nucleus is $1 - 0.014 = 0.986$. Now, in a 180μ path we have $180/27 = 6.6$ - or, roughly, 7 possible positions of nuclei on the average. Thus, the probability of not hitting any nucleus in the total path is $(0.986)^7 = 0.9$ and, the probability of hitting one or more nuclei with at least one particle in the total path is $1 - 0.9 = 0.1$. Now, 1 pCi of activity will emit 0.37 particles/sec. or 3.2×10^4 particles/day. Combining this with the above, we see that if Q is the activity of any one source in picocuries located in the alveoli, it will then require D days on the average to kill all 1240 epithelial cells within the range of the alpha particles as given by $QD = 1240/(3.2 \times 10^4)(0.1) = 0.4$. This is plotted in the accompanying graph. While a few cell nuclei in the critical volume are hit by more than one particle, delivery times longer than D days for a given Q simply means that most all cells will be hit more than once (overkill region). Times shorter than D days obviously lead to underkill as shown.

Since estimates of cell turnover times in the lung range from 5 days to 80 days, this graph tells us that even with cell turnover, the effects of this type radiation in terms of epithelial cell death will be the same for activities ranging upwards from about 0.005 pCi. There is no apparent reason to suspect any sort of a precipitous change in the biologic effects at or near 0.07 pCi as has been suggested on the basis of dose (in rads).

In fact, this target treatment states that if turnover is neglected, two plutonium particles of a given activity located at different points in the lung will kill twice as many cells as one particle containing all the activity. While this effect has been found to hold approximately for tumor production in rat skin (Passonneau, 1952) extrapolation to lungs is not warranted because of turnover, particle migration, and dependence of initial distribution on particle size. Nevertheless, there is no apparent reason to support the concept of increased risk with particle activity above a certain level ($\sim .005$ pCi).

V. Irradiation of Rat Skin - Epithelial Cells

Albert, et al, (1967) found that irradiating the skin of rats with alpha particles produced tumors of the hair follicles only when the range was 0.35 mm or larger. Neither 0.12 mm penetration by alpha particles nor 0.16 mm penetration by protons produced any detectable tumors. Similarly, 0.2 Mev electrons did not produce tumors but 0.7 Mev electrons did (Glucksman, 1963). Since the papilla which produces the cells for the medulla of the hair, the cortex, the cuticle and the internal root sheath is located at this depth, it is most suspect of being the primary site of the induced tumors. Even though many of these cells were in the telagen phase, they are capable of rapid regeneration. However, some tumors also apparently arose from the sebaceous gland located further up the hair. Another observation was that no higher production of tumors was noted when the Bragg peak ($\times 5$ in dose in rads) was located at or near the papilla. While the authors chose

to interpret this as meaning that the irradiation of the whole follicle was needed to induce a tumor, it would seem more logical to accept this as proof of the single particle "hit" hypothesis which does not depend on dose in rads. Finally and most interesting was the observation that no tumors originated in the heavily irradiated epithelial cells between the hair follicles.

Let us for the moment consider only those epithelial cells in the basal layer of the skin. Data by Withers (1967) indicates a population density in normal mouse skin of 1.4×10^6 cells/cm². This in turn leads to a cell area of about $100\mu^2$ or cell size of about 10μ on a side which is close to that measured for most epithelial cells. Further, the average nuclear size is about $3\mu \times 3\mu$, which produces a cross sectional area of about $10\mu^2$.

Now, the alpha particle density used by Burns, Albert and Heimbach (1968) was $10^8/\text{Cm}^2$ or 1 particle/ μ^2 for a dose of 520 rads. If X is the average number of primary ionizations per target, then e^{-X} is the probability of no primary ionizations occurring in the target. It follows then for $X = 10$ that the number of nuclei not struck by an alpha particle is $N_s = (1.4 \times 10^6)e^{-10} = 60$ nuclei/cm². From Withers data, 1300 rads of x-rays to the mouse skin will also leave 60 survivors per cm² which leads to an RBE of 2.5 for the killing of epithelial cells by alpha particles if one measures the dose in rads. This is reasonably close to RBE values reported for death and other biologic effects produced by alpha particles (Burns, Albert, Heimbach, 1968). In passing,

it is interesting that Withers reported that 10 to 20 surviving cells were capable of preventing ulceration by proliferating to cover a 1 cm^2 area during a 10 day post irradiation period. Most important in the alpha particle experiments by Albert, et al, all doses delivered to the rats killed about 10^6 epithelial cells each. Since 6 cm^2 was exposed per rat and 210 rats were irradiated, than about 10^9 cells were killed without producing one tumor. Thus the chance that one alpha particle passing through one epithelial cell will produce a tumor is less than 10^{-9} assuming the probability of producing a tumor is proportional to the number of cells killed. This is equivalent to assuming tumors result from "near" misses which in turn produce appropriate genetic changes. Since a 5.3 Mev alpha will, on the average in the lung, penetrate only one cell, the probability of its producing a tumor is less than 10^{-9} if the lung epithelial cells are like those in mouse skin. Applying this to the hot particle concept as discussed earlier, it follows that the probability of producing a tumor near such a particle is less than 1.2×10^{-6} . Further, this is not dependent on the activity of the plutonium oxide particle since for the ranges discussed no more than 1200 cells will be killed per oxide particle. Only the number of particles will be important. Also note that if the density or proximity of cells is important in forming a tumor, the above probability limit becomes even smaller since the epithelial cells in the skin are very close packed relative to lung tissue.

VI. Irradiation of Rat Skin - Hair Follicles

Now, let us consider the hair follicles as a model for tumor production in the lungs. Even though there is no structure in the lungs that corresponds to the papilla in the skin, we shall assume only for the sake of argument that the epithelial cells in the alveoli are similar in tumorigenicity to those potentially dividing cells in the papilla. This can be partially rationalized on the basis that the cells in the bulb of the hair follicle are an epidermal derivative (Bullough and Lawrence, 1958). Now, the bulb of an average rat follicle is about 100μ in diameter and 150μ long while the average cell in the papilla is quite similar in size to the epithelial cells described above (Burns, 1975). Although not all the bulb consists of potentially dividing cells, cells with measurable mitotic indices have been found in the root sheath extending 170μ upward from the papilla. Thus we shall assume the bulb to be approximated by a sphere 100μ in diameter filled with close packed potentially dividing cells. Such cells would number about 1000 in a typical bulb.

Now, there are 2500 follicles/cm² or a total of 2.5×10^6 cells/cm² involved in follicles. This means that for an incident alpha particle dose of 10^8 /cm² (520 rads) we should have about 100 surviving cells distributed amongst 2500 follicles. Even if one surviving cell could regenerate a follicle, just like one epithelial cell can regenerate skin (Withers, 1967), this would account for only a 4% follicle survival compared with a scored 85%. Implications, of course, are (1) that many more cells are

involved in a follicle than assumed above; (2) that active cells migrate from unirradiated volumes to reform follicles and/or (3) that wholly killed follicles can still be scored as "normal" due to the fact that most of the cells remain in a resting phase and are not challenged to divide. Rationale (3) is much more appealing. Of more importance here is that the alpha irradiation data indicates that one tumor is induced per 9000 abnormal hair follicles or one tumor per 6×10^4 equally irradiated hair follicles. Further, for any of the doses used in these experiments, virtually all the potentially dividing cells in each follicle bulb are "killed" or rendered incapable of division. Thus, if we assume that 1000 closely packed cells or some fraction thereof must be killed to form a nidus for a tumor, then tumor production should not depend on dose in these experiments. Since it obviously does depend on dose, the assumption of the necessity of a nidus of killed cells to produce a tumor is not valid.

On the other hand, it is much more reasonable to assume that the probability of producing a tumor is simply proportional to the total number of killed cells since this will also be a measure of near misses that may create the appropriate genetic aberrations. In the above example, we see that for 1000 cells per follicle, we should have a probability of creating one tumor per 6×10^7 killed cells. This leads to a probability of $(\frac{1}{6}) \times 10^{-7} = 1.6 \times 10^{-8}$ for a 5 Mev alpha particle in the lung to produce a tumor, assuming, of course, that the epithelial

cells are identical to those in the papilla. Thus, for the plutonium oxide particles that kill at most 1200 cells per particle, we see a tumor induction probability of about 2×10^{-5} per particle regardless of its activity. While this value is higher by a factor of at least 100 than has been observed in either animals or man (Bair, et al, 1974) most important is that the assumption of this model says that uniformly distributed activity has a much greater tumorigenicity than if concentrated in a hot particle. Consider a 0.1 pCi particle. Its chances of inducing a tumor in lungs containing cells like those in rat skin papilla increase to 2×10^{-5} in 4 days after which it remains constant. If that activity should be uniformly distributed in the lungs, then the chance steadily increases with time becoming 2×10^{-4} at 4 days and 5×10^{-3} at 100 days. This illustrates an important difference between the hot particle approach and the uniform distribution. In the former, for a fixed cell population, the chances of tumor production remain constant because all viable cells in the vicinity of each particle can be "killed" only once. On the other hand, for the uniform distribution the chances of tumor production increase with time since cells are being continuously "killed". Cell turnover and possible particle migration will change the picture slightly but considering the previous discussion and the fact that observable rat skin tumors appeared as early as 30 days after irradiation, it is most unlikely that there are cells of this type and sensitivity in the lungs.

One last exercise of interest is to determine the area of a possible single hit target for tumor production in the rat hair follicle. If A represents the target area and D the dose in particles/ unit area then AD is the average number of primary events occurring in the target. As before, the probability that a target will not be hit is e^{-AD} producing for the number of survivors $N_s = N_0 e^{-AD}$. From this we see $A = 1/D_{37}$ where D_{37} is that dose required to reduce N_s to $0.37 N_0$.

Consider now the data shown in Fig. 8 of Heimbach, et al, (1969) where percent of abnormal follicles is plotted against dose (in rads). Since the number of tumors is directly (and linearly) related to the number of abnormal follicles, one minus the percent of abnormal follicles is a measure of the number of survivors (i.e. those that don't get tumors). Plotting $\log N_s$ Vs. dose as shown in the accompanying graph, we see that because the curve has only a slight shoulder showing an almost negligible extrapolation number but still implying possible repair, the straight line portion may still be used to determine A .

From the best straight line fit, we find $D_{37} = 1000$ rads or an incident dose of about 2×10^8 particles/cm². This leads to $A = 0.5 \mu^2$. The meaning of this is that the probability of one cell producing a tumor is finite only when this particular area is hit by one alpha particle. This might be an equivalent area just outside the whole nuclear membrane that will allow the cell to stay viable when hit but yet allow penetration by free radicals and/or delta rays to produce possible non-lethal genetic aberrations.

Since the probability of producing a tumorous cell by one alpha particle is 6×10^{-8} , we see that the number of rat skin tumors produced by dose D is $N_t = (N_0)(6 \times 10^{-8})(1 - e^{-AD})$ where N_0 is the total number of cells involved in all the irradiated follicles.

VII. Conclusions

On the basis of fundamental dosimetric and radiobiologic considerations, it is concluded that the "hot particle" concept and Geesaman Hypothesis are invalid. The specific reasons follow:

(1) It is incorrect to base any considerations of biologic effects of alpha particles on dose in rads particularly when small sensitive sites are involved.

(2) Local ("point") concentrations of radioactivity cannot kill or affect any more than a fixed number of cells after a relatively short interval of time regardless of their total activity. This is due to the finite range of the alpha particles and the fact that a cell can only be killed once. Thus, there is no increased risk for tumor induction with activity of a hot particle above a certain level (~ 0.005 pCi).

(3) The concept of the necessity of a nidus of dead cells to form a tumor does not hold for tumors induced in the rat skin since it predicts that the production of tumors would be independent of dose for the doses used in the experiments.

(4) For a cell population fixed in position, the hot particle concept implies that any resulting tumors must be produced in the first few days after deposition until replacement or migration occurs. Time scales of tumor production in

man and animal do not support this.

(5) There is no evidence - histological or radiobiological - that any structures exist in the lung similar to the cells in the bulb of the rat hair follicle. Thus, the probability of producing tumors in rat skin cannot be extrapolated to the human lung.

(6) Models based on current radiobiological theory and experiments not only satisfactorily explain existing animal data, but imply that calculations based on uniformly distributed radioactivity represent the highest risk for induction of tumors in the human lung.

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00936

RADIATION BIOCHEMISTRY SECTION INDEX

- A. Investigation of DNA Replication In Vitro.....B 1
- B. Studies with Plasmodia.....B 4
- C. Induction, Isolation and Characterization of Strains
Conditionally Defective in DNA Synthesis.....B 15
- D. The Effects of Various Drugs on P. polycephalum
Plasmodia and Amoebae.....B 19

Nucleic Acid Metabolism in *Physarum polycephalum* With Particular
Reference to DNA Replication and Sensitivity to Ionizing Radiation

Physarum polycephalum is particularly suited to studies of DNA replication and the effects of radiation on this and other cell-cycle related processes because of the naturally synchronous nuclear divisions within any plasmodium and the fact that each mitosis is immediately followed by a clearly defined S period. Our studies of DNA replication have involved the use of in vitro systems (necessary for the eventual identification of the separate components required) as well as the whole plasmodia. Ultimately, we hope to isolate mutant strains which are defective in DNA replication under restrictive conditions and to use such mutants in the investigation of DNA replication. P. polycephalum is relatively resistant to ionizing radiation with regard to mitotic delay (measured in plasmodia) and to lethality (measured using the uninucleate amoebal cells). It is hoped that the isolation and subsequent study of radiation-sensitive mutants will reveal information as to the reasons for the radiation resistance of this organism.

A. Investigation of DNA Replication In Vitro

E.N. Brewer and J. Rosenthal

We have reported previously that synthesis of about 2% of the total genome could be carried out in homogenates of P. polycephalum at an initial rate of approximately 15% of that found in intact plasmodia (Brewer and Ting, J. Cell. Physiol. in press, 1975; C00-78-312). More recently we have found that the extent of DNA synthesis in homogenates of S-phase plasmodia is greatly stimulated by the presence of dextran in the homogenizing medium. The initial

rate of synthesis is increased about 1.7 times in the presence of dextran, and the reaction continues for a much longer period of time, so that the overall extent of synthesis after a 2 hr incubation period is about 15% of the genome. Essentially all of the endogenous DNA-synthetic activity found in homogenates made in the presence of dextran is recovered in the nuclear fraction (obtained by centrifugation of homogenates at 4300 x g for 15 min). The activity of dextran-free homogenates is stimulated by addition of dextran, resulting in about 50% of the activity obtained by homogenizing in the presence of dextran. The stimulation obtained by addition of dextran requires both the nuclear and post-nuclear supernatant fractions of the dextran-free homogenate. When the DNA synthesized in vitro was centrifuged in alkaline sucrose density gradients, two species were found. The smaller of these (10 S) was present in homogenates prepared at various times in the S period. Since this species did not accumulate in vivo, (Brewer, J. Mol. Biol. 68, 401, 1972; C00-78-260) (Brewer, Evans, and Evans, J. Mol. Biol., 90, 335, 1974, C00-78-311), it is possible that the 10 S species accumulates in vitro because of some type of deficiency in this system. This possibility will be the subject of future investigation. For further details of these experiments, see Brewer, Biochem. Biophys. Acta, in press, 1975, C00-78-334.

We have suggested previously that the DNA polymerase activity found in extracts of P. polycephalum may be involved in the DNA replication process in vivo (Fed. Proc. 32, 452, 1973, C00-78-297).

This activity, assayed with an exogenous (salmon sperm DNA) template appears to have characteristics similar to the endogenous DNA-synthetic activity of homogenates of Physarum. Thus, the presence of Mg^{++} and EGTA in the homogenizing medium and of ATP in the incubation medium is required in both systems; the pH optimum and inhibition by NaCl and NEM are similar; and treatment of plasmodia with radiation or cycloheximide (Brewer, Fed. Prod. 32, 452, 1973, C00-78-297) results in a similar decrease in activities observed with either endogenous or exogenous templates. Sucrose density gradient sedimentation profiles of the soluble DNA polymerase activities demonstrates the presence of a high-molecular weight (8-10 S) DNA polymerase species, as well as a minor peak representing a lower-molecular weight (4-6 S) polymerase species (Brewer, to be submitted, C00-2486-343).

B. Studies with Plasmodia

1. The Metabolism of Thymidine Triphosphate in Physarum Polycephalum

H. H. Evans and S.R. Littman

The level of incorporation of ^3H -thymidine into DNA is frequently used as an index of the amount of DNA synthesis occurring in various organisms. The amount of ^3H incorporated, however, is dependent upon the specific activity of thymidine triphosphate (TTP), the proximal precursor. This specific activity can be affected by various experimental treatments. On the other hand, the determination of the specific activity of TTP, in addition to the determination of the amount of ^3H incorporated into DNA, allows the calculation of the absolute amount of DNA synthesis occurring under various conditions. We have carried out this type of experiment, using P. polycephalum, and have found that the decrease in ^3H -thymidine incorporation into DNA following treatment of this organism with cycloheximide or γ -irradiation can be partially accounted for by a decrease in the specific activity of TTP following these treatments.

Methods

Plasmodia were timed for mitosis and incubated in the presence of ^3H -thymidine (100 $\mu\text{Ci/ml}$; 6 Ci/mMole; Schwarz/Mann). After the incubation the samples were harvested and scraped into cold 10% TCA. The samples were allowed to stand in an ice bath for 30 min and were then homogenized and centrifuged. The pellets were washed with cold 10% TCA and hydrolyzed in hot perchloric acid. The hydrolysates were assayed for DNA content by the diphenylamine reactions (Biochem. J. 67, 315, 1956) and for ^3H content by liquid scintillation counting. The supernatants from the original centrifugation were extracted 3 times

with ethyl ether, and 50 mmoles of tris buffer (pH 8) were added. The samples were then frozen and lyophilized. Subsequently the frozen samples were dissolved in 1 ml distilled water and their pH was adjusted to 7.8-8.0. The specific activity of TTP in each extract was assayed by an adaptation of Walters et al. (Biochim. Biophys. Acta 319, 336 1973) modification of the method of Solter and Handschumacher (Biochim. Biophys. Acta 174, 585, 1969). Aliquots of each extract were added to a reaction mixture containing excess amounts of (α - ^{32}P) dATP, d(AT) template, and M. luteus DNA polymerase¹ and were incubated for 60 min to allow completion of the polymerization reaction². The incorporation of ^{32}P from the labeled dATP into acid-insoluble material is dependent on and proportional to the TTP content of the tissue extract³. Mixtures containing known amounts of TTP (10-300 nM) in place of the tissue extract served in each experiment for the construction of a standard curve, subsequently used to determine the amount of TTP in each extract. Addition of tissue extracts to mixtures containing known amounts of TTP did not produce significant inhibition. The amount of ^3H in the TTP was determined by the ^3H content of the acid-insoluble material following the in vitro enzymatic incubation, and the specific activity of TTP in cpm/pmole was calculated. The ^3H incorporated into DNA by the plasma was then divided by the specific activity of the TTP to determine the pmoles of TMP-moieties incorporated into DNA.

¹ d(AT) template and DNA polymerase were obtained from Miles Laboratories and (α - ^{32}P)dATP from New England Nuclear.

² No degradation of d(AT) was found to occur in 60 min.

³ UTP, ATP, CTP, and dCTP were found neither to substitute for TTP or dATP or to influence the reaction.

Results

1. Level of TTP and its Specific Activity (following incubation with ^3H -thymidine) at various times during the division cycle.

The pool of TTP was found to increase early in the S period, decrease late in S, and increase again during the G_2 period (Fig. 1). Very similar fluctuations in the TTP pool during the division cycle of P. polycephalum have been reported by Bersier and Braun (Biochim. Biophys. Acta 340, 463, 1974). The specific activity of TTP, following a 15 min incorporation in the presence of ^3H -thymidine (100 $\mu\text{Ci/ml}$) at various time in the division cycle is also shown in Fig. 1 . The specific activity was found to increase markedly in late S, and drop again in the middle of the G_2 period.

2. The effect of cycloheximide on TTP metabolism

Variation in cycloheximide concentration. The pool size of TTP was found to increase markedly upon incubation in cycloheximide, reaching a value of over 4 times the control level at a cycloheximide concentration of 5 $\mu\text{g/ml}$. As the concentration of cycloheximide was increased from 5-50 $\mu\text{g/ml}$ the pool of TTP decreased to and remained at about twice the control level (Fig. 2a). Similar results have been reported by Bersier and Braun (Exp. Cell Research 84, 436, 1974). The specific activity of the TTP (following a 15 min incubation in the presence of ^3H -thymidine) decreased to about 70% of the control level at a cycloheximide concentration of 1 $\mu\text{g/ml}$ but did not decrease further as the concentration was raised to 50 $\mu\text{g/ml}$ (Fig. 2b). No significant effect on DNA synthesis was observed (as measured by the pmoles of TMP-moiety incorporated) at cycloheximide concentrations below 10 $\mu\text{g/ml}$, but be-

Fig. 1

Fig. 1 The Pool Size and Specific Activity of TTP at Various Times in the Cycle

Plasmodia were timed for M_2 and sectors were transferred to medium containing 3H -thymidine (100 $\mu Ci/ml$) at various times thereafter, incubated for 15 min, harvested, and treated as described in the text. The data represent the mean values of all appropriate control values reported in these experiments. The standard deviation of each mean is indicated by the off-line dots and dashes. The efficiency of counting was approximately 35%.

Fig. 2

Fig. 2 The Effect of Various Concentrations of Cycloheximide on
TTP Metabolism

Plasmodia were timed for M_2 and 5 minutes later sections were placed in medium containing 3H -thymidine (100 μ Ci/ml) $\pm$ cycloheximide and the drug was added at the concentrations indicated. The plasmodia were incubated for 15 min and were then harvested into 10% TCA and treated as described in the text. The standard deviation of each mean is indicated by the off-line dots and dashes.

tween 10-50 $\mu\text{g/ml}$, the level was 60-65% of the control (Fig. 2c).

When only ^3H -thymidine incorporation was measured and no correction for the alteration in the specific activity of TTP was made, the apparent effect of cycloheximide on DNA synthesis was greater (Fig. 2c).

The fact that the specific activity of TTP did not vary inversely with the pool size of TTP indicates that cycloheximide has an effect on nucleoside metabolism as well as upon DNA synthesis (see below).

Incubation of Plasmodia in the presence of ^3H -thymidine at various times after cycloheximide addition. The results shown in Table 1 indicate that the amount of TTP increased in the first 30 min after cycloheximide (10 $\mu\text{g/ml}$) addition to a level about twice that of the control. The specific activity of the TTP, however, determined following successive 15 min pulses in the presence of ^3H -thymidine, fell to a level below that expected on the basis of the increase in the size of the TTP pool. The amount of TMP-moiety incorporated into DNA decreased progressively with time in the presence of cycloheximide (60%, 20% and 12% of the control in successive 15 min intervals). The marked reduction in TTP specific activity with time after drug addition again indicates that nucleoside metabolism (as well as DNA synthesis) is affected by the drug, and that the effect changes with time after drug addition.

Incubation of plasmodia in the presence of ^3H -thymidine $\pm$ cycloheximide at various times during the division cycle. The TTP pool was increased at all times in the presence of cycloheximide (10 $\mu\text{g/ml}$) (Table 2). The specific activity of TTP was decreased in the presence of cycloheximide and the effect was greatest during the first hour of the S period. The amount of TMP-moiety incorporated into DNA was reduced

Table 1. TTP Metabolism at Various Times Following Cycloheximide Addition

Time of Incubation with ³ H-thymidine (min after cycloheximide addition)	Pool Size (pmoles TTP/ μ g DNA)		Specific Activity TTP cpm/pmole		DNA Synthesis pmoles TMP incorporated/ μ g DNA	
	Control	Cycloheximide	Control	Cycloheximide	Control	Cycloheximide
0 - 15	9.0 $\pm$ 1.1	16.2 $\pm$ 3.4	593 $\pm$ 223	420 $\pm$ 60	18.0 $\pm$ 8.1	11.2 $\pm$ 2.5
15 - 30	10.3 $\pm$ 1.0	20.9 $\pm$ 0.3	472 $\pm$ 34	89 $\pm$ 4	16.8 $\pm$ 4.2	3.5 $\pm$ 0.4
30 - 45	6.7 $\pm$ 0.9	18.7 $\pm$ 8.1	506 $\pm$ 29	73 $\pm$ 36	19.8 $\pm$ 2.6	2.5 $\pm$ 1.2

Plasmodia were transferred to medium $\pm$ cycloheximide (10 μ g/ml) 5 min after mitosis. Sections were transferred to medium containing ³H-thymidine (100 μ Ci/ml) $\pm$ cycloheximide at 0, 15, or 30 min following the first drug addition and were incubated, harvested and treated as described in the text. The standard deviation is shown following each mean. The efficiency of ³H counting was approximately 35%.

Table 2. Effect of Cycloheximide on TTP Metabolism at Various Times in the Division Cycle

Time of Incubation with ^3H -Thymidine $\pm$ cycloheximide (min post mitosis)	Pool Size		Specific Activity TTP		DNA Synthesis	
	pmoles TTP/ μg DNA		cpm/pmole		pmoles TMP incorporated/ μg DNA	
	Control	Cycloheximide	Control	Cycloheximide	Control	Cycloheximide
5 - 20 (Early S)	7.3 $\pm$ 2.0	14.2 $\pm$ 5.2	686 $\pm$ 204	496 $\pm$ 220	18.8 $\pm$ 7.2	13.7 $\pm$ 4.9
60 - 75 (Mid S)	5.2 $\pm$ 0.7	9.8 $\pm$ 2.3	836 $\pm$ 39	424 $\pm$ 30	23.1 $\pm$ 4.9	22.2 $\pm$ 3.7
120 - 135 (Late S)	4.2 $\pm$ 0.8	6.2 $\pm$ 0.3	1672 $\pm$ 147	1342 $\pm$ 152	11.7 $\pm$ 0.6	8.2 $\pm$ 0.9
300 - 315 (G2)	7.2 $\pm$ 0.6	8.3 $\pm$ 1.0	697 $\pm$ 44	660 $\pm$ 83	0.8 $\pm$ 0.1	1.0 $\pm$ 0.2

Sections of plasmodia were transferred to media containing ^3H -thymidine ($100\mu\text{Ci/ml}$) $\pm$ cycloheximide ($10\mu\text{g/ml}$) and at various times after mitosis (as indicated above). The sections were incubated for 15 min and were then harvested and treated as described in the text. The standard deviation is shown following each mean. The efficiency of ^3H counting was approximately 35%.

by the drug 15 min and 2 hours post-mitosis, but did not appear to be reduced 1 or 5 hours following mitosis. DNA synthesis occurring 5 hours post-mitosis consists primarily of nucleolar and mitochondrial DNA synthesis (Braun and T. Evans, Biochim. Biophys. Acta 182, 511, 1969). These processes are only slightly inhibited in the presence of cycloheximide (Werry and Wanka, Biochim. Biophys. Acta 287, 1972). The lack of inhibition of DNA synthesis by cycloheximide 60-75 min following mitosis may represent experimental error or could be due to the fortuitous encompassing of a period during which DNA synthesis is not dependent upon protein synthesis (Muldoon et al., Biochim. Biophys. Acta 247, 310, 1971). More data is needed in order to decide between these alternatives.

3. The Effect of Irradiation on TTP Metabolism

As shown in Fig. 3, γ -irradiation was found to increase the pool size of TTP and to decrease its specific activity correspondingly. The amount of TMP-moiety incorporated into DNA was reduced slightly by the irradiation to 70-90% of the control level. When only ^3H -thymidine incorporation was measured, and no correction made for the reduction in the specific activity of TTP, the apparent effect of γ -radiation on DNA synthesis was greater. An expansion of the dCTP pool and an increase in the specific activity of dCTP following incubation of irradiated Chinese hamster cells in the presence of ^3H -deoxycytidine has been reported by Walters et al. (Radiat. Res. 60, 173, 1974).

Discussion

These results show that the specific activity of TTP changes following exposure of P. polycephalum to cycloheximide or ionizing radiation.

Fig. 3

Fig. 3. Effect of γ -radiation on TTP Metabolism

Plasmodia were timed for M_2 , and 5 minutes thereafter 1 half of each was exposed to 10 kR ^{60}Co γ -irradiation. Irradiated and control halves were then placed in medium containing ^3H -thymidine (100 $\mu\text{Ci/ml}$). Sectors of each half were harvested 15, 30 and 60 min thereafter and were treated as described in the text. The standard deviation of each mean is indicated by the off-line dots and dashes. The efficiency of ^3H counting was approximately 35%.

Unless this change is taken into consideration, the level of incorporation of ^3H -thymidine into DNA is not a reliable index of DNA synthesis. The results show that both treatments cause an increase in the pool size of TTP, and that cycloheximide appears to exert an additional effect on ^3H -thymidine metabolism. Thus, this drug might alter the conversion of thymidine to TTP or cause a change in the amount of thymidine metabolized by pathways other than that leading to TTP formation. Such alterations could vary with time after cycloheximide addition: for instance in the first 15 min less thymidine degradation might occur in the presence of the drug, whereas at later times the conversion of thymidine to TTP might be inhibited. The increase in the pool size of TTP is also of interest and could arise from decreased utilization or increased production of this compound. These various possibilities will be the subject of further investigation.

2. The Effect of Gamma Radiation on DNA Chain Elongation in Intact Plasmodia of Physarum

E.N. Brewer and J. Rosenthal

Irradiation of plasmodia during the S period results in a slight decrease in the rate of DNA synthesis in vivo (Nygaard, Brewer, Evans, and Wolpaw, in Advances in Radiation Research, in press C00-78-260). To investigate the mechanism of this effect, a single plasmodium was incubated in growth medium containing ^3H -thymidine for 10 min at the beginning of the S period, transferred to medium with unlabeled thymidine ($5 \times 10^{-5}\text{M}$) for five min, and then half of the plasmodium was exposed to 40 kR ^{60}Co γ -radiation. Sectors of control and irradiated plasmodia were harvested either immediately after the irradiation or following a 3-hour incubation in fresh growth medium. Sedimentation profiles after centrifugation in alkaline sucrose gradients demonstrated that while the control progeny strands reached the mature size during the 3-hour incubation, the irradiated strands did not (Table 3). Possible reasons for this effect include: a decreased elongation rate; the presence of gaps in the progeny strands as observed following UV-radiation (Rupp and Howard-Flanders, J. Mol. Biol. 31, 291, 1968); or an inhibition of the initiation of the synthesis of replicating units. These possibilities will be the subject of future investigation.

Table 3

Effect of Gamma Radiation (40 kR) on Elongation of DNA Subunits in Physarum.

<u>Treatment</u>	<u>Recovery (hr)</u>	<u>Sed. Coeffic.*</u>
Unirradiated	0	38.5
"	3	60
Irradiated	0	32
"	3	44

* The sedimentation coefficient was based on a marker DNA (60 S) of mature DNA subunits (G_2 phase) of Physarum.

3. Attempt to Purify mRNA by Isolating Poly A-containing RNA Fractions of *P. polycephalum*

H. H. Evans

Shake cultures of microplasmodia were incubated for 30 min with either ^3H -uridine or ^3H -adenosine. The cultures were then harvested and polysomes isolated according to the method of Brewer (Biochim. Biophys. Acta 277, 39, 1972). RNA was isolated from the polysomes by phenol extraction at pH 7 and pH 9 according to the method of Brawerman et al. (Biochemistry 11, 637, 1972). The poly A content of the two fractions was determined by binding to poly U adsorbed to glass fiber filters according to the method of Sheldon et al. (Proc. Nat. Acad. Sci. U.S. 69, 417, 1972). Of the total ^3H , about 2% was found to bind to the poly U filters. The RNA fractions were then centrifuged in sucrose gradients (3-20% sucrose, 0.1 mM MgCl_2 , 0.01 M Na acetate, SW 50.1 rotor, 3 hours, 4°C). Following centrifugation, the tubes were punctured at the bottom and fractions collected. Aliquots of each fraction were tested for optical density, total ^3H content, and ^3H which bound to poly-U filters. No discrete fraction was present which had a high percentage of poly-U binding material. However, each fraction contained some ^3H which bound to the filters. It is possible, therefore, that *P. polycephalum* has poly A-containing RNA fractions of differing molecular weights or that the RNA is degraded during the isolation procedure. Further investigation of this problem as an approach to messenger purification has been postponed.

C. Induction, Isolation and Characterization of Strains Conditionally Defective in DNA Synthesis

T.E. Evans, H.H. Evans, L.C. Leicht, and J.E. Lewis

Our work with the amoebal stage of the life cycle of Physarum polycephalum has progressed considerably during the past year. Two of us (Helen Evans and Tom Evans) returned to Cleveland in August after a very instructive period at the University of Wisconsin, Madison, and have re-established this haploid cell system in our laboratories.

As proposed previously, we intend to induce, isolate and characterize mutant strains of P. polycephalum which carry temperature-sensitive defects in chromosomal DNA synthesis. Such strains will subsequently be used in the study of the mechanism and control of DNA replication and of the nature and function of proteins involved in these processes. Also, we are developing a screen for isolating radiation-sensitive strains for use in studying the remarkable radiation resistance of this organism. Our progress in these areas of work with the amoebae is indicated below.

Screening for Temperature-Sensitive Mutants of P. polycephalum

We have been screening survivors of chemically mutagenized cultures for temperature sensitivity. At present we have tested over 7,000 unselected colonies both by replica plating and by toothpick transfer procedures. Several "leaky" variants have been identified (Fig. 4).

Throughout this mutant hunt we continue to make adjustments in our procedures. The principal changes to the protocol (described in detail in last year's Annual Report) are the following:

Strains In our previous work, we used strain 4-37, a mating type two (mt_2) heterothallic strain constructed for efficient life cycle

Figure 4 . Effect of temperature shift-up on the growth of strain 4-37 and three temperature-sensitive derivatives.

Cells were grown on *E. coli* and plated at a density of 10^5 per millipore filter plate at time zero. At the times indicated by the vertical arrows, the filters were transferred onto liquid buffer petri plates prewarmed to 31° . Each data point represents the total number of cells per culture plate at the time indicated as determined by direct counting in a hemacytometer. The control (4-37) curve is superimposed onto the other graphs for comparison purposes (dashed line). It can be seen that for all three variants (judged to be temperature-sensitive in macrocolony formation), the phenotype is not expressed until at least two generations after shift-up.

characteristics from the Wis-1 isolate by Dr. David Jacobson of Duke University. Recently, we have begun working with LU-648, an mt_1 strain with a *Colonia* genetic background constructed by Drs. Cooke and Dee (Genet. Res. Camb. 24, 175, 1975) by extensive backcrossing of Wis-1 amoebae with a *Colonia* isolate (LU-688 is the mt_2 counterpart). All strains are now maintained on formalin-killed *E. coli* (strain K-12) which aids in detecting contaminants. Stocks are stored in a -70° Revco freezer after controlled freezing ($3^\circ/\text{min}$) in a liquid nitrogen refrigerator (see COO-2486-345 appended). Normal plating efficiencies have been maintained for over three months with this storage procedure.

Mutagens: We are now using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as well as ethylmethane sulfonate (EMS). Both of these alkylating agents are effective in inducing cycloheximide-resistant (cyc^r) variants in our laboratory, although MNNG is far more toxic to the cells than is EMS.

Screening: Due to considerable variability in the degree of mutagenesis attained (as judged by survival and cyc^r induction data), we store the outgrown cells in a refrigerator until the survival and cyc^r selection plates have been read. Only when an effective mutagenesis is indicated are the outgrown cells harvested and plated for colonies. This procedure also allows us to reject an experiment due to contamination problems before an excessive amount of time is wasted.

Several changes have been made in the replica plating procedure. We have found that the use of glass (rather than plastic) petri dishes for the master plate increases the efficiency of colony transfer. Also, we now use a water bath, rather than an air incubator, for incubating

plates at the restrictive temperature (30°). Thermistor measurements at various places in our air incubators revealed large temperature variations throughout the chamber as well as significant variations with time at individual stations. Neither condition obtains in water baths as long as the plates are held in fully submersed containers; we use coffee cans (in plastic bags) weighed down with lead bricks.

The third replica plate (supplemented with inositol, choline and adenine) has been dropped from the screen since the efficiency of this third transfer is consistently poor. We are working on a mechanical device for improving the replica plating procedure and hope that multiple screening will become practical when we have optimized the method. Currently, therefore, all screens that require replica plating are carried out individually.

Biochemical Screening of Amoebae

We have continued to investigate conditions wherein radioisotopically-labeled precursors can be incorporated by amoebae. Some success has been achieved, as described below. We intend to use this approach for testing temperature-sensitive variants for specific defects in DNA replication and are therefore working to optimize the system for this purpose.

The incorporation of [^3H]-deoxyadenosine, -deoxycytidine, -deoxyuridine, and -thymidine [$\pm$ amphotericin B (0.8 $\mu\text{g/ml}$)] was tested using strain LU-648 grown on formalin-killed E. coli and supported on millipore filters. When logarithmic growth was attained on liver infusion agar, the millipore filters were transferred to petri dishes containing 10 $\mu\text{Ci/ml}$ of the labeled precursor in 15 ml of dilute buffered saline, pH 5. The cells were harvested either 3 or 20 hours after transfer and

were extracted by a modification of the Schmidt-Thannhauser procedure. Small but significant amounts of radioactivity were incorporated into the DNA and RNA fractions in the case of all of the precursors. [^3H]-Thymidine was the most efficient and specific DNA precursor [0.01% (46,000 dpm) of the added radioactivity was incorporated into DNA in 3 hrs and 0.035% (125,000 dpm) in 24 hours, while "incorporation" into the DNA fraction by formalinized bacteria alone amounted to less than 0.001% (2600 dpm) of the added radioactivity]. Amphotericin B did not affect the incorporation. Of the other precursors, [^3H]-deoxyadenosine and [^3H]-deoxyuridine were incorporated into DNA about 40% as efficiently as [^3H]-thymidine, while the incorporation of [^3H]-deoxycytidine into DNA amounted to only about 10% that achieved with [^3H]-thymidine. In the case of [^3H]-thymidine and [^3H]-deoxyuridine six times as much radioactivity was incorporated into DNA as into RNA, while in the case of [^3H]-deoxyadenosine, and [^3H]-deoxycytidine, the incorporation into DNA and RNA was approximately equal.

Response of Amoebae to ^{60}Co Radiation

Earlier work with strain $\text{M}_3\text{C VII (II}_e\text{)}$ indicated that the amoebae of P. polycephalum are highly resistant, in terms of survival, to γ -radiation (see 1973 Annual Report, C00-78-300). Further, these data described a survival curve having a large D_q . We have repeated these experiments using strain LU 648 (and under vastly improved plating conditions). The survival curves from these recent experiments show no detectable shoulder and a high D_0 (112 KR; see Figure 5). Further work in progress includes testing of various strains and plating conditions. Since all strains of P. polycephalum are interfertile (as long as the mating type alleles differ), truly different survival responses could be studied from the molecular genetic point of view within the same species (See Proposed Research Program, 1975-1976).

Figure 5 . The response of LU-648 to ^{60}Co radiation.

Log-phase cells were harvested in phosphate buffer (0.05 M, pH 6.8-7.0), counted and adjusted to a final concentration of $5 \times 10^6/\text{ml}$. Samples of the suspension were taken at the indicated doses, diluted and plated for colony formation.

D. The Effects of Various Drugs on *P. polycephalum* Plasmodia and Amoebae

T.E. Evans, H.H. Evans, R.A. Biehler, L.C. Leicht and S.R. Littman

Very few drugs have been found which are effective in vivo inhibitors of macromolecular synthesis in *P. polycephalum*. Thus for reasons of expanding our experimental potential in this system, we have continued to test drug action by monitoring precursor incorporation and growth of plasmodia. This screening process now includes measuring the growth and viability of the haploid amoebae in response to drugs which seem of potential value as genetic selection and/or enrichment agents.

During the past year, several drugs have been tested and the results are presented below:

Daunorubicin, MPB, and DNP

Daunorubicin ($1-4.5 \times 10^{-5}M$) was found to stimulate the incorporation of 3H -thymidine into DNA by S phase plasmodia. Mercaptopyridyl ethyl benzimidazole ($1 \times 10^{-3}M$) exerted a slight inhibitory effect on the incorporation of 3H -thymidine into DNA and of 3H -leucine into protein while slightly stimulating the incorporation of 3H -uridine into RNA by S phase plasmodia. Dinitrophenol ($2 \times 10^{-4}M$) caused a slight inhibition of the incorporation of 3H -thymidine into DNA without affecting the incorporation of 3H -leucine or 3H -uridine into protein and RNA, respectively, if the incorporation was measured in the first 15 min following drug addition. At later times, or using higher drug concentrations, the incorporation of all three precursors was inhibited. Whether these drugs affect precursor specific activity or affect macromolecular synthesis directly is not known. However, since no marked effects were observed, further experiments on these drugs are

not planned.

Phleomycin

Phleomycin at a concentration of 100 $\mu\text{g/ml}$ was found to inhibit the incorporation of ^3H -thymidine into DNA by microplasmodia to about 50% of the control level within 4 hours after drug addition. No breakdown of DNA was observed up to 26 hours after drug addition (Table 4).

Table 4 . Effect of Phleomycin on ^3H -thymidine Incorporation

Time of Incubation with ^3H -thymidine (min after drug addition)	Specific Activity of DNA (cpm/ μg)			DNA Content $\mu\text{g DNA}/\mu\text{g protein}$	
	Control	Treated	% Control	Control	Treated
0-15	4444	3675	82	0.025	0.026
60-75	5128	3916	76	0.027	0.025
240-255	4783	2575	54	0.028	0.025
450-465	4100	2315	56	0.030	0.024
1560-1575	1637	772	47	0.026	0.022

A 60 ml shake culture of microplasmodia was divided in half and phleomycin was added to one half to a concentration of 100 $\mu\text{g/ml}$. 5 ml aliquots were removed from the control and treated flasks at the times indicated, ^3H -thymidine was added to both to a level of 10 $\mu\text{Ci/ml}$, and the cultures were incubated for 15 min (in 50 ml Erlenmeyer flasks on a reciprocal shaker). The microplasmodia were then harvested by centrifugation, washed with cold acid and hydrolyzed with hot perchloric acid. The hot acid hydrolysate was assayed for ^3H content by scintillation counting and for DNA content by the diphenylamine reaction (Biochem.

(Table 4 legend cont'd)

J. 62, 315, 1956). The insoluble material remaining after hot acid hydrolysis was dissolved in 1N NaOH and assayed for protein content by the method of Lowry, et al. (J. Biol. Chem. 193, 265, 1961).

The effect of phleomycin upon the growth of microplasmodia was also investigated using culture turbidity as the growth parameter. Measurements were taken using a modified Klett-Summerson colorimeter. The use of a magnetic stirring device along with magnetic stirring bars in side-armed nephelometer flasks results in homogeneity. Readings are proportional to microplasmodia concentration. Untreated cultures double every 10 - 13 hrs in this system. In the experiment presented in Fig. 6, control cultures doubled every 10 hrs; a culture with 50 μ g phleomycin/ml doubled every 12 hrs and exhibited a 20 hour delay by mid-log phase; a culture with 100 μ g phleomycin/ml doubled every 34 hrs. Thus, phleomycin appears to slow growth of microplasmodia without affecting the culture yield.

The effects of phleomycin on growth and viability of amoebae are indicated in Fig. 7. The cell number data are in agreement with those reported by Haugli (Ph.D. Thesis, U. of Wisconsin, 1971) and have been further confirmed by us in other experiments. Although continuous treatment with phleomycin kills P. polycephalum amoebae in a concentration-dependent way, the cells recover if the drug is removed (significant survival levels are observed by colony counting). The inhibition of growth is rapid (much less than one generation time).

Phleomycin-Cycloheximide

Since phleomycin acts in a reversible fashion, we have been testing the drug for possible use in indirect mutant enrichment procedures.

Figure 6 . Growth of *P. polycephalum* microplasmodia in the presence of phleomycin-containing medium.

Equal dilutions of log-phase microplasmodia shake cultures were transferred to nephelometer flasks containing growth medium (••) and to flasks containing growth medium plus 50 (◦◦) or 100 (××) µg phleomycin per ml.

Figure 7 . Effects of phleomycin on growth and survival of P. polycephalum amoebae.

At the time indicated by the arrow, exponentially growing cultures were transferred onto dilute neutral medium (••) and onto dilute neutral medium containing 30 µg/ml (××) and 100 µg/ml (○○) phleomycin. The numbers in parentheses represent the per cent viability determined at the last harvest point.

Following a protocol similar to that described by Gorman and Dove (Molec. gen. Genet. 133, 345, 1974), an initial experiment was designed to ascertain the killing effectiveness of phleomycin on growing and non-growing (cycloheximide-inhibited) cells. While the growing cells were killed as predicted from the above results, the cycloheximide inhibition was transient, necessitating a determination of population growth and viability in various concentrations of cycloheximide (see below). We are now repeating the phleomycin-cycloheximide experiment using a cycloheximide concentration (10 μ g/ml) which should effectively block growth (cell division) in a fully reversible fashion.

Cycloheximide

The level of cycloheximide chosen for the phleomycin-cycloheximide experiment (5 μ g/ml; see Gorman and Dove, op. cit.) did not fully block the growth of the culture, but merely caused a delay of about 10-15 hours followed by a recovery to a somewhat lower exponential growth rate. This "slip-through" was confirmed in another experiment testing the effects of various concentrations of cycloheximide (Fig. 8).

Griseofulvin

The action of griseofulvin was investigated since it appeared that sensitivity to this drug might serve to select mutants with altered microtubular functions (Gull and Trinci, Protoplasma 81, 37, 1974). Griseofulvin was found to inhibit the growth of microplasmodia, as measured by culture turbidity (see above).

Stock solutions of griseofulvin were made up in dimethyl sulfoxide (DMSO). (A final concentration of DMSO of up to 1% was found to

Figure 8 . Effects of cycloheximide on growth and survival of P. polycephalum amoebae.

At the time indicated by the arrow, exponentially-growing cultures were transferred onto dilute neutral medium containing various cycloheximide concentrations: . . control; + 4ug/ml; o o 8 ug/ml; x x 16 ug/ml. The numbers in parentheses represent the per cent viability determined at the last harvest point.

have little or no effect on growth rate.) At a griseofulvin concentration of 20 $\mu\text{g/ml}$, the growth rate was normal (10-12 hr doubling time) for 20-30 hours and then decreased. Final culture yield was similar to the controls. A concentration of 50 $\mu\text{g/ml}$ immediately depressed the growth rate and the final yield was sometimes depressed as well. At 100 $\mu\text{g/ml}$, griseofulvin immediately affected the growth rate and consistently lowered the final culture yield. Macropasmodial surface cultures also exhibited a depression of growth rate in the presence of griseofulvin (50 $\mu\text{g/ml}$). However, the growth rate of the haploid myxamoebae was not significantly affected by griseofulvin (50 $\mu\text{g/ml}$). Taken together, these results indicate that griseofulvin would be unsuitable as a mutant selection agent with this organism. Colchicine, colcemid, metronidazole, podophyllotoxin and rotonone (which also affect microtubular function) have also been found to be unsuitable as selective agents.

DNA REPLICATION BY A POSSIBLE CONTINUOUS-DISCONTINUOUS MECHANISM IN
HOMOGENATES OF PHYSARUM POLYCEPHALUM CONTAINING DEXTRAN

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SUMMARY

Nuclear DNA synthesis in homogenates of Physarum is greatly stimulated by the presence of dextran in the homogenizing medium. In this cell-free system, the DNA precursor is incorporated approximately equally into two classes of DNA intermediates. One of these is similar in size to that observed previously in the intact organism, i.e. its sedimentation rate in alkaline sucrose density gradients increases, presumably by chain elongation, as the organism progresses through the S phase. The other class (approx. 10 S) is similar to "Okazaki" fragments. Thus, nuclear DNA synthesis in homogenates of Physarum may occur by a continuous-discontinuous mechanism. Substantial DNA-synthetic activity is obtained by the addition of dextran to dextran-free homogenates. Maximal activity in this system requires the presence of both the nuclear and post-nuclear supernatant fractions. It is possible that a partial separation and recombination of a DNA polymerase and the endogenous template is effected by this procedure.

INTRODUCTION

We have shown previously that the nuclear DNA of Physarum polycephalum (av. mol. wt. 2.3×10^8 d) is comprised of single-strand subunits (4×10^7 d) which may be the fundamental units of DNA replication in this organism^{1,2}. These subunits appear to be replicated in vivo* by continuous elongation of progeny subunits, without the appearance of smaller replication intermediates².

Lynch et al.³ have reported a stimulatory effect of dextran (a polysaccharide containing predominantly α , $1 \rightarrow 6$ glycosidic linkages) on the synthesis of DNA in nuclei isolated from rat liver. These investigators suggested that dextran might act by reducing the leakage from nuclei of components of the replicative system.

In the present communication, we report the stimulatory effect of dextran on DNA synthesis in homogenates and isolated nuclei of Physarum. Synthesis is increased several-fold over that reported previously for homogenates of Physarum prepared in the absence of dextran⁴. DNA synthesis in the present cell-free system appears to be replicative, and may occur by a continuous-discontinuous mechanism. It is suggested that DNA replication in the intact organism occurs by a similar mechanism, but that the small (Okazaki) intermediates formed by the discontinuous mode of synthesis on the $3' \rightarrow 5'$ template strands are joined too rapidly to allow their observation by the methods we have utilized^{1,2}.

METHODS AND MATERIALS

Cultivation of the organism

Macroplasmodia of Physarum polycephalum, strain M₃C, were prepared from stock cultures of microplasmodia as described previously^{5,6}. Within such plasmodia, synchronous nuclear divisions occur at 8-9-hr intervals⁷. DNA

*Throughout this paper, the term in vivo refers to the intact organism.

synthesis begins immediately after telophase⁸, and elongation of progeny subunits continues for at least 4 hr².

Preparation of homogenates and isolated nuclei of *Physarum*

Plasmodia were homogenized at 30 min after MIII (third synchronous metaphase following fusion of microplasmodia) in ice-cold homogenizing medium (12.5 ml per plasmodium) using a Potter-Elvehjem homogenizer equipped with a teflon pestle. Unless otherwise indicated, the homogenizing medium contained 0.028 M magnesium acetate, 0.06 M EGTA (ethyleneglycol-bis-[β -aminoethyl ether]N,N'-tetraacetic acid), and dextran as indicated under RESULTS. The pH was adjusted to 7.6 with NaOH. Homogenates were centrifuged for 5 min at 50 x g, and plasmodial debris removed with a pasteur pipette. Nuclei were obtained by centrifugation of the homogenates at 1500 x g (- dextran) or 4300 x g (+ dextran) for 10 min.

Nuclear DNA synthesis *in vitro* as determined by the incorporation of [³H]dATP into an acid-insoluble product

To 0.5-ml aliquots of homogenates or nuclear preparations (containing approx. 5 μ g DNA) were added 0.05 ml of a solution containing 0.22 M ATP, 1.7 mM dGTP, dCTP, and dTTP, and 0.23 mM dATP (including 8.3 μ M [³H]dATP), pH 7.2.. Following incubation for 2 hr at 35°C, duplicate 0.2-ml aliquots were assayed for incorporation of [³H]dATP into DNA. To each aliquot was added 2.5 ml of 0.25 N perchloric acid, and the resulting precipitates were recovered by centrifugation at 10,000 x g, taken up in 1 ml of 0.4 N NaOH, and re-precipitated with 2.5 ml of 0.5 N perchloric acid. Acid-insoluble material was then collected for liquid scintillation counting on fiber-glass filters (Gelman Type E) as described previously⁴.

Alkaline sucrose density gradient centrifugation of DNA strands synthesized *in vitro*

Homogenates containing dextran (12.5%) were prepared and incubated as

described above. Following incubation at 35°C for 45 min, 1.5 vols of Mohberg medium⁹ (0.25 M sucrose, 0.01 M CaCl₂, 0.1% Triton, 0.1 M Tris, pH 7.2) were added and nuclei centrifuged down at 4300 x g. The nuclear pellet was resuspended in the same volume of Mohberg medium, and 0.5-ml aliquots (or 0.4 ml + 0.1 ml of [¹⁴C]thymidine-prelabeled nuclei isolated by Mohberg's method) were pelleted at 1500 x g, resuspended in 0.2 ml of 0.15 M NaCl-0.015 M Na citrate containing 0.005 M EDTA and 2% Sarkosyl, and layered over 5-20% sucrose gradients in 0.1 N NaOH. Sedimentation profiles of the labeled DNA were determined as described previously^{1,4}.

DNA polymerase assay utilizing an exogenous template

Homogenates of Physarum contain a DNA polymerase activity (assayed using an exogenous template) which appears to be that responsible for nuclear DNA replication (utilizing the endogenous template) in such homogenates^{10,11}. To determine the level of this activity in preparations obtained from Physarum, plasmodia were homogenized in 12.5 ml of homogenizing medium without dextran (in later experiments plasmodial halves were homogenized in this vol of homogenizing medium), and plasmodial debris removed as described above. To 0.5-ml aliquots of such homogenates, or of post-nuclear supernatant fractions prepared from homogenates by centrifugation at 1500 x g for 10 min, was added 0.05 ml of a suspension of salmon sperm DNA in H₂O (10 mg/ml) followed by 0.05 ml of the standard incorporation medium containing [³H]dATP. Incubation was for 120 min, and DNA synthesis utilizing the exogenous template was determined as described above. Under these conditions (no dextran), little synthesis on the endogenous template is observed.

Materials

Dextran (mol. wt. 70,000, radioimmunoassay grade), [³H]dATP (10-12 Ci/mole), ATP (ultrapure) and unlabeled deoxyribonucleoside triphosphates

were purchased from Schwarz/Mann, [^{14}C]thymidine (50 mCi/mmol) from New England Nuclear, and DNA (salmon sperm, type III) and EGTA from Sigma. Sarkosyl NL 97 (sodium laurylsarcosinate) was a generous gift of the CIBA/Geigy Corp.

RESULTS

DNA replication in homogenates of *Physarum*

We have reported recently the synthesis of DNA in homogenates of *Physarum* prepared in the absence of dextran. In this cell-free system, DNA synthesis occurred at an initial rate of about 15% of the in vivo rate, precursor incorporation increased linearly for about 7-8 min, and an overall synthesis of about 2% of the total genome could be demonstrated after a 60-min incubation period⁴. As seen in Fig. 1, DNA synthesis, utilizing the endogenous template, in homogenates of *Physarum* is stimulated considerably by the inclusion of dextran (10-15% w/v) in the homogenizing medium (modified somewhat from that described previously⁴). At the same time, the DNA polymerase activity of such homogenates (assayed using an exogenous template) appears to be inhibited by dextran. Endogenous activity is similarly inhibited at the higher dextran levels.

The results shown in Fig. 1 suggest either that a DNA polymerase(s) itself is inhibited by dextran, or that it becomes less accessible to the exogenous template owing, perhaps, to its increased affinity for the endogenous template (it seems unlikely that dextran acts by inhibiting the breakdown of the reaction product by nuclease activity since this effect might be expected with both templates). To distinguish between these possibilities, a plasmodium was homogenized during the S phase in the dextran-free medium and nuclei were removed from the homogenate by centrifugation. As in the case of the homogenate, the activity of the DNA polymerase(s) present in

the post-nuclear supernatant fraction is inhibited by the addition of dextran to this fraction (Table I).

The time course for DNA synthesis in homogenates of Physarum utilizing the endogenous template is shown in Fig. 2. The rate of synthesis is constant (approx. 25% of the in vivo rate) for about 40-45 min, and synthesis continues at a decreasing rate for at least another 30-60 min.

Thus, a total of about 15% of the total genome undergoes replication in homogenates of Physarum containing dextran, during a 120-min incubation

For maximal activity, period. DNA synthesis in the cell-free system requires the presence of Mg^{++} , EGTA, and all four deoxyribonucleoside triphosphates, in addition to dextran. The extent of synthesis in homogenates prepared from G_2 -phase plasmodia is <5% that of S-phase cultures. Homogenates of cultures pretreated for 30 min in vivo with cycloheximide (10 μ g/ml) exhibit only about 60% the level of synthesis observed in homogenates of untreated controls (Table II).

Apparent dissociation and recombination of the endogenous template and a DNA polymerase(s)

Substantial DNA-synthetic activity (compared to homogenates prepared using medium containing dextran) can be obtained by the addition of dextran to dextran-free homogenates. Approx. half of this activity is associated with the nuclear pellet obtained from such homogenates (following addition of dextran). However, maximal activity in this reconstituted system requires the presence of both the nuclear and the post-nuclear supernatant fractions. By contrast, essentially all of the DNA-synthetic activity found in homogenates of Physarum containing dextran is associated with the nuclear fraction of these homogenates (Table III).

Alkaline sucrose density gradient profiles of DNA labeled in vitro

The sedimentation profiles of DNA synthesized in homogenates obtained at different times in S are shown in Fig. 3. It can be seen that about half of the incorporated radioactivity is associated with increasingly heavier (i.e. faster-sedimenting) single-strand DNA as cultures are harvested later in the S period. However, approx. half of the incorporated radioactivity is found in a peak having a sedimentation coefficient of about 10 S, regardless of the time in S at which the culture is homogenized. In order to determine whether these radioactive peaks represent newly-synthesized DNA, a homogenate was prepared from a plasmodium at 30 min after metaphase and mixed with the standard incubation medium. After a 45-min incubation period, half of the reaction mixture was treated with DNase (250 µg/ml) for an additional 60 min, and the alkaline sucrose density gradient patterns obtained for each. As seen in Fig. 4, both radioactive peaks disappeared from the gradient (i.e. became acid-soluble) upon treatment with DNase. Similarly, incubation of the complete reaction mixture (without DNase) for 0 min results in the absence of acid-insoluble radioactivity in alkaline sucrose density gradients (data not shown), indicating that neither radioactive peak is due to anomalous binding of the labeled precursor to DNA or to some other macromolecular species.

DISCUSSION

There are several lines of evidence to suggest that DNA synthesis observed in homogenates and isolated nuclei of Physarum is of a replicative type. First, homogenates prepared from S-phase plasmodia are >20-fold more active than are those prepared from G₂-phase cultures. Second, homogenates obtained from cultures pre-treated with cycloheximide are less active in

2

DNA synthesis than are those prepared from control cultures (Table II). Studies on the inhibition of DNA replication in vivo suggest that cycloheximide strongly inhibits DNA chain elongation, rather than the initiation of synthesis of DNA subunits, in this organism¹². Third, only those DNA strands which are being elongated in the intact organism are labeled in homogenates as judged by alkaline sucrose gradient sedimentation profiles, i.e. parental (template) strands are not labeled under these conditions (Fig. 3). Finally, synthesis in the cell-free system requires Mg^{++} , ATP, and all four deoxyribonucleoside triphosphates (Table II).

Under the present conditions, DNA synthesis in homogenates prepared from plasmodia at 30 min after metaphase (early S period) occurs at an initial rate of about 25% of the in vivo rate, incorporation increases linearly for about 45 min, and after a 2-hr incubation period, approx. 15% of the total genome has been replicated (Fig. 2). This value is several-fold higher than that reported for other eukaryotic cell-free systems^{3,13}.

DNA replication in homogenates of Physarum is greatly stimulated by the presence of dextran (10-15%) in the homogenizing medium (Fig. 1). Lynch et al.³ suggested that dextran (or Ficoll) inhibits nuclear swelling and leakage from nuclei of components of the replicative system. The present data suggest that the mechanism of dextran stimulation may be a more complex one, since addition of dextran to dextran-free homogenates results in substantial recovery of DNA-synthetic activity. It is possible, therefore, that dextran acts by providing an environment which somehow stabilizes the replication complex. This suggestion is also supported by the fact that cell-free synthesis continues for a much longer period of time in the presence (Fig. 2) than in the absence⁴ of dextran.

In view of the fact that the extent of DNA synthesis is more than

ten-fold higher in the presence than in the absence of dextran, it is tempting to suggest that a substance which performs a similar function may play a role in the DNA replication process in the intact organism. At the same time, it is surprising that DNA polymerase activity, per se, is inhibited by dextran (Fig. 1, Table I).

This observation suggests the possibility that the stimulation of endogenous DNA synthesis might be even greater than that reported herein if a dextran substitute, one which does not inhibit DNA polymerase activity, could be found.

A partial separation of the enzyme (post-nuclear supernatant fraction) from the endogenous template (nuclear pellet) may be effected by homogenization in the absence of dextran, and DNA-synthetic activity can be restored by recombining these two fractions in the presence of dextran (Table III). It is possible, however, that the stimulatory effect of the post-nuclear supernatant fraction on nuclear DNA synthesis is due to a stimulatory factor other than the DNA polymerase(s) present therein (Table I). Clarification of this point awaits further study.

In homogenates of Physarum, [³H]dATP is incorporated to approximately the same extent into two discrete size classes of DNA as evidenced by their sedimentation profiles in alkaline sucrose density gradients (Fig. 3). One of these DNA species appears to sediment at a rate which depends upon the time in the S period at which cultures are homogenized. This DNA peak is also broader at later times in S. In these respects, the heavier DNA species appears to be very similar to DNA progeny strands pulse-labeled in vivo². On the other hand, the lighter (10 S) DNA species sediments at the same rate regardless of the time at which cultures are homogenized. The sedimentation coefficient of this DNA is similar to that of "Okazaki"

fragments¹⁴. Thus, nuclear DNA synthesis in homogenates of Physarum may occur by a continuous-discontinuous mechanism. It is possible that DNA synthesis in the intact organism occurs by the same mechanism, but that the joining of DNA intermediates is too rapid to be detectable by the methods we have employed². If so, the results seen in Fig. 3 imply that one or more enzyme functions responsible for joining of the intermediate DNA fragments (DNA ligase?) are missing or inactive in the present cell-free system. Evidence suggesting a continuous-discontinuous mechanism for DNA synthesis in mammalian cell-free systems also has been obtained recently^{16,17}.

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TABLE I

EFFECT OF DEXTRAN ON DNA POLYMERASE ACTIVITY

Half of a single plasmodium was homogenized in the dextran-free medium (12.5 ml) at MIII + 30 min (S phase). Nuclei were removed from the homogenate by centrifugation at 1500 x g for 10 min. Dextran was added to an aliquot of the post-nuclear supernatant to a concentration of 12.5%. To a second aliquot (control) was added H₂O to compensate for the volume change resulting from the addition of dextran (approx. 10% volume). DNA synthesis utilizing the exogenous template was determined as described in the text. Average deviations of duplicate determinations are indicated.

Cell-free system	Dextran added	Cpm $\pm$ av. dev.
Post-nuclear supernatant fraction	-	8176 $\pm$ 203
"	+	4618 $\pm$ 31

TABLE II

CHARACTERISTICS OF DNA SYNTHESIS IN HOMOGENATES OF PHYSARUM

Plasmodia were homogenized during either the S or G₂ phase of the division cycle. Homogenization and incubation conditions are described under METHODS. All results shown are averages of at least two independent experiments. For cycloheximide pretreatment, one-half of a single plasmodium was transferred to fresh nutrient medium immediately following telophase, and the other half transferred to the same medium containing 10 µg/ml cycloheximide. Homogenates of both halves were prepared 30 min later.

<u>Culture Homogenized</u>	<u>Incubation Medium</u>	<u>Relative Incorporation</u>
S-phase	Complete	100
"	- Mg ⁺⁺	1
"	- EGTA	3
"	- ATP	4
"	- dGTP, dCTP, dTTP	4
" (cycloheximide-pretreated)	Complete	60
G ₂ -phase	Complete	4

TABLE III

DNA-SYNTHETIC ACTIVITY OF VARIOUS CELL-FREE PREPARATIONS IN THE PRESENCE
OF DEXTRAN ADDED BEFORE OR AFTER HOMOGENIZATION

Plasmodia were homogenized at MIII + 30 min in homogenizing medium with or without dextran, and incorporation of [^3H]dATP determined as described under METHODS. In some cases, dextran was added subsequent to homogenization in the absence of dextran. Nuclei were gently resuspended in homogenizing medium or the post-nuclear supernatant fraction, to which dextran had been added to a final concentration of 12.5%. Average deviations for duplicate determinations are indicated.

Experiment	Fraction	Dextran*	Cpm $\pm$ av. dev.
I	Homogenate	+	17,063 $\pm$ 378
	Nuclei	+	16,141 $\pm$ 1,090
	Supernatant	+	400 $\pm$ 16
II	Homogenate	+	11,919 $\pm$ 159
	Homogenate	(+)	6,207 $\pm$ 194
	Nuclei (in homog. med.)	(+)	2,806 $\pm$ 317
	Nuclei (in supernatant fraction)	(+)	5,763 $\pm$ 98
	Supernatant only	(+)	486 $\pm$ 14

* Parentheses indicate that dextran was added to dextran-free preparations.

FIGURE LEGENDS

Fig. 1. Effect of dextran on DNA synthesis in homogenates of Physarum using endogenous and exogenous templates. Sectors (1/6) of a single plasmodium were homogenized at MIII + 30 min (S phase) in 2.1 ml of the homogenizing medium containing various amounts of dextran as indicated, and incorporation of [3 H]dATP determined as described under METHODS. Sectors of a second plasmodium were homogenized and incubated in the same way, except that 0.5 mg of salmon sperm DNA (Sigma Type III) was added to each 0.5 ml of homogenate prior to incubation. Incorporation data were normalized for variations in the relative sizes of plasmodial sectors by determining the pigment content¹⁵ of each. The data shown are averages of duplicate determinations with average deviations represented by vertical bars.

O, - exog. DNA; ●, + exog. DNA.

Fig. 2. Time course for endogenous DNA synthesis in homogenates of Physarum. A single plasmodium was homogenized at MIII + 30 min (S-phase), and aliquots incubated for various times as described under METHODS, except that the magnesium acetate concentration in this experiment was 0.002 M, and the ATP concentration of the added incubation medium was 0.15 M. Average deviations of duplicate assays are indicated.

Fig. 3. Alkaline sucrose density gradient profiles of nuclear DNA

synthesized in homogenates of Physarum. Plasmodia were homogenized at 15 min (a) or 60 min (b) after metaphase III, incubated for 45 min, and alkaline sucrose density gradient sedimentation profiles of the labeled DNA determined as described under METHODS. ¹⁴C-prelabeled (non-replicating) DNA (mol. wt. 4×10^7 d) was included as a molecular-weight marker¹. Frac-

tions were collected into glass tubes, and the labeled DNA was precipitated with 2.5 ml of 0.25 M perchloric acid and collected for scintillation counting by filtration. —, ³H; — — —, ¹⁴C.

Fig. 4. Effect of DNase on the alkaline sucrose density gradient profile of DNA labeled in vitro. A single plasmodium was homogenized at MIII + 30 min and incubated for 45 min under the standard conditions. Half of the labeled homogenate was then treated with DNase as described under METHODS. DNA of each fraction was precipitated with acid and counted as described for Fig. 3. —, control; — — —, DNase-treated.

FIG. 1

FIG. 2

FIG. 4

Anoxic Radiosensitization of Mammalian Cells by Dehydroascorbate

by

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We have determined that the oxidizing agent dehydroascorbate (DHA) sensitizes anoxic Ehrlich ascites tumors (EAT cells) in vivo as well as anoxic V-79 Chinese hamster lung cells in vitro. DHA does not appear to be toxic to EAT cells when the total time of exposure to radiation plus drug is 15 minutes or less. However, in experiments with V79 cells longer times are needed for producing anoxic conditions, there is an indication that select killing of anoxic cells may occur.

DHA causes only small changes in cellular NAD(P)H with V-79 or EAT cells suspended in complete medium or in PBS with added glucose in the presence of oxygen. However, when DHA was injected intraperitoneally into mice bearing large, partially anoxic Ehrlich ascites tumors, the cellular NAD(P)H was found to be oxidized and there was a 30% reduction of the GSH content. The intratumor glucose concentration in eight-day old ascites tumors is known to be low. For this reason DHA may be a more potent sensitizer of EAT cells in vivo than of V79 cells exposed to complete growth medium.

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00937
POSSIBLE ROLE FOR A HIGH-MOLECULAR WEIGHT DNA POLYMERASE
IN THE DNA REPLICATION PROCESS IN PHYSARUM

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SUMMARY

The DNA polymerase activity of homogenates of Physarum exhibits similar requirements for Mg^{++} , EGTA, ATP and pH, and is similarly inhibited by NaCl and NEM, whether assayed using the endogenous (nuclear) template or an exogenous (salmon sperm) template. The soluble DNA polymerases present in such homogenates can be resolved into a major peak (approx. 8-10S) and a minor peak (4-6 S) by sucrose density gradient sedimentation under conditions of ionic strength which support maximal polymerase activity. It is suggested that the 8-10 S polymerase species may be the principal DNA chain elongation enzyme in Physarum.

INTRODUCTION

DNA polymerases have been isolated from a variety of eukaryotic cell types. Historically, DNA polymerase activity has been found principally in two enzyme species exhibiting sedimentation coefficients of approx. 3.3 S (nuclear polymerase) and 6-8 S ("cytoplasmic" polymerase)¹. The possible relationship between these polymerase species and the DNA replication process occurring within the intact cell is uncertain, although the activity of the 6-8 S species increases somewhat during the S period² and reflects, in general, the growth rate of the parent cell population^{3,4}. More recently, evidence has been reported to suggest that the 6-8 S species may be derived from a higher-molecular weight (8-11 S) form of the enzyme under conditions of high ionic strength⁵⁻⁸.

DNA replication in homogenates of Physarum is similar in several respects to the replication process occurring in the intact organism. This endogenous activity, and the DNA polymerase activity of such homogenates determined using an exogenous template, can be assayed essentially under mutually exclusive conditions⁹. In the present communication, we present evidence suggesting that the DNA polymerases present in such homogenates may be involved in the DNA replication process in this eukaryotic organism.

METHODS AND MATERIALS

Cultivation of the organism

Macroplasmodia of Physarum polycephalum, strain M₃C, were prepared from shaken cultures of microplasmodia as described previously^{10,11}. Within such multinucleate plasmodia, synchronous nuclear divisions occur every 8-9 hr¹². DNA synthesis begins immediately after telophase and, although bulk DNA synthesis is complete within 3 hr as measured by incorporation of [³H]thymidine into an acid-insoluble product¹³, DNA chain elongation continues for at least

4 hr¹⁴.

Preparation of homogenates of Physarum

Homogenates of Physarum plasmodia were prepared essentially as described previously⁹. To determine DNA synthesis utilizing the endogenous nuclear template, plasmodia were homogenized, except as indicated otherwise, in an ice-cold medium (12.5 ml per plasmodium) containing 0.028 M magnesium acetate, 0.075 M EGTA (ethylene glycol-bis-[β -aminoethyl ether]N,N'-tetraacetic acid), pH 7.7, and dextran (12.5% w/v). To determine DNA synthesis using an exogenous template, plasmodia were homogenized (25 ml per plasmodium) in the same medium (but without dextran), pH 7.4. In both cases, plasmodial debris was removed with a pasteur pipette following centrifugation at 50 x g for 5 min.

Nuclear DNA synthesis *in vitro*

To 0.5-ml aliquots of homogenates were added 0.05 ml of a solution containing 0.22 M ATP, 1.7 mM dGTP, dCTP and dTTP, and 0.23 mM dATP (including 8.3 μ M [³H]dATP), pH 7.2. For the DNA polymerase assays, 0.05 ml of a suspension of salmon sperm DNA in water (10 mg/ml) was also added to the homogenates. Following incubation for 2 h at 35° C, duplicate 0.2-ml aliquots were assayed for incorporation of [³H]dATP into DNA. To each aliquot was added 2.5 ml of 0.25 N perchloric acid, and the resulting precipitates were recovered by centrifugation at 10,000 x g, taken up in 1 ml of 0.4 N NaOH, and re-precipitated with 2.5 ml of 0.5 N perchloric acid. Acid-insoluble material was then collected for liquid scintillation counting on fiber-glass filters (Gelman Type E).

Determination of sedimentation coefficients of the soluble DNA polymerases of Physarum

Individual plasmodia were homogenized in 4 ml of the dextran-free homogenizing medium at 30 min after M III (early S). Homogenates were centrifuged at 4300xg for 10 min and 0.2 ml of each of the supernatant fractions was layered over ice-cold 5-20% sucrose gradients (4.6 ml) in the dextran-free homogenizing medium. The gradients were centrifuged at 4° C in the SW 39 rotor of the model L2-65B ultracentrifuge for 19 hr at 30,000 rpm, and 6-dp fractions were collected from the bottom of the tubes. To each fraction (about 0.16 ml) was added 0.09 ml of the homogenizing medium followed by 0.025 ml of the DNA suspension and 0.025 ml of the standard incubation medium. Following a 2-hr incubation period, 0.2-ml aliquots were assayed for incorporated [³H]dATP as described above.

Materials

Dextran (mol. wt. 70,000, radioimmunoassay grade), [³H]dATP (10-12 Ci/mmole), ATP (ultrapure), unlabeled deoxyribonucleoside triphosphates, and N-ethyl maleimide (NEM) were purchased from Schwarz/Mann, and DNA (salmon sperm, type III), EGTA, and bovine serum albumin (BSA) from Sigma.

RESULTS

Comparison between the DNA-synthetic activities of homogenates of Physarum utilizing either the endogenous or an exogenous template

The replication of the endogenous template and the copying of an exogenous template in homogenates of Physarum can be studied essentially under mutually exclusive conditions⁹. The requirements of both of these activities for the presence of Mg⁺⁺ in the two homogenizing media are depicted in Fig. 1. It can be seen that the concentration of Mg⁺⁺ which supports maximal DNA synthesis is somewhat higher when the endogenous template is utilized. However, the optimal levels are within 25%. EGTA and

ATP are also required for both synthetic activities, and both exhibit similar pH optima (Figs. 2, 3, and 4, respectively). The effect of two inhibitors of cell-free DNA synthesis was also determined. The results suggest that both NaCl (Fig. 5) and NEM (Fig. 6) cause a similar inhibition of both activities.

Sedimentation profiles of the soluble DNA polymerases of *Physarum*

The soluble DNA polymerases present in the post-nuclear supernatant fraction of homogenates prepared from S-phase cultures of *Physarum* were sedimented through a 5-20% sucrose gradient prepared from the standard dextran-free homogenizing medium. Assays of individual gradient fractions revealed the presence of a major peak of activity representing a high-molecular weight (approx. 8-10S) polymerase, and a minor peak representing a lower-molecular weight (4-6S) polymerase species.

DISCUSSION

Homogenates of *Physarum* containing dextran are able to carry out the synthesis of about 15% of the total genome of this organism. DNA synthesis in this cell-free system is similar in several respects to the replicative chain elongation process occurring in the intact organism at the time of homogenization⁹. In the absence of dextran and in the presence of an exogenous template, homogenates of *Physarum* exhibit substantial DNA polymerase activity. The requirement for Mg^{++} , EGTA, and ATP for maximal synthetic activity utilizing either the endogenous or an exogenous template are similar (i.e. within 25%) in all cases, and the optimal pH for both activities is within 0.3 pH units. The apparent difference in the profiles of the curves shown in Figs. 1-4 might be owing to a variety of factors. For example, (a) the uptake of DNA precursors into intact nuclei may require somewhat different conditions from those necessary for the DNA replication process itself; (b) the presence or absence

of dextran in the medium in which DNA-synthetic activity is determined may have an affect on the requirements for other factors involved in the replication process; thus, the results of Figs. 1-4 are not inconsistent with the hypothesis that the DNA polymerase(s) found in homogenates of Physarum may play a role in the nuclear DNA replication process. Evidence in support of this hypothesis may be found in the close similarities between the inhibition of both types of synthesis by NaCl and NEM (Figs. 5 and 6, respectively). In addition, treatment of the intact organism with cycloheximide¹⁵ and with gamma radiation/ (Brewer, unpublished) results in similar decreases in the level of DNA synthesis found in homogenates of the treated cultures utilizing both templates. From these observations it seems reasonable to conclude that the DNA polymerase(s) present in dextran-free homogenates of Physarum may participate in the replication of the endogenous template in homogenates containing dextran.

The sucrose density gradient sedimentation profiles of the soluble DNA polymerases of Physarum reveal the presence of a major polymerase species of high-molecular weight (approx. 8-10S) and a minor species of lower molecular weight (approx. 4-6 S). The fact that the sucrose density gradients were prepared using the standard dextran-free homogenizing medium, i.e. sedimentation of the polymerases was carried out in a milieu which supports DNA synthesis, suggests that the polymerase profile observed (Fig. 7) may reflect the sedimentation properties of the naturally-occurring enzyme species. Thus, the 8-10S polymerase may be the principal DNA chain elongation enzyme in intact plasmodia of Physarum. It is possible that the 4-6 S polymerase may play a role in the replication process as well. Alternatively, the latter species may be a subunit or a conformational isomer of the 8-10 S polymerase. It is possible also that both peaks represent more than one DNA polymerase activity. This possibility is under investigation.

The present results are in general agreement with the recent findings of others that eukaryotic DNA polymerases of high molecular weight can be isolated under conditions of low ionic strength⁵⁻⁸. On the other hand, the Physarum high-molecular weight polymerases may be stable even to high salt concentrations¹⁶.

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FIGURE LEGENDS

Fig. 1. Effect of Mg^{++} concentration on DNA synthesis in homogenates of Physarum in the presence and absence of an exogenous template.

Sectors (1/6) of a single plasmodium were homogenized each in 2.1 ml of homogenizing media containing dextran and various Mg^{++} concentrations as indicated. Sectors of a second plasmodium were homogenized each in 4.2 ml of dextran-free media. All sectors were homogenized in early S phase, approx. 30-35 min after M III. Salmon sperm DNA was added to the latter set of 6 homogenates prior to incubation. DNA synthesis in the presence or absence of the exogenous template was determined as described in the text. Incorporation data were corrected for variations in the relative sizes of plasmodial sectors by determining the pigment content¹⁷ of each. The horizontal bars indicate average deviation of duplicate assays. ●—●, endogenous template; ○—○, exogenous template.

Fig. 2. Effect of EGTA on DNA synthesis in homogenates of Physarum in the presence and absence of an exogenous template. Experimental conditions are as described for Fig. 1 except that the Mg^{++} concentration was held constant (0.028 M) and the EGTA concentration was varied as indicated. ●—●; endogenous template; ○—○, exogenous template.

Fig. 3 Effect of ATP concentration on DNA synthesis in homogenates of Physarum in the presence and absence of an exogenous template. Single plasmodia were homogenized at M III + 30 min (early S period) in either the dextran-containing or the dextran-free homogenizing media. To 0.5-ml aliquots of each of the homogenates was added 0.05 ml of incubation medium containing various amounts

ATP. The ATP concentrations shown are the final concentrations present in the complete reaction mixture. The horizontal bars indicate average deviation of duplicate assays. ●—●, endogenous template; ○—○, exogenous template.

Fig. 4. Effect of pH of homogenizing media on DNA synthesis in homogenates of Physarum incubated in the presence or absence of an exogenous template. Experimental details were as described for Fig. 1 except that the Mg^{++} (0.028 M) and EGTA (0.075 M) concentrations of the homogenizing media were held constant, while the pH was varied as indicated. ●—●, endogenous template; ○—○, exogenous template.

Fig. 5. Inhibition of DNA synthesis by NaCl in homogenates of Physarum incubated in the presence or absence of an exogenous template. Experimental conditions were similar to those described for Fig. 1, except that the standard homogenizing media contained NaCl as indicated. ●—●, endogenous template; ○—○, exogenous template.

Fig. 6 Inhibition of DNA synthesis by NEM in homogenates of Physarum incubated in the presence or absence of an exogenous template. Experimental conditions were similar to those described for Fig. 1 except that the standard homogenizing media contained NEM as indicated. ●—●, endogenous template; ○—○, exogenous template.

Fig. 7. Sucrose density gradient profiles of the soluble DNA polymerases of Physarum. Experimental details are described in the text. The arrow indicates the position of the BSA marker (4.4 S).

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 7

PROCEDURE FOR LIQUID NITROGEN STORAGE OF *P. polycephalum* AMOEBAE

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Materials: 2.0 ml sterile plastic freezing vials¹
 PBS buffer pH 6.8-7.0
 Dimethyl sulfoxide (DMSO)

Make up 10% v/v solution of DMSO² in PBS, allowing 1 ml per vial to be frozen. Allow several vials for each strain to be stored, and the correct number of vials to fully load the freezer core (our model holds 9 vials per batch).

The amoebae to be stored are stationary phase plated on N+C/10 (pH7) with formalinized bacteria.

Harvest loop of amoebae, suspend in 1 ml DMSO-PBS in vial. Enumerate with hemacytometer---there should be at least 10^5 /ml.

Have freezer neck plug and freezing core at room temperature.³ Allow at least 45 minutes between batches for the plug and core to come to room temperature. Load vials on core.

Freeze at the rate of $-3^\circ\text{C}/\text{min}$. At this rate it takes 30 minutes to cool to -70°C . After 1 hour, transfer to liquid nitrogen on aluminum canes---use hemostat and towels to protect hands and vials in making this transfer. After several hours (or overnight) the vials may be transferred to the Revco freezer (-72°C).

Recovery. Transfer vials rapidly from liquid nitrogen or Revco to 30°C water bath. Plate on appropriate media. We have had viable recovery of 70% after one week's storage, and 60% after three month's storage (single points). Neither figure is significantly different from untreated control viability.

PARAMETERS TESTED

Log phase cells, 25°C incubator; Stationary phase cells, refrigerated; 0.05 M phosphate buffer; PBS buffer, with and without DMSO; 30-minute permeation of DMSO in ice bath prior to freezing; 30-minute precooling in ice bath; slow thaw at room temperature in plastic vial tray; quick thaw in 30°C water bath.

Parameters Giving Best Viability: PBS with DMSO; quick thaw.

Neutral Parameters: precooling; prepermeation; growth phase of cells.

Some Parameters not Tested: cell concentrations; live vs. formalinized *E. coli*; other freezing rates.

¹COSTAR PRO-VIAL catalog number 235-1, Cooke Laboratory Products

²Our DMSO is a new bottle, handled with "sterile" technique. DMSO may be autoclaved full strength at 114°C for 10 min.

³We use a Union Carbide Biological Freezer Type BF-5

THE INHIBITION OF DNA STRAND ELONGATION BY CYCLOHEXIMIDE
IN *PHYSARUM POLYCEPHALUM*

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ABSTRACT. The mechanism of the inhibition of nuclear DNA replication by cycloheximide in *Physarum polycephalum* has been investigated by means of alkaline sucrose density gradient centrifugation techniques. The results suggest that the rapid decrease in the incorporation of [^3H]thymidine into DNA after exposure to cycloheximide is caused by an inhibition of the elongation of progeny strands. No effect was detected on either the initiation of the synthesis of DNA replication units or on the ligation of DNA fragments produced by ionizing radiation. It has been concluded that cycloheximide treatment inhibits the elongation of progeny strands within replication units, presumably by inhibiting the synthesis of short-lived proteins necessary for this process.

INTRODUCTION

In many types of eukaryotic cells protein synthesis appears to be necessary not only for the entry of cells into the S period but also for the continuation of DNA replication (1-7). It has been suggested that protein synthesis may be necessary for the initiation of the synthesis of the many DNA replication units comprising the eukaryotic genome (4, 8-10), whereas evidence for an inhibition of the elongation of growing DNA strands within replication units has also been presented (11-14). We have examined these alternatives using *P. polycephalum*, a myxomycete in which each synchronous mitosis is followed by the S period. Earlier studies have shown that cycloheximide inhibits both the initiation and continuation of DNA replication in this organism (15), and that required pro-

teins appear to be synthesized at defined times during the S period (16).

METHODS

The organism was cultured as described previously (16). For measurement of the extent of DNA synthesis, macroplasmodia were prelabeled with [^{14}C] thymidine, and at various times after mitosis sectors of single macroplasmodia were transferred to medium containing [^3H]thymidine and incubated for 15 min. $\pm$ cycloheximide (10 $\mu\text{g}/\text{ml}$). Samples were harvested and washed with cold 4% TCA in 50% acetone, and then extracted at 85° two times with 0.6N HClO_4 . The $^3\text{H}/^{14}\text{C}$ ratio was used as a measure of the amount of DNA synthesized per unit of DNA present in the sample. For alkaline sucrose density gradient centrifugation, labeled portions of plasmodia were harvested and nuclei were isolated (17) and layered on top of alkaline sucrose gradients and centrifuged according to methods described previously (18).

RESULTS AND DISCUSSION

When sectors of plasmodia were transferred to media containing [^3H]thymidine $\pm$ cycloheximide, incorporation into DNA during the first 15 min. in the presence of the inhibitor was 45-60% of the control. Very little incorporation occurred after 15 min. in the presence of the drug. Treatment of the plasmodia with cycloheximide at any time during the S period was found to inhibit elongation of progeny strands pulse-labeled at the beginning of the S period, as determined by alkaline sucrose density gradient centrifugation (Fig. 1). The initiation of the synthesis of replication units was indicated by the non-superimposability of ^3H and ^{14}C density gradient profiles after successive 15 min incubations in the presence of differentially labeled precursors (19). Cycloheximide was found to have no detectable effect on initiations as determined in this manner (Fig. 2). The results in Fig. 3 show that single-strand breaks caused by γ -irradiation during S can be repaired in the presence of cycloheximide, indicating that the synthesis of protein is not required for ligation of DNA fragments and that ligation per se is not directly affected by cycloheximide. Thus, these results indicate

that in *P. polycephalum* cycloheximide prevents nucleotide addition, i.e. elongation within replicating units; presumably by preventing the synthesis of short-lived proteins necessary for this process.

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Fig. 1. Effect of cycloheximide on the elongation of progeny strands. Sectors of a single plasmodium were incubated with [^3H]thymidine for the first 15 min. of the S period, then rinsed and incubated in non-radioactive medium containing unlabeled thymidine ($1 \times 10^{-4}\text{M}$) for two hours. Cycloheximide was added to the medium at the times indicated by the open circles, and was present until harvest, which was at the end of the 120 min. chase period in all cases. Sectors were harvested, combined with sectors of a second plasmodium prelabeled with [^{14}C]thymidine and harvested during the G_2 period, and nuclei were co-isolated, layered on alkaline sucrose gradients and centrifuged as described previously (18). Molecular weight values of the peak [^3H] fractions were calculated, using the peak fraction of the [^{14}C]-prelabeled non-replicating DNA as a marker (4×10^7 d).

Fig. 2. The effect of cycloheximide on the initiation of the synthesis of replication units. Sectors of plasmodia were placed in medium containing [^{14}C]thymidine at various times after mitosis and incubated for 15 min. The sectors were then rinsed and immediately placed in medium containing [^3H]deoxyadenosine, non-radioactive thymidine $\pm$ cycloheximide, incubated another 15 min. and harvested. Nuclei were isolated, lysed on top of alkaline sucrose gradients and centrifuged. Sedimentation was from right to left. (a). Incubated with [^{14}C]thymidine for 0-15 min. following mitosis and with [^3H]deoxyadenosine in the following 15 min.; (b). Incubated as in (a) but with cycloheximide present during the [^3H]deoxyadenosine labeling period; (c). incubated 30-45 min. following mitosis with [^{14}C]thymidine and with [^3H]deoxyadenosine 45-60 min. following mitosis; (d). Incubated as in (c) but with cycloheximide present during the [^3H]deoxyadenosine labeling period; (e). Incubated with [^{14}C]thymidine 60-75 min. following mitosis and with [^3H]deoxyadenosine 75-90 min. following mitosis; (f). Incubated as in (e) but with cycloheximide present during the [^3H]deoxyadenosine labeling period. Cpm/fraction for the peak tubes were 200-1200 cpm ^3H and 130-400 cpm ^{14}C .

Fig. 3. Effect of cycloheximide on the repair of single-strand breaks (produced by gamma irradiation) during the S period. Plasmodia were prelabeled either with [^3H]thymidine or [^{14}C]thymidine. At the beginning of the S period, sectors of each were treated with 10,000 R [^{60}Co] gamma radiation. Sectors were then either harvested immediately or allowed to incubate for 3 hours in the presence or absence of cycloheximide. Differentially labeled irradiated and non-irradiated nuclei were co-isolated, lysed on top of alkaline sucrose gradients and centrifuged. The vertical line indicates the sedimentation position of the peak fraction of the DNA from unirradiated S-phase plasmodia. (a). Harvested immediately following irradiation; (b). Irradiated and incubated for 3 hours in the absence of cycloheximide; (c). Irradiated and incubated for 3 hours in the presence of cycloheximide. Cpm/fraction in the peak tubes ranged from 700-1700. Sedimentation was from right to left.

Fig 1

Evans, Evans
& Brewer

RADIOACTIVITY PER FRACTION RELATIVE TO PEAK TUBE
(cpm ^{14}C ----; cpm ^3H —)

Fig. 2

FRACTION NUMBER

Fig. 2

Fig. 2

Fig 3

Fig 5

CELLULAR RADIOBIOLOGY SECTION INDEX

Nuclear-cytoplasmic Relations in Radiation Sensitivity.....	C 1
The Relationship of Protein Synthesis to Radiation-Induced Mitotic Delay.....	C 3
Biochemical Effects of Chemical Radiosensitizers.....	C 10

A. Nuclear-cytoplasmic Relations in Radiation Sensitivity

1. The Effects of Radiation on Centrioles

We previously obtained considerable indirect evidence that radiation interferes with either the replication or the separation of centrioles. (Reviewed by Rustad in "Developmental Aspects of the Cell Cycle" Eds., I. Cameron, G. Padilla and A.M. Zimmerman, Academic Press, 1971, COO-78-213). We have also demonstrated that UV-radiation inhibits the amoeba-flagellate transformation in Naegleria (Yuyama, Ph.D. Thesis, CWRU, 1967, COO-78-153) and that both U.V. and γ -radiation inhibit the regeneration of the oral ciliature in Stentor (Burchill, J. Exptl. Zool. 169, 471, 1968, COO-78-186; Burchill and Rustad, J. Protozol. 16, 303, 1969). Similarly, Hodge and Nachtwey demonstrated that the field of oral kinetosomes regresses following X-irradiation of Tetrahymena which had been previously synchronized by heat shocks (Radiat. Res. 52, 603, 1972). The duration of the radiation-induced mitotic delay was equal to the time required for the regression plus redifferentiation of this ciliary field. Therefore, we have been investigating the effects of radiation on the formation of cilia, kinetosomes and centrioles.

a. The Post-Irradiation Regeneration of Cilia in Tetrahymena

(Rustad)

In harmony with the observation that post-irradiation inhibition of protein synthesis blocks mitosis at a time when unirradiated Physarum are no longer sensitive to cycloheximide (Section 2 below), we find that post-irradiation

exposure of Tetrahymena to cycloheximide blocks reciliation at a time when the unirradiated cells are no longer sensitive to the drug. Analogous differences in patterns of sensitivity to caffeine are seen in normally regenerating and irradiated Tetrahymena.

b. Kinetosomal ("Centriolar") Replication in Irradiated Tetrahymena

(Rustad)

We find that the central body kinetosomes of Tetrahymena are visible with phase contrast and Normarski differential interference microscopy after DeTerra's fixation procedure or simple fixation in 5% neutral formalin. However, the more densely-packed anterior and posterior kinetosomes which are located on surfaces curving along the optic axis cannot always be counted. Although the counting errors would probably not exceed 10%, we have gone back to trying various protargol staining methods. If these fail we can still use interference microscopy to obtain a general answer (albeit not a quantitative one) to the question of whether or not radiation affects the multiplication of kinetosomes.

c. Centriole Replication in Mammalian Cells

(Rustad)

Dr. Elton Stubblefield of the M.D. Anderson Hospital recently introduced us to a modification of the technique for visualizing centrioles in Don C. hamster cells which permits a 100% harvesting of cells grown as fibroblasts. Trypsinization and osmotic swelling before exposure to Kane's fixative and resuspension in 0.5% digitonin yields good preparations

of V79 and CHO cells. As yet, variations on this basic technique have not yielded satisfactory preparations of L5178Y mouse cells. If possible we wish to use the latter because Dr. Ursula Ehmann of the University of California Medical School wishes to collaborate on the experiments using her L5178Y parent strain and a radiosensitive mutant which often undergoes multipolar divisions before dying.

B. The Relationship of Protein Synthesis to Radiation-Induced Mitotic Delay

For a number of years we have been examining our hypothesis that radiation interferes with the synthesis of "mitotic proteins" and/or creates the need for the synthesis of "recovery proteins" (e.g., Rustad and Burchill, Radiat. Res. 29, 203, 1966, COO-78-133; Failla and Rustad, Int. J. Radiat. Biol. 17, 385, 1970; COO-78-206; Oleinick, Radiat. Res. 51, 638, 1972; COO-78-249; Rustad and Greenberg, Exptl. Cell Res. 75, 285, 1972; COO-78-237). A variety of experimental approaches may help elucidate these hypotheses.

1. Analysis of Rates of Protein-Synthesis by Pre-Loading Sea Urchin Eggs with Amino Acids (Rustad)

In order to avoid amino acid permeability problems, we began to study protein synthesis by "preloading" unfertilized eggs with ³H-labeled amino acids (See: Epel et al., 1969 in "The Cell Cycle," ed. Padilla et al., Academic Press). The results in previous years were too erratic to allow one to draw any conclusions.

Last summer the sources of variability were eliminated, and we established that prefertilization irradiation of the eggs with 10 kR did not inhibit the normal postfertilization increase in

the rate of protein synthesis. Thus, direct inhibition of bulk protein synthesis cannot be involved in radiation-induced mitotic delay in the sea urchin egg.

The procedure was also employed in a collaborative study with Dr. Seymour Zigman of the University of Rochester on the effects of near UV photoproducts of tryptophan on the metabolism and early embryonic development of the sea urchin.

An abstract entitled "How cell growth is inhibited by near UV light photoproducts of tryptophan" by S. Zigman, T. Yulo, G. Griess, B. Antonellis, and R. Rustad, was published in Biol. Bull. 147, 504, 1974 (COO-78-333).

2. The Relationship of Terminal RNA and Protein Synthesis in the Cell Cycle to Radiation-Induced Inhibition of Mitosis in Physarum

(Oleinick, Rustad and Brewer*)

The entry of Physarum nuclei into metaphase becomes insensitive to cycloheximide (CH) ten minutes earlier than to γ -radiation, i.e., the "CH-marker" occurs earlier in prophase than the " γ -marker". Gamma-irradiation of plasmodia which have passed the CH marker and even the γ -marker leads to further sensitivity to CH. Thus, the data reveal a " γ -CH marker" in the mitotic cycle of this organism.

a. An abstract entitled "Synthesis of Mitotic Proteins During G_2 in Physarum polycephalum" by R.C. Rustad, E.N. Brewer and N.L. Oleinick has been published in J. Cell Biol. 63, 293a (1974) (COO-78-336).

b. An abstract entitled "A Radiation-Induced Cell Cycle Marker for Cycloheximide Sensitivity" by E.N. Brewer, N.L. Oleinick and

* Radiation Biochemistry Section

R.C. Rustad, is in press in Radiat. Res. 62, 536, 1975
(COO-2486-340).

c. A paper entitled "A New Mitotic Cycle Marker" by R.C. Rustad,
N.L. Oleinick and E.N. Brewer has been accepted for publication
in Exptl. Cell Res. (COO-78-337).

d. The previous experiments were performed at a dose of 1060 rads
of γ -radiation. Recently we have found that plasmodia irradiated
after the "CH marker" with doses as low as 4.3 rads of X-radia-
tion can subsequently be blocked in prophase when exposed to
cycloheximide (Fig. 1)

3. Postfertilization Sensitivity of NH_3 -Stimulated Eggs to γ -Radiation
-Induced Mitotic Delay (Rustad)

Since 1969 we have been systematically investigating in sea
urchin eggs the action of prefertilization stimulation of protein
synthesis on the reduction of post-fertilization mitotic delay
induced by γ -radiation (Rustad et al., Biol. Bull. 137, 412-413,
1969; COO-78-205). We were previously able to "cure" the mitotic
delay which usually arises when sperm are γ -irradiated (Rustad and
Greenberg, Exptl. Cell Res. 75, 285-289, 1972; COO-78-237). However,
not until two summers ago did we finally find a reliable stimulating
agent (NH_3) which consistently eliminates the mitotic delay induced
by irradiating sperm (Rustad and Goldman, manuscript submitted for
publication, 1974; COO-78-327).

Normally, the sensitivity to γ -radiation-induced mitotic delay
increases continuously for about 15 minutes after fertilization
(apparently because progressively less time is left for recovery
prior to a radiation-sensitive mitotic event). Thereafter, the

Fig. 1

sensitivity decreases to zero in two distinct steps which we have proposed represent the times at which two separate radiation-sensitive mitotic events occur. (R.C. Rustad, Radiat. Res. 42, 498, 1970, COO-78-204).

If the prestimulation of unfertilized eggs leads to a build-up of enzymes for the repair of radiation damage to the sperm, these eggs might show an attenuated pattern of the normal variations in sensitivity during the mitotic cycle following fertilization with normal sperm. Under favorable conditions cells irradiated early in the mitotic cycle might recover almost completely, whereas those irradiated just before the radiation-sensitive mitotic stages would still exhibit considerable division delay. In contrast, if the stimulation leads to the completion before fertilization of the radiation-sensitive mitotic events, then the fertilized eggs would never be susceptible to the induction of γ -radiation-induced mitotic delay.

Eggs of the sea urchin Arbacia punctulata were exposed to ammoniated sea water and fertilized. Different samples of the fertilized eggs were irradiated at intervals thereafter. In some experiments, there was no time-dependent increase in radiation sensitivity in the ammonia-treated eggs, while the unstimulated eggs exhibited the usual increase. The uniform response could occur if the damage leading to the delay of the first radiation-sensitive mitotic event (but not the second) were reparable during the first 15 minutes after fertilization, and ammonia stimulated the egg to complete that mitotic event before irradiation.

tion. In other experiments, the ammonia-treated, irradiated eggs divided before the control eggs. Thus, all of the radiation-sensitive mitotic events appeared to have been completed within 5 minutes after fertilization and possibly even prior to sperm entry.

An abstract entitled "Reduction of radiation-induced mitotic delay in sea urchin eggs following prefertilization ionic stimulation" by R.C. Rustad, E.I. Cassen and S.D. Gharib was published in Biol. Bull. 147, 497, 1974 (C00-78-332).

4. A Cell-Free Protein Synthesizing System for the Analysis of the Radiation Damage to Sub-Cellular Components of *Tetrahymena*

(Oleinick)

Sub-lethal doses of γ -radiation lead to a transient, dose-dependent decrease in the polysome content of *Tetrahymena pyriformis* (G.S. Kuncio, R.C. Rustad, and N.L. Oleinick, Biochem. Biophys. Res. Commun. 48, 457, 1972, C00-78-277; N.L. Oleinick, R.C. Rustad and G.S. Kuncio, Biochim. Biophys. Acta 336, 215, 1974, C00-78-313). We have initiated a study of protein synthesis in cell-free preparations from *Tetrahymena* in order to analyze the in vivo and in vitro radiosensitivity of the several sub-cellular components involved in ribosomal polypeptide synthesis. Some properties of this system were reported in the Annual Report for 1974 (C00-78-325).

During the course of this investigation, it became apparent that the results of this study might be complicated if the high-speed supernatant fraction which is used as a source of enzymes contained an enzymatic activity capable of transferring amino acids into peptide linkage in a non-ribosome dependent reaction. Such activ-

ities which usually transfer specific amino acids onto the N-terminus of already completed proteins have been found in a variety of other cells and tissues (e.g., E. coli, Kaji, Kaji, and Novelli, J. Biol. Chem. 240, 1185 and 1192, 1965; rat liver, Hird, McLean and Munro, Biochim. Biophys. Acta 87, 219, 1964; rabbit liver, Soffer and Horinishi, J. Mol. Biol. 43, 163, 1969).

Accordingly, we prepared a ribosome-free high-speed supernate from exponentially growing Tetrahymena by centrifugation of sonicated cells at 10,000 x g for 10 min and then at 150,000 x g for 4 hours. The resulting supernatant fraction was passed through a column of Sephadex G-25 equilibrated with standard Tetrahymena buffer (0.01 M tris, pH 7.6; 0.01 M MgAc₂; 0.05 M KCl) to remove free amino acids and other low molecular weight compounds. This material, designated HSS, was assayed for its ability to transfer radioactively-labeled amino acids into hot acid-insoluble form (Table I).

It can be seen in Table I that Tetrahymena contains the amino acid transfer activity. The enzyme is active with arginine and in this respect is similar to the arginine transfer activity from rat liver (Soffer and Horinishi, op. cit.). It shows no activity with methionine or leucine. Therefore, assays of ribosome-dependent incorporation of methionine and leucine will not be complicated by incorporation through non-ribosomal reactions. Other amino acids have not yet been assayed.

The arginine transfer reaction is slightly stimulated by addition of exogenous tRNA, ATP, and bovine serum albumin, but there is no absolute requirement for these materials. Further purification

of the enzymatic activity will be necessary before its requirements can be determined. In addition, since some similar activities from other cells transfer the amino acid into N-terminal positions, the product will be assayed by a modified Edman procedure (Oleinick, Fed. Proc. 34, 521, 1975) for the fraction of incorporated arginine which is N-terminal.

Table 1. Properties of the Arginine Transfer Reaction in Tetrahymena pyriformis

	cpm
Complete	1267
-HSS	183
-ATP	1192
+2xATP	1555
+ATP, PEP, PK	1470
-tRNA	1314
+2xtRNA	1723
-BSA	1320
+2xBSA	1491
+5xBSA	1637

The complete reaction mixture contained the following components in a final volume of 0.2 ml: 10 mM tris buffer, pH 7.6; 10 mM $MgCl_2$; 50 mM KCl; 25 mM ATP; 20 ng tRNA; 0.3 mg BSA; 0.1 ml HSS (56.6 mg protein); and 5 μ l arginine $-^3H$ (10 μ Ci/ml; 10 Ci/mmol). When indicated, the reaction mixture was supplemented with 5.0 mM phosphoenolpyruvate (PEP) and 0.2 mg pyruvate kinase (Sigma). Incubation was for 60 min at 28°, after which 50- μ l samples were placed on 2-cm filter paper discs and processed for hot-acid-insoluble radioactivity by the procedure of Mans and Novelli (Biochem. Biophys. Res. Commun. 2, 540, 1960).

C. Biochemical Effects of Chemical Radiosensitizers

1. Inhibition of Protein Synthesis by Menadione

(Oleinick and Biaglow*)

Menadione (vitamin K₃) acts in most cell types at the level of the mitochondrial respiratory chain by shuttling electrons to oxygen, bypassing the usual electron carriers, thereby increasing oxygen consumption while decreasing oxidative phosphorylation (J.L. Webb, Enzyme and Metabolic Inhibitors, Vol. 3, Academic Press, New York, 1963). It is also one of a class of electron affinic compounds which can sensitize cells to the lethal action of x-irradiation (J.S. Mitchell, in Biochemistry of Quinones, ed. R.A. Morton, Academic Press, New York, 1965, pp. 503-537).

Considerable evidence has accumulated suggesting that protein synthesis is required for the repair of some types of radiation damage (Oleinick and Rustad, Adv. Radiat. Biol., Vol. 6, Academic Press, New York, 1975, in press; COO-78-338). Hence, one mechanism for chemical sensitization of radiation damage might involve interference with protein synthesis-dependent repair systems. The inhibition of protein synthesis has therefore been analyzed in exponentially-growing Tetrahymena and CHO cells.

An abstract "Inhibition of Protein Synthesis by Menadione Without an Alteration of Electron Transport" by N.L. Oleinick and J.E. Biaglow was published in J. Cell Biol. 63, 250a, 1974 (COO-78-335). A preliminary draft of a manuscript by N.L. Oleinick and J.E. Biaglow was previously submitted (COO-78-329).

* Radiation Biochemistry Section

We have expanded this study in Tetrahymena and will soon submit the revised manuscript "Menadione: Inhibition of Protein Synthesis in Tetrahymena Independent of Effects on Respiration" for publication. The study indicates that menadione inhibits protein synthesis by a mechanism other than the loss of ATP and possibly by direct binding to or adduct formation with non-protein sulfhydryls or a ribosomal or protein sulfhydryl. Tetrahymena lacks the enzyme menadione reductase and hence, in this organism, there is no menadione-induced increase in oxygen consumption. The loss in polysomes is rapidly reversible upon removal of menadione and resuspension of the cells in fresh medium.

In CHO cells, menadione also induces a decrease in polysome content (Fig. 2). The kinetics are biphasic. The early rapid phase is very similar to the single phase in Tetrahymena, suggesting that menadione may interact directly with components of the protein synthetic machinery. The second slower phase is not found in Tetrahymena and may result from a loss of ATP. Resuspension of washed cells in fresh medium does not result in recovery of the polysomes within the next 20 min.

2. Inhibition of DNA Synthesis by Menadione

(Biaglow* and Oleinick)

Bosmann and McMinn (Chem. Biol. Interact. 3, 230, 1971)

earlier reported that warfarin and vitamin K₁, quinone analogs of menadione, interfered with DNA and RNA synthesis as well as protein synthesis in HeLa cells. The mechanism of these actions is unknown,

*Radiation Biochemistry Section

Fig. 2

Legend to Fig. 2

Chinese hamster ovary cells were grown in suspension culture at 37° in McCoy's 5A medium. Cells were studied in exponential growth at 4×10^5 /ml. Menadione was dissolved in DMSO and added to the cultures at sufficient dilution so that the concentration of DMSO never exceeded 1% (v/v), which had no effect on protein synthesis. In part a, cells were harvested after 10 min exposure to various concentrations of menadione. In part b, cells were treated with 10^{-4} M menadione for varying lengths of time. After 30 min (arrow), some cells were washed free of the drug and resuspended in fresh medium.

The harvested cells were washed in cold phosphate-buffered saline, lysed, and the post-mitochondrial supernatant fraction centrifuged through 20-50% sucrose density gradients to determine the percentage of ribosomes in polysomes as described previously (N.L. Oleinick, R.C. Rustad and G.S. Kuncio, *Biochim. Biophys. Acta* 336, 215, 1974, C00-78-313).

and in mammalian cells it is difficult to separate direct interactions from losses of synthetic activity secondary to decreases in high energy phosphate. In Tetrahymena, menadione inhibits DNA synthesis as measured by ^3H -thymidine incorporation in the concentration range 10^{-9} - 10^{-4} M (Fig. 3) in which no effects on energy metabolism are discernible. Therefore, in this organism menadione may interact directly with DNA or protein components involved in DNA synthesis.

3. The Involvement of NPSH and Protein-SH in the Repair of Radiation Damage (Biaglow* and Rustad)

Preliminary autoradiographic studies indicate that the radiosensitizers menadione (K_3), diamide, and NEM (N-ethylmaleimide) inhibit unscheduled DNA synthesis (repair synthesis?) in V79 lung cells, Chinese hamster ovary cells (CHO) and in HeLa cells. In contrast, the sensitizers that act only under anaerobic conditions (mimicking oxygen) e.g., nitrofurazone and the nitroimidazole derivatives, flagyl and Ro-04780, do not inhibit unscheduled DNA synthesis. The autoradiographs from the first six of a more extensive series of experiments on V79 cells are currently being exposed.

4. Dose and Dose-Rate Responses of RNA and Protein Synthesis in Tetrahymena pyriformis (Oleinick and Rustad)

a. Ribosomal Functions⁺

Polysome content and rate of amino acid-incorporation have been measured in exponentially-growing cultures of the ciliate

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⁺ Supported in part by NIH Grant CA15378.

Fig. 3

Legend to Fig. 3

Exponentially growing cultures of Tetrahymena pyriformis were concentrated five-fold in conditioned medium. To 1-ml aliquots of the cell suspension were added the indicated concentrations of menadione in 10 μ l of DMSO and ^3H -thymidine (10 $\mu\text{Ci/ml}$). Samples of 50 μ l were taken every 5 min for 30 min. Each sample was applied to a 2 cm square of Whatman 3MM filter paper, which was washed in ice-cold 5% trichloroacetic acid, ethenol, and ether and the precipitated radioactivity determined by liquid scintillation spectrometry. The rates of incorporation were determined by least squares analysis of the sequential samples from each culture and compared to the control culture.

protozoan Tetrahymena pyriformis after doses of 0-120 krad of ^{60}Co gamma-radiation delivered at three different dose rates. The polysome content decreases exponentially with increasing dose at all three dose rates. Curiously enough, the sensitivity to dissociation was lower at the intermediate dose rate than at either the high or low one. Simple target theory analysis of the data indicates a target size for polysome dissociation in the range between a single ribosome and a disome. Amino acid-incorporation is more sensitive to radiation than is polysome content. Data obtained at the highest dose rate suggest that there is a second sensitive target which is considerably smaller than the more sensitive one.

A manuscript entitled "The Sensitivity of Ribosomal Functions in Tetrahymena pyriformis to Varying Doses and Dose-rates of Gamma-radiation" by N.L. Oleinick and R.C. Rustad has been submitted for publication (COO-2486-347).

b. RNA Synthesis**

Radiation-induced disturbances in RNA synthesis were investigated in exponentially growing Tetrahymena. Sublethal doses of γ -radiation lead to a transient, dose-dependent decrease in the rate of total RNA synthesis measured by ^3H -uridine incorporation, without an alteration of ^3H -ur-

** Supported by NIH Grant CA-15378 and included here because of its obvious relationship to the other studies in this area.

idine uptake by the cells. The rate of ^3H -uridine incorporation decreases exponentially with dose. In contrast, the duration of inhibition of RNA synthesis is linearly dependent on dose. Target theory calculations suggest that the sensitive molecule has a molecular weight of approximately 2×10^7 Daltons.

A manuscript entitled "Radiation-Induced Inhibition of RNA Synthesis in Tetrahymena pyriformis" by S.G. Ernst, R.C. Rustad, and N.L. Oleinick has been submitted for publication.

A NEW MITOTIC CYCLE MARKER

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Summary. The entry into metaphase of Physarum nuclei becomes insensitive to cycloheximide ten minutes earlier than to γ -radiation, i.e., the "CH-marker" occurs earlier in prophase than the " γ -marker". Gamma-irradiation of plasmodia which have passed the CH marker and even the γ -marker leads to further sensitivity to CH. Thus, the data reveal a " γ -CH marker" in the mitotic cycle of this organism.

One method of analyzing the sequence of division-related events in the cell cycle is to determine the time at which cells become refractile to inhibitors of mitosis. The latest time prior to division at which the cell remains sensitive to a given agent (e.g., the cell will not divide) may be defined as a cell cycle "marker". This method has been used extensively to show that many types of cells remain sensitive to a G_2 or prophase block by inhibitors of protein synthesis for some time after they become refractile to inhibitors of RNA synthesis [1].

Some previous studies on the relative temporal positions of the markers for inhibitors of protein synthesis and for X- or γ -radiation revealed that they occurred near each other in the mitotic cycle of mammalian cells [2-5], plant cells [6], sea urchin eggs [7], and Tetrahymena [cf. references 8-11]. We have recently reported the relative positions of the γ -radiation and cycloheximide prophase markers in plasmodia of the acellular slime mold Physarum polycephalum [12]. Entry of nuclei into metaphase remains sensitive to γ -radiation for some ten minutes after the cycloheximide marker has been passed. This same sequence of events was established independently in Chinese hamster ovary cells [13]. Chemical inhibitors of protein synthesis can also induce a mitotic inhibition at metaphase [12, 14], while radiation does not [12]. The present study is concerned only with the inhibition of mitosis at prophase.

Previously, we had shown that irradiation of sea urchin eggs extends the period in the mitotic cycle during which the cells remain sensitive to the induction of mitotic delay by puromycin and vice versa [7]. This observation suggested that additional markers might exist for combinations of mitotic inhibitors, such that at a time when the cell was no longer sensitive to mitotic inhibition by either or both of the agents singly, the application of one agent might expose a previously undetectable period of sensitivity to the second agent.

The clear separation of the prophase markers for cycloheximide (approximately 30 min before metaphase) and for γ -radiation (approximately 20 min before metaphase) in Physarum [12] allowed us to test this hypothesis directly. Accordingly, we irradiated plasmodial sectors of Physarum after the cycloheximide marker and either before or after the radiation marker, and then exposed the irradiated sectors to cycloheximide. Since the exact temporal location of the markers varies slightly from one plasmodium to another, the position of each plasmodial sector in the mitotic cycle relative to the cycloheximide and γ -radiation markers was determined by treating other sectors of the same plasmodium with each of the two agents individually. When unirradiated sectors of the same plasmodium are no longer sensitive to cycloheximide, post-irradiation exposure to the drug causes an apparent prophase block or a delay of entry into metaphase in excess of that observed with radiation alone (Fig. 1a). This phenomenon is observed even when the plasmodia are irradiated after the γ -radiation marker (Fig. 1b). A few minutes later, however, the combined treatment no longer leads to an extended prophase delay (Fig. 1c); *i.e.*, the plasmodium has progressed past the " γ -CH marker". Analysis of some twenty plasmodia in this manner revealed that this new mitotic cycle marker occurs five to ten minutes later than the γ -radiation marker (Fig. 2).

The biochemical basis for these markers remains to be established. Since cycloheximide appears to be a specific inhibitor of protein synthesis, the pre-mitotic loss of sensitivity to this drug has usually been interpreted as signifying the completion of all protein synthesis necessary for entry into metaphase. This assumption seems well-founded for two reasons: first, the marker for actinomycin D, a selective inhibitor of RNA synthesis, occurs, as expected, earlier than that for cycloheximide [12,15]; and second, in a variety of cell types, the markers for two chemically different inhibitors of protein synthesis,

cycloheximide and puromycin, are located in the same region of the/cycle [2-7, 9,11-15], making it unlikely that they are both acting on a biochemical process other than protein synthesis.

The radiation dose employed (1060 rads) was sufficiently low that target theory would not predict inactivation of a significant fraction of some protein species, and the release of lysosomal enzymes would not be anticipated [16]. If cycloheximide is indeed acting selectively on protein synthesis, then it appears that irradiation after the synthesis of all proteins necessary for entry into metaphase is complete creates the need for synthesis of new proteins [cf. ref. 7]. These proteins could be enzymes necessary for repair of radiation damage or normal mitotic proteins which have to be resynthesized to replace others whose activity was lost owing to radiation damage to a structure much larger than a single protein molecule, e.g., components of the mitotic apparatus or condensing chromosomes. The observations of Highfield and Dewey (personal communication) that the mitotic index of asynchronously dividing CHO cells decreases sooner after both irradiation and exposure to cycloheximide than after either treatment alone is consistent with our hypothesis.

While it may prove difficult to determine the biochemical basis for the present observations, one thing is clear. We have discovered a new cell cycle marker in Physarum: a time in prophase at which plasmodia that have lost their sensitivity to a delay or block in prophase either by cycloheximide or by γ -radiation will still exhibit prophase sensitivity when irradiated and subsequently exposed to cycloheximide.

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Fig. 1. Abscissa: minutes before or after control metaphase; ordinate: delay of metaphase relative to control (min).

Plasmodia of Physarum polycephalum were cultured on filter paper supports in 100 mm Petri plates at 27° in semi-defined medium [17]. Under these conditions the nuclei divided synchronously at intervals of about 8 hours. At the time indicated by the arrow ($\nearrow$), a plasmodium was cut into several sectors which were treated as follows: (1) A control sector was left in growth medium. (2) One sector was transferred to medium which contained cycloheximide (50 μ g/ml). (3) The remainder of the plasmodium was irradiated for 10 sec. in a U.S. Nuclear Corp. ^{60}Co Irradiator (Model GR-9); total dose = 1060 rads determined with LiF thermoluminescent dosimeters. The irradiated plasmodium was returned to growth medium, and at 3-4 minute intervals thereafter, pieces of it were transferred to cycloheximide-containing medium, where they remained for the duration of the experiment. One irradiated piece was not exposed to cycloheximide. The time of metaphase was determined in samples of each plasmodial sector by observing alcohol-fixed smears by phase contrast microscopy [18]. The time at which metaphase was observed in the control (untreated) piece is referred to as zero time on the abscissa. The delay of metaphase in the sectors which were treated only with cycloheximide ($\blacksquare$) or radiation ($\blacktriangle$) but not both are plotted vs. the time at which the treatment was initiated. For the pieces which were both irradiated and drug-treated ($\odot$), the abscissa gives the time relative to the control metaphase at which sectors were transferred to cycloheximide-containing medium, and the ordinate either the observed delay of metaphase relative to the control or the appearance of a prophase block (metaphase not observed in the course of the experiment, 3.5 hrs. past the control metaphase in panel a,

2.5 hrs. in panel b). a) Irradiation performed while the plasmodium was still sensitive to radiation-induced prophase delay, but after the time of cycloheximide-induced prophase block; b) Irradiation at a time when the plasmodium no longer exhibited significant sensitivity to either cycloheximide or radiation alone; c) Irradiation when the plasmodium was essentially insensitive to cycloheximide and radiation, both singly and in combination.

Fig. 2 Abscissa: approximate time before metaphase (minutes).

Sequence of prophase markers in Physarum polycephalum. Each marker represents the last time at which the entry of nuclei into metaphase may be delayed or blocked by treatment with the agent(s) indicated.

CH = cycloheximide alone; γ = gamma-radiation alone; γ -CH = gamma-radiation followed by cycloheximide.

Fig. 2 Rustad, Oleinick + Brewer

The Sensitivity of Ribosomal Functions in Tetrahymena
pyriformis to Varying Doses and Dose-rates of Gamma-radiation

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ABSTRACT

Polysome content and rate of amino acid-incorporation have been measured in exponentially-growing cultures of the ciliate protozoan Tetrahymena pyriformis after doses of 0-120 krads of ^{60}Co gamma-radiation delivered at three different dose rates. The polysome content decreases exponentially with increasing dose at all three dose rates. Curiously enough, the sensitivity to dissociation was lower at the intermediate dose rate than at either the high or low one. Simple target theory analysis of the data indicates a target size for polysome dissociation in the range between a single ribosome and a disome. Amino acid-incorporation is more sensitive to radiation than is polysome content. Data obtained at the highest dose rate suggest that there is a second sensitive target which is considerably smaller than the more sensitive one.

INTRODUCTION

Gamma-irradiation of exponentially growing Tetrahymena pyriformis leads to a rapid, transient dissociation of polysomes and concomitant increase in monosomes (1). Further studies in this laboratory are compatible with the hypothesis that radiation inhibits both the initiation of protein synthesis and the systematic elongation and termination of peptide chains (2). Following irradiation with sub-lethal doses of ⁶⁰Co gamma-radiation, polysomes reform in a biphasic manner with respect to time (2). Some of this recovery appears to depend upon the synthesis of new RNA (1).

The initial dissociation of polysomes occurs during or immediately after the irradiation and increases with increasing exposure at constant exposure rate (1). While the cells are in the irradiator, amino acid incorporation apparently stops as does the recovery from polysome dissociation previously induced by puromycin (2). The question arose whether the observed effects were strictly a function of the total dose delivered or whether they were simply a reflection of the time the cells were in the irradiator. The present study is concerned with the responses of polysomes to various doses of radiation delivered at three different dose rates. Although polysome dissociation occurs very rapidly upon exposure to radiation, the major response is dose-dependent and not merely time-dependent.

MATERIALS AND METHODS

A U.S. Nuclear Corp. ^{60}Co Irradiator (Model GR-9) was used. In previous experiments, we had relied upon an earlier calibration and calculated incident exposure rates uncorrected for the geometry of the sample vessels (2). For the present series of experiments accurate calibration was accomplished with LiF thermoluminescent dosimeters irradiated under conditions identical to those of the experiments. The unattenuated dose rate averaged 4.5 krad/min. Two cylindrical blocks of lead with cavities to hold receptacles containing the cells were used to attenuate the dose. For irradiation at 4.5 krad/min, the cells were placed in a 125-ml Erlenmeyer flask; for 1.85 krad/min, a 6.0-cm diameter bottle was used in a 1.1-cm thick lead cylinder; for 0.96 krad/min, a 3.2-cm diameter cellulose nitrate tube was used in a 2.6-cm thick lead cylinder.

Tetrahymena pyriformis strain GL-C were grown at 28° in a medium, PPL, which consisted of 1% proteose-peptone (w/v) (Difco), 0.1% liver extract (Nutritional Biochemicals Corp.) and 0.13% $\text{Na}_3\text{PO}_4 \cdot 12 \text{H}_2\text{O}$ as previously described (2). Experiments were performed on exponentially growing cultures at $4-8 \times 10^4$ cells per ml. Aliquots (15-25 ml) from a large culture were transferred to appropriate vessels and irradiated. At all times the temperature of the cells was maintained at 28° either in a water bath or in the lead blocks which had previously been equilibrated at this temperature. Immediately after irradiation, the cultures were harvested by pouring them over frozen crushed medium containing 5 µg/ml cycloheximide (2). The cells were washed, lysed, and polysomes prepared and analyzed on sucrose density gradients as previously described (2). Unirradiated control cultures were treated exactly as were the experimental cultures. The data are presented as "% polysomes", which is the percentage of ribosomes in polysomes determined by integration of appropriate areas under the recorder tracings of 260 nm ultraviolet absorption. In addition, some data are presented "% of control".

The incorporation of L-leucine [$4,5\text{-}^3\text{H}$] (Schwarz/Mann) into acid-insoluble material was determined as previously described (2).

RESULTS

In the first series of experiments, the polysome content of Tetrahymena was determined after irradiation with doses up to 120 krad delivered at 4.5 krad/min (Fig. 1). The dose response curve can be described by two exponential components, the first with a D_{37} of approximately 143 krad and the second with a much higher D_{37} (~ 700 krad). At doses below 50 krad, the response appears to be exponentially dependent on dose.

In the second series of experiments, only doses below 50 krad were studied. The polysome content of Tetrahymena was determined after irradiation with 10, 20, and 40 krad, delivered at dose rates of 0.96, 1.85, and 4.5 krad/min. The combined results of 6 separate experiments, each employing 10-12 gradients, are presented in tabular form (Table I) and graphically (Fig. 2). For each dose and dose-rate, the mean and the standard deviation of all polysome distributions have been calculated. These data have been normalized by setting each control polysome distribution equal to 100%. For simplicity only the means have been plotted in Fig. 2. Also plotted are the average values for means of "% of control" at each dose. The lines have been determined in each case by least squares analysis of the mean values.

The radiation-induced dissociation of polysomes is clearly dose-dependent at all three dose rates. The values at each dose have been compared by Student's t test for unpaired variates, and the P values are given in Table I. The slight variations with dose rate in % polysomes and "% of control" at 0 and 10 krad are not significant. However, most differences of % polysomes with respect to dose rate for doses of 20 and 40 krad are significant to the 99% confidence level, while some values are significantly different only to the 90% confidence level. When the data are normalized to the respective control values, it is clear that the dose

-response curve determined at 1.85 krads/min is significantly different (to at least the 95% confidence level) from the other two, which do not differ significantly from each other. The D_{37} 's for the loss of polysomes were 76, 165 and 92 krads for dose rates of 0.96, 1.85, and 4.5 krads/min, respectively, and 97 krads for the average values shown in Fig. 2.

We previously showed that irradiation leads to a nearly complete block in amino acid incorporation during the exposure period but that the incorporation rate returns to normal within 3-5 min (2). The data presented in Fig. 3 show that this is true at dose rates of 0.96 and 4.5 krads/min. The intermediate dose rate data presented in this figure show a return to normal incorporation rate shortly after the irradiation. However, during the exposure period it is uncertain whether amino acid incorporation was blocked, reduced in rate by a factor of 4 or continued normally for about two minutes and then was blocked. Two other experiments indicated a complete blockage at this dose rate. Hence, the lowest dose rate employed in this study is sufficient to inhibit the systematic elongation of peptides.

DISCUSSION

There have been numerous reports of post-irradiation disturbances in the rate of amino acid incorporation in many types of cells (reviewed in ref. 3). We have previously demonstrated the almost immediate inhibition of amino acid incorporation in Tetrahymena during irradiation at a dose rate of 4.7-5.0 krads/min. Present experiments suggest a cessation of protein synthesis at dose rates as low as 0.96 krads/min (fig. 3). Protein synthesis also appears to be inhibited in synchronized Tetrahymena (4). However, the kinetics are quite different, and it may be impossible to compare that data with data from exponentially growing cells, because the division synchrony was achieved by repetitive heat shocks (4).

Our first investigations of radiation-induced fluctuations of the polysome content of Tetrahymena revealed a decrease followed by an increase to normal and supranormal levels of polysomes (1). These observations may explain discrepancies in the literature as to whether radiation increases, decreases, or has no effect on polysome content of various cell types (reviewed in ref. 3). The decrease and return to normal were also found with an improved polysome isolation procedure but no statistically significant overshoot could be demonstrated when some 90% of the ribosomes of control cells were in polysomes (2). Paul and Zimmerman (5) irradiated heat-shock-synchronized Tetrahymena from 10 to 15 min after the last 30 min heat shock and found no immediate radiation-induced alteration in the polysome profiles. However, the increase in percentage polysomes that normally occurs as the cells recover from heat shock was inhibited by irradiation. Heat shocks dissociate polysomes in many cell types (e.g., 6), including Tetrahymena (Kuncio, Oleinick and Rustad, unpublished).

Polysome integrity appears less radiosensitive at the intermediate dose rate (1.85 krads/min) than at the lower and higher dose rates (Fig. 2 and Table I). A hypothetical model which could explain the data is that at low dose rates radiation dissociates polysomes, at intermediate dose rates damage accumulates sufficiently fast to stimulate a repair system, and at high dose rates radiation saturates or

or inactivates this repair system. Calkins (7) has interpreted survival curves of Tetrahymena as indicating that radiation can trigger a system for the repair of potentially lethal damage. UV-irradiation of E. coli before infection leads to a subsequent increase in the repair of potentially lethal damage in exogenously UV-irradiated phage (8).

The dose response curve in the range of 0 to 120 krad can be described by two noninteracting exponential equations. It might also be possible to derive other empirical equations to describe the data and even attempt to relate some to an equilibrium between rates of dissociation and reassociation of monosomes. Since there is no concrete evidence for reassociation during irradiation the data have been analyzed only by conventional target theory.

Within the limitations of the assay method, at 4.5 krad/min the polysome content appears to be exponentially dependent on dose up to about 50 krad (Fig. 1). Therefore, the data in Fig. 2 have been treated as straight lines, the slopes of which have been determined by least squares analysis. If it is assumed that the primary target is spherical and that a single ionizing event causes the dissociation of one ribosome from a polysome (or prevents the attachment of one ribosome), then target theory can be used to estimate the target diameter and molecular weight from the D_{37} values of the experiments in Table I and Fig. 2. Application of the approximate relationship:

$$MW = \frac{0.72 \times 10^{12}}{D_{37} \text{ (rads)}} \text{ Daltons (ref. 9)} \quad \text{Equation 1}$$

yields an average molecular weight of 7.4×10^6 Daltons (Table II, line 2). Graphical interpolation from Fig. 1 of Hutchinson and Pollard (9) yields an average target diameter of 29 nm (Table II, line 3) and a molecular weight of 8.1×10^6 Daltons (Table II, line 4). Ionizations occurring within 1.5 nm of a target may lead to indirect effects (E.C. Pollard, personal communication). If these events can inactivate the target, its actual diameter would be 3 nm less than the value shown in Table II, line 3. The corrected target diameter would then be 26 nm and the corrected average

molecular weight would be 5.8×10^6 Daltons (Table II, lines 5 and 6, respectively). Since we do not know whether or not the target can be inactivated by indirect effects, we do not know which molecular weight is the more likely.

The target would not appear to be as large as an entire chromosome or even a DNA unit length which is 10^8 to $>10^9$ Daltons in most cells (e.g., 10-12). Conversely, mRNAs, tRNAs, and initiation and elongation factors would appear to be too small. In Tetrahymena the cytoplasmic ribosomes are 18 nm in diameter (13) and have molecular weights of approximately 4×10^6 Daltons (14). The average value which we have calculated suggests that the target may be somewhat larger than a single ribosome. Therefore, the target may be a ribosome plus mRNA or some other associated structure, such as a segment of a membrane.

The second dissociation component of the graph shown in Fig. 1 has a D_{37} on the order of 700 krads. Application of equation 1 would suggest that the second target is smaller, having a molecular weight of approximately 10^6 Daltons. Molecular species involved in protein synthesis which fall in this size category include the small ribosomal subunits and some mRNAs and messenger ribonucleoprotein particles.

The D_{37} of amino acid incorporation in cell-free systems varies from 20-270 krads, depending on the isolation procedure and the cell of origin (15-17). Kućan (15) calculated the molecular weight of the target to be 2.7×10^6 Daltons which is the accepted value of an E. coli monosome. Kempner and Pollard (18) exposed E. coli to γ -radiation and deuterons and estimated from the reduction of the incorporation of amino acids into acid-insoluble material that the target for the inhibition of this incorporation was $3-4 \mu\text{m}$ long yet less than 2 nm in diameter. Pollard (19) subsequently suggested that this target represented mRNA. In contrast, the incorporation of methionine appeared to involve a target of the approximate dimensions of a ribosome (18), leading him to further comment that the latter effect could be related to the initiation of protein synthesis by N-formyl-methionyl-tRNA (19).

From preceding studies (1,2) and the present ones, we can present a general description of the effects of γ -radiation on the protein synthetic machinery of

Tetrahymena. Amino acid incorporation effectively stops within one transit time (the time for a ribosome to traverse the length of an average mRNA, which in Tetrahymena is approximately 2 min). This inhibition appears to involve an effect on the polysomes since the uptake of radioactive amino acids by the cell is not inhibited. The data imply that elongation of the nascent polypeptide chain stops as a consequence either of the cessation of translocation or of an uncoupling of translocation from the synthesis of peptide bonds. The initiation of protein synthesis may also be inhibited. There is a dose-dependent dissociation of some 30 to 40% of the ribosomes from polysomes. Target theory analysis suggests that the sensitive site is approximately the size of, but slightly larger than, a single ribosome. At high doses a second smaller target appears to be significantly inactivated. Within minutes after the irradiation is stopped, ribosomes exhibit reassociation into polysomes with two-component kinetics. The reassociation appears to involve only pre-existing ribosomes (unpublished observations), but does not proceed normally in the presence of an inhibitor of RNA synthesis so some species of newly synthesized RNA may be required.

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TABLE I

The Effect of Dose and Dose-Rate on the Polysome Content of *Tetrahymena pyriformis*

Dose Rate (krads/min)	Total Dose (krads)	Polysome Content							
		% Polysomes ^a	P _{0.96} ^b	P _{1.85}	P _{4.5}	% of Control ^c	P _{0.96}	P _{1.85}	P _{4.5}
0.96	0	82.5 ± 7.5 (4)	—	0.37	0.13	100.0 ± 9.1 (4)	—	—	—
	10	73.9 ± 8.4 (2)	—	0.65	>0.9	89.6 ± 10.2 (2)	—	0.89	0.63
	20	56.9 ± 5.2 (7)	—	<0.01	<0.01	69.0 ± 6.3 (7)	—	<0.01	0.15
	40	49.7 ± 5.3 (5)	—	<0.01	0.06	60.2 ± 6.4 (5)	—	<0.01	0.39
1.85	0	87.1 ± 4.9 (5)	0.37	—	0.41	100.0 ± 5.6 (5)	—	—	—
	10	79.8 ± 7.4 (2)	0.65	—	0.58	91.6 ± 8.5 (2)	0.89	—	0.47
	20	71.4 ± 5.2 (7)	<0.01	—	0.08	82.0 ± 6.0 (7)	<0.01	—	0.03
	40	68.3 ± 2.8 (6)	<0.01	—	<0.01	78.4 ± 3.2 (6)	<0.01	—	<0.01
4.5	0	89.5 ± 3.4 (6)	0.13	0.41	—	100.0 ± 3.8 (6)	—	—	—
	10	74.4 ± 4.1 (2)	>0.9	0.58	—	83.1 ± 4.6 (2)	0.63	0.47	—
	20	66.2 ± 5.0 (9)	<0.01	0.08	—	74.0 ± 5.6 (9)	0.15	0.03	—
	40	56.9 ± 4.6 (6)	0.06	<0.01	—	63.6 ± 5.1 (6)	0.39	<0.01	—

^a The percentage of total ribosomal material in the polysomal ($\geq$ dimer) region of sucrose density gradients. The results are given as the mean $\pm$ standard deviation; the number in parentheses is the number of determinations.

^b For each dose, the % Polysomes or % of Control values at each dose rate were compared to the corresponding parameters at each other dose rate by Student's t-test for unpaired variates, and the P values are recorded.

^c For each dose rate, the % Polysomes in the unirradiated cells has been set equal to 100%.

TABLE II

Calculation of Target Diameter and Molecular Weight

	<u>Dose Rate (krads/min)</u>			
	<u>0.96</u>	<u>1.85</u>	<u>4.5</u>	<u>Average</u>
1. D_{37} (krads)	76	165	92	97
2. $MW \times 10^{-6}$ (Daltons) ^a	9.5	4.4	7.8	7.4
3. Target diameter (nm) ^b	31	24	30	29
4. $MW \times 10^{-6}$ (Daltons) ^c	9.9	4.6	8.9	8.1
5. Target diameter (nm), corr. ^d	28	21	27	26
6. $MW \times 10^{-6}$ (Daltons) ^e	7.3	3.1	6.5	5.8

^a Calculated by the relationship $MW = \frac{0.72 \times 10^{12}}{D_{37} \text{ (rads)}}$ from ref. 9.

^b Interpolated from Fig. 1 of ref. 9.

^c Calculated from target diameter in line 3 by the relationship
 $MW = \text{volume} \times \text{density} (1.05 \text{ g/cm}^3) \times 6.023 \times 10^{23}$

^d Diameter, line 3 minus 3.0 nm. See text.

^e Calculated as in c from diameter in line 5.

FIGURE LEGENDS

- Fig. 1. The response of the polysome content of Tetrahymena to increasing doses of radiation at 4.5 krad/min. Cell cultures were irradiated at 4.5 krad/min, harvested, and processed as described in Materials and Methods and in ref. 2.
- Fig. 2. The effect of increasing dose on the polysome content of Tetrahymena at different dose rates. Exponentially growing cells were exposed to ^{60}Co γ -rays at the indicated doses and dose rates. Immediately after irradiation, the cultures were harvested and processed for determination of polysome content as described in Materials and Methods. For each dose rate, the % polysomes in the unirradiated cells has been set equal to 100%. $\bullet$ --- $\bullet$, 0.96 krad/min; $\blacksquare$ ----- $\blacksquare$, 1.85 krad/min; $\blacktriangle$ --- $\blacktriangle$, 4.5 krad/min; $\circ$ — $\circ$, Average of means of determinations at the three dose rates.
- Fig. 3. The rate of amino acid incorporation into irradiated Tetrahymena. Exponentially growing cultures (25 ml) were given ^3H -leucine (5 $\mu\text{Ci/ml}$) at time zero. Five min later, part of the culture (10 ml.) was irradiated at the indicated dose rate. Total irradiation time in each case was 9 min 25 sec. The number in the bar indicates the total dose delivered. Throughout the course of the experiment, 1 ml. aliquots of control (0) and irradiated ($\bullet$) cultures were transferred to tubes containing 0.1 ml 1N NaOH at room temperature to lyse the cells and hydrolyze amino acyl-tRNA. After at least 15 min, the samples were neutralized with 0.1 ml 1N HCl, 0.1 ml bovine serum albumin (1 mg/ml) was added as carrier, and protein was precipitated with 1.2 ml 10% trichloroacetic acid containing a 10,000-fold excess of unlabeled leucine. The acid-insoluble material was washed, filtered, and counted for radioactivity as previously described (2).

^3H -Leucine Incorporated (dpm/ml) $\times 10^{-3}$

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- COO-78-281 Prenatal Evaluation in a Case of Familial Y Chromosome Long Arm Deletion (Yq-)
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- COO-78-298 The Erythromycins
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Nancy L. Oleinick and Ronald C. Rustad
Submitted for publication.

OTHER STAFF ACTIVITIES

W.F. Schmidt, G. Bakale and W. Tauchert, "Excess Electrons in Liquid Methane Solutions II". Proceedings of the 42nd Annual Conference on Electrical Insulation and Dielectric Phenomena, National Academy of Sciences, Washington, D.C. (1974).

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H.M. Rosenberg and E.C. Gregg, "Mitotic Delay and Killing: Independent Phenomena?" to be presented at the Radiation Research Society in Miami, Florida (May 1975).

M.G. Ort, E.C. Gregg, P.S. Rao and R. Kwienzien, "Detecting Small Contrast Changes Using Radioopaque Dyes and Image Subtraction Techniques" to be presented at the American Association of Physicists in Medicine in San Antonio, Texas (August 1975).

M.G. Ort and E.C. Gregg, "Characteristics and Limitations of Photographic Films Used in Subtraction Radiography" to be presented at the Society of Photooptical Instrumentation Engineers in Atlanta, Georgia (September 1975).

J.E. Biaglow, O.F. Nygaard and C.L. Greenstock, "Electron Transfer in Ehrlich Ascites Tumor Cells in the Presence of Nitrofurans". Accepted for publication Biochem. Pharmacol.

MEETINGS ATTENDED, PAPERS PRESENTED

1. American Society of Microbiologists, Chicago, Illinois, May, 1974 - H.H. Evans*, T.E. Evans* and A.H. Evans* (Radiation Biochemistry).
2. American Society of Biological Chemists/Biophysical Society, Minneapolis, Minnesota, June, 1974 - H.H. Evans* and T.E. Evans* (Radiation Biochemistry).
3. Fifth International Congress for Radiation Research, Seattle, Washington, July, 1974 - E.C. Gregg, W.H. Voelker, H.M. Rosenberg (Radiation Physics), H.H. Evans*, T.E. Evans*, O.F. Nygaard* (Radiation Biochemistry), R.C. Rustad, N.L. Oleinick* (Cellular Radiobiology).

Papers presented: "Effect of Biologically Important Solutes on Charge-Carrier Decay Processes in n-Hexane" by E.C. Gregg, R.D. McCreary and G. Bakale (COO-78-317).

"An AC Conductivity Cell for Pulse Radiolysis of Liquids" by J. Park, W.H. Voelker, E.C. Gregg (COO-78-320).

"Temporal Order of DNA Replication Following γ -Radiation of Physarum polycephalum" by T.E. Evans and H.H. Evans (COO-78-322).

"Redox Reactions of Anoxic Radiosensitizers" by J.E. Biaglow, O.F. Nygaard and C.L. Greenstock (COO-78-316).

"Metabolic Limitations in the Use of Radiosensitizers" by O.F. Nygaard and J.E. Biaglow (COO-78-318).

"Recovery from Gamma-Radiation-Induced Dissociation of Polysomes in Tetrahymena pyriformis" by N.L. Oleinick and R.C. Rustad (COO-78-319).

"Comparative Sensitivities of Sea Urchin Eggs, Sperm and Zygotes to Radiation-Induced Mitotic Delay" by R.C. Rustad and R. Goldman (COO-78-321).

4. American Society for Photobiology, Vancouver, British Columbia, July, 1974 - O.F. Nygaard (Radiation Biochemistry), R.C. Rustad (Cellular Radiobiology).
5. American Association of Physicists in Medicine, Kansas City, Missouri, August, 1974; Chicago, Illinois, December 1974-E.C. Gregg* (Radiation Physics).
6. General Scientific Meetings of the Marine Biological Laboratory, Woods Hole, Massachusetts, August, 1974 - R.C. Rustad, B. Antonellis, I.I. Cassen and S.D. Gharib (Cellular Radiobiology).

Papers presented: "Reduction of Radiation-Induced Mitotic Delay in Sea Urchin Eggs Following Prefertilization Ionic Stimulation" by R.C. Rustad, E.I. Cassen and S.D. Gharib (COO-78-332).

"How Cell Growth is Inhibited by Near UV Light Photoproducts of Tryptophan" by S. Zigman, T. Yulo, G. Griess, B. Antonellis and R. Rustad (COO-78-333).

7. American Society for Cell Biology, San Diego, California, November, 1974 - R.C. Rustad (Cellular Radiobiology).

Papers Read by Title: "Synthesis of Mitotic Proteins During G₂ in Physarum polycephalum" by R.C. Rustad, E.N. Brewer and N.L. Oleinick (COO-78-336).

"Inhibition of Protein Synthesis by Menadione Without an Alteration of Electron Transport" by N.L. Oleinick and J.E. Biaglow (COO-78-335).

8. ICN-UCLA Conference on DNA Synthesis and Its Regulation, Squaw Valley, California, March, 1975 - H.H. Evans*, T.E. Evans* and E.N. Brewer (Radiation Biochemistry).

Paper presented: "The Inhibition of DNA Strand Elongation by Cycloheximide in Physarum polycephalum" by H.H. Evans, T.E. Evans and E.N. Brewer (COO-2486-339).

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