# DNA REPLICATION AND THE REPAIR OF DNA STRAND BREAKS IN NUCLEI OF PHYSARUM POLYCEPHALUM

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# DNA REPLICATION AND THE REPAIR OF DNA STRAND BREAKS IN NUCLEI OF PHYSARUM POLYCEPHALUM


## Abstract

Nuclei isolated from Physarum are able to replicate approx. 15% of the total genome in a manner which is qualitatively similar to the DNA replication process occurring in the intact organism. Such nuclei, however, are defective in the joining of Okazaki intermediates in vitro. Two DNA polymerase species, isolated from nuclei or intact plasmodia of this organism, can be separated by sucrose density gradient centrifugation. Total DNA polymerase activity is low in nuclei isolated during mitosis. A heat-stable glycoprotein material present in aqueous nuclear extracts stimulates DNA synthesis in well-washed nuclei. A sub-nuclear preparation active in DNA synthesis in vitro has been obtained from isolated nuclei of Physarum.

Radiation-induced DNA double-strand breaks are rejoined in intact plasmodia and isolated nuclei of Physarum in a cell cycle-dependent manner. This phenomenon does not appear to be due to an intrinsic difference in nuclear DNA endonuclease activity at different times of the mitotic cycle. DNA strand breaks and repair induced by the carcinogen 4-nitroquinoline-1-oxide is similar in several respects to that resulting from exposure of the organism to ionizing radiation.

Temperature sensitive strains of Physarum have been constructed and preliminary genetical and biochemical characterizations have been carried out. Two of the strains appear to be conditionally defective in DNA metabolism.

An isogenic ploidal series of amoebae has been prepared and characterized as to UV and ionizing radiation sensitivity (in terms of cell survival). There is a direct relationship between ploidy and resistance to UV whereas ploidal change does not appear to affect the response to ionizing radiation.

# DNA REPLICATION AND THE REPAIR OF DNA STRAND BREAKS IN NUCLEI OF PHYSARUM POLYCEPHALUM

## 1. DNA Replication in vivo and In Cell-free Preparations of Physarum (E.N. Brewer)

The mechanism by which a cell initiates those events which commit the cell to replicate its DNA, and consequently to divide, remain a mystery. There are several possible approaches to a solution of this problem. Ours has been directed toward identifying the constitutents of the DNA replication complex on the assumption that the control of the DNA replication process might be associated with an alteration in the activity or availability of one or more of these constituents (or to the presence or absence of inhibitors of the replication process present therein). This approach requires isolation of an intact replication complex, active in DNA synthesis, which can be dissociated and recombined in vitro. For these studies we chose the slime mold Physarum polycephalum, whose nuclei undergo naturally synchronous mitosis and initiation of the DNA replication process.

In order to determine whether the activity which we hoped to demonstrate for cell-free preparations was a true reflection of the process occurring in vivo, we first investigated certain aspects of the DNA replication process as it occurs in the intact organism. We found, for example, that parental -strand DNA sedimented in a relatively narrow band in both neutral and alkaline sucrose density gradients, exhibiting peak molecular weights of approx $8.6 \times 10^7$ (native) and $3.8 \times 10^7$ (denatured), respectively (Brewer, J. Mol. Biol., 68, 401-412, 1972, COO-78-260; Brewer, Radiat. Res., 79, 368-376, 1979, Appendix C). Pulse-labeling experiments demonstrated that synthesis of these DNA units requires essentially the entire S period (Brewer, Evans and Evans, J. Mol. Biol., 90, 335-342, 1974, COO-78-311).

DNA strand elongation was blocked abruptly by exposure of the organism to cycloheximide at any time during S (Evans, Littman, Evans, and Brewer, J. Mol. Biol., 101, 169-184, 1976, COO-78-324). These characteristics of the DNA replication process in vivo, as well as the differences in the rate of DNA synthesis between the S and $G_2$ periods, were used as the basis of comparison for establishing the authenticity of DNA synthesis obtained with cell-free preparations.

As a first step toward attainment of DNA synthesis in an isolated DNA replication complex, we first demonstrated that synthesis in isolated nuclei of Physarum was qualitatively similar to that occurring in the intact organism (Brewer and Ting, J. Cell. Physiol., 86, 459-470, 1975, COO-78-312). Based on a report by Lynch et al. (Lynch, Umeda, Uyeda and Lieberman, Biochim. Biophys. Acta, 287, 28-37, 1972) that DNA synthesis in isolated nuclei of rat liver was increased in the presence of certain carbohydrate polymers, we were then able to show that homogenates and isolated nuclei of Physarum, in the presence of dextran, were able to carry out the synthesis of approx. 15% of the total genome at an initial rate of 25% of the in vivo rate (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334).

Since the appearance of the latter report we have attempted to determine conditions under which the joining of Okazaki intermediates could be demonstrated, and have proceeded to sub-fractionate isolated nuclei and to begin to identify certain of the constituents of the DNA replication complex. These recent activities are described in detail below.

A. Attempts to Demonstrate Joining of Okazaki Intermediates in vitro

Considerable effort was expended in an attempt to find conditions under which the Okazaki intermediates synthesized in isolated nuclei could be shown to combine to produce fully mature DNA progeny strands in vitro. This

effort met with partial success, in that some increase (3-fold) in size of these small DNA intermediates was observed, but the complete maturation of these fragments could not be attained under a variety of conditions (Brewer, Fed. Proc., 35, 1418, 1976, COO-2486-352). In the course of this study, we observed that the joining of Okazaki intermediates could be blocked in vivo by immersing the organism in the plasmodial homogenizing medium (Annual Report, 1975-1976, COO-2486-365). It is possible, therefore, that a constituent of the homogenizing medium (EGTA?) inhibits joining of these DNA fragments. In this connection, it is interesting that DNA strand rejoining in vivo following gamma irradiation is also inhibited in the presence of EGTA (Annual Report, 1975-1976, COO-2486-365). It is possible that a divalent cation ($Ca^{++}$?), while necessary for ligation of Okazaki intermediates, also stimulates DNA endonuclease activity in vitro, thereby producing DNA strand breaks and inhibiting DNA chain elongation (Brewer and Ting, J. Cell. Physiol., 86, 459-470, 1975, COO-78-312). Further experimentation will be required to resolve this problem.

B. The DNA Polymerases of Physarum

When nuclei of Physarum are isolated in the absence of dextran, they lose a substantial portion of their DNA synthesis activity. Part of this activity can be restored by recombining such nuclei with the post-nuclear supernatant fraction to which dextran has been added (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334). The latter fraction contains considerable DNA polymerase activity, which can be resolved by sucrose density gradient centrifugation into two peaks of activity (Brewer, unpublished manuscript, COO-2486-343) corresponding to the DNA polymerases α and β reported for mammalian cells (Weissbach, Annu. Rev. Biochem., 46, 25-47, 1977). An effort

was made to determine which of these two entities (or both) might be involved in the DNA chain elongation process in nuclei of Physarum. Various sucrose density gradient fractions (to which dextran had been added) containing either or both of these activities were combined with dextran-free nuclei. Although DNA synthesis in such nuclei is stimulated by the total post-nuclear supernatant fraction containing both polymerases (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334), little stimulation was observed with individual or combined sucrose density gradient fractions (Table I). This observation suggests the possibility that additional factors necessary for DNA synthesis may be present in the unfractionated post-nuclear supernatant fraction.

Table I. Attempted stimulation of DNA synthesis in dextran-free nuclear preparations by sucrose density gradient fractions of the post-nuclear supernatant fraction containing dextran.

| Fraction No. | Polymerase | Cpm ± av. dev. |
|---|---|---|
| None | -- | 2823 ± 181 |
| 1-10 | -- | 3262 ± 78 |
| 11-18 | α(6-8 S) | 3342 ± 82 |
| 19-26 | β(3.3 S) | 3215 ± 172 |
| 27-31 | -- | 2945 ± 445 |
| 11-26 | α + β | 2609 ± 2 |

Two macroplasmodia were homogenized in 3 ml of the dextran-free homogenizing medium, and 0.2 ml of the post-nuclear supernatant fractionated by sucrose density gradient centrifugation (Brewer, unpublished manuscript, COO-2486-343). Fractions (0.16 ml each) of 3 separate gradients were combined, 1.8 ml mixed with 0.25 g. dextran, and recombined with nuclei prepared by centrifugation of 0.5 ml of a fresh dextran-free homogenate at 1500 x g. Incorporation of [$^3$H]dATP into DNA was determined as described previously (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334).

We also attempted to determine whether there were any differences between the total DNA polymerase activity of Physarum nuclei and of the "cytoplasmic" fraction (post-nuclear supernate) prepared at different times of the nuclear division cycle. As can be seen in Table II, considerably less DNA polymerase activity was found in the nuclear fraction of a dextran-free homogenate prepared at metaphase, as compared with nuclei obtained from S- or $G_2$-phase homogenates. On the other hand, a correspondingly greater activity was present in the "cytoplasmic" fraction of homogenates obtained during metaphase. These findings suggest the intriguing possibility that the DNA polymerase(s) of Physarum may shuttle from nuclei to cytoplasm during mitosis, in order, perhaps, to be modified in some way, and return to the nuclei at about the time of initiation of DNA replication. This hypothesis remains to be tested.

Table II. DNA polymerase activity of nuclear and "cytoplasmic" fractions of dextran-free homogenates prepared at different times in the nuclear division cycle.

| Fraction of homogenate | Time of Cycle | cpm ± av. dev. |
|---|---|---|
| Nuclear | S | 4139 ± 309 |
| " | M | 2037 ± 54 |
| " | $G_2$ | 3448 ± 73 |
| "Cytoplasmic" | S | 14,990 ± 939 |
| " | M | 28,168 ± 2450 |
| " | $G_2$ | 17,001 291 |

Plasmodia sectors (1/6) were homogenized at M + 30 min (S), M - 30 min ($G_2$) or at M, in 4.2 ml of the dextran-free homogenizing medium, and the homogenates centrifuged at 1500 xg for 10 min. The nuclear pellets were resuspended in the same volume of dextran-free medium and DNA polymerase activity present in both fractions was determined, using an exogenous DNA template, as described previously (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334). Cpm's were normalized for differences in size (protein content) between plasmodial sectors.

C. Isolation of Stimulatory Factor for DNA Synthesis in Isolated Nuclei of Physarum

As a first step toward the goal of nuclear sub-fractionation, isolated nuclei of Physarum were washed thoroughly with the plasmodial homogenizing medium (containing dextran). This procedure resulted in the loss of considerable DNA-synthesis activity in such nuclear preparations. A portion of this activity could be restored by adding back the washes (or plasmodial extracts) which had been heated for 5 min at 100°C. The stimulatory activity was not that of DNA polymerase α or β, therefore, both of which are heat-labile in Physarum. Further purification of heated extracts showed that this activity resides in a previously unreported glycoprotein material having a molecular weight of approx. 30,000. The mechanism of stimulation is not known. Additional characteristics of the stimulatory substance are described in the accompanying paper (Brewer, Biochim. Biophys. Acta, 564, 154-161, 1979, Appendix A).

D. DNA Synthesis in a Sub-Nuclear Preparation Isolated from Nuclei of Physarum

Our long-term goal has been to isolate the DNA replication complex from nuclei of Physarum in order to identify the constituents of this complex by dissassociating it and recombining fractions. We have isolated recently such a sub-nuclear preparation active in DNA synthesis. The characteristics of this activity are similar to those of isolated nuclei (and of the intact organism). This study is described more fully in the accompanying manuscript (Brewer and Busacca, submitted for publication, Appendix B).

## II. The Repair of DNA Strand Breaks *in vivo* and in Isolated Nuclei of *Physarum* (E.N. Brewer and G.S. Kuncio)

### A. Rejoining of Radiation-Induced DNA Strand Breaks *in vitro*

We reported earlier (Brewer and Nygaard, Nature New Biol., 229, 108-110, 1972, COO-78-267) that nuclear DNA single- and double-strand break repair (and mitotic delay) following gamma-irradiation of *Physarum* is cell-cycle dependent, with less rejoining (and greater mitotic delay) occurring in S-phase, as compared to $G_2$-phase, plasmodia. As an approach to elucidating the biochemical mechanism of this repair process, considerable effort was made to demonstrate rejoining of DNA single-strand breaks in isolated nuclei of *Physarum*. Despite the fact that we were unable to demonstrate this activity *in vitro*, we discovered that isolated nuclei were able to effect the repair of radiation-induced *double-strand* breaks, and that this process was similarly cell-cycle dependent. Moreover, the DNA of nuclei isolated from unirradiated S-phase plasmodia was more susceptible to DNA strand breakage than was that of $G_2$-phase nuclei (Brewer, Radiat. Res., 79, 368-376, 1979, Appendix C). One of the hypotheses put forward to explain these results was that S-phase nuclei might possess a greater intrinsic DNA endonuclease activity than do those present in $G_2$-phase plasmodia. This suggestion was tested by determining the comparative levels of endonuclease activity in nuclear extracts (sonicates) prepared during both phases of the mitotic cycle. As can be seen in Fig. 1, no significant difference in the level of this activity could be discerned in such extracts. It appears unlikely, therefore, that the difference in the efficacy of DNA strand-break repair between S- and $G_2$-phase nuclei resides in endonuclease levels *per se*. It remains to be determined whether this difference might be due, rather, to differences in DNA ligase activity, or perhaps to differences in the accessibility of the template itself to endonucleolytic attack.

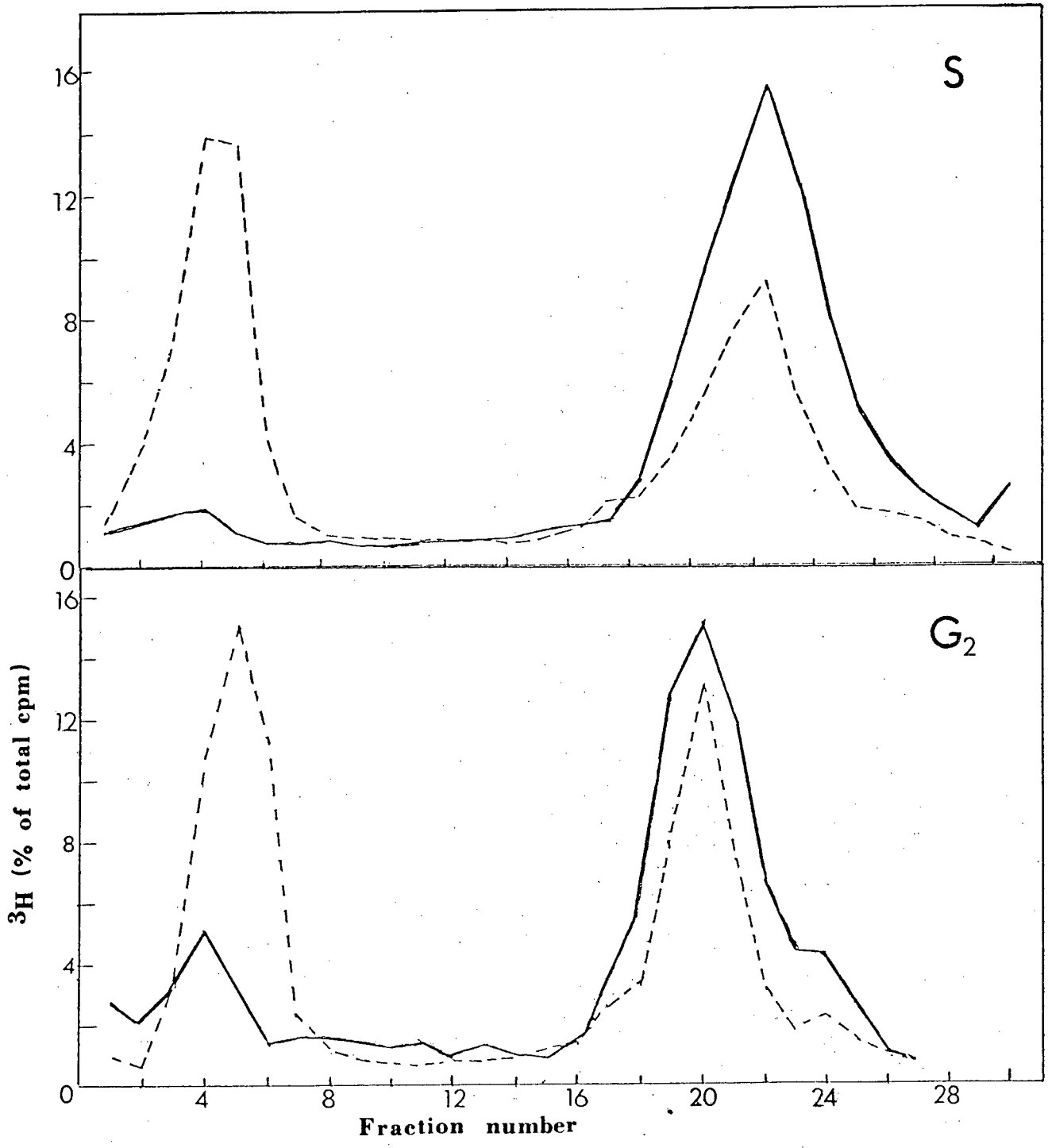


Fig. 1. Endonuclease activity of nuclear extracts prepared from S- or $G_2$-phase plasmodia. Nuclei from a single macroplasmodium were isolated (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334), then resuspended in 1 ml of buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5), and sonicated for 20 sec (Branson Model LS 75 Sonifier, postion 2). Samples (10 µl) were mixed with 10 µl [$^3$H]ϕX174RF DNA (supercoiled) and incubated at 37°C for 3 min., then layered over 5-20% alkaline sucrose gradients and centrifuged in the SW 50.1 rotor at 50,000 rpm for 90 min. Top of gradient is to the right. -----, 0 min incubation; _____, 3 min incubation.

B. Rejoining of 4-Nitroquinoline-1-Oxide-Induced DNA Strand Breaks in vivo

In the previous Annual Report (COO-2486-369) we described our preliminary findings concerning the production of DNA strand breaks following exposure of intact plasmodia of Physarum to the carcinogen 4-nitroquinoline-1-oxide (4-NQO). These studies have been extended. It appears that this type of DNA damage and its repair are similar in many respects to that brought about by ionizing radiation. 4-NQO-induced DNA single-strand breaks are repaired to a lesser extent during the S period, and greater mitotic delay results following exposure to the drug during this phase of the mitotic cycle. These findings are described in further detail in the accompanying manuscript (Kuncio and Brewer, submitted for publication, Appendix D).

III. Construction and Complementation Testing of ts/ts Plasmodia (T.E. Evans)

The following simplified procedure is used for construction of homozygous ts diploid plasmodia. ts Mutants derived from strain LU 648 ($mt_1$, fus $A_1$) are mated with CW 202 ($mt_2$, fus $A_2$); heterozygosity at the fus A locus results in a fusion phenotype that allows unambiguous and rapid confirmation that the plasmodium is diploid as a result of mating between the two amoebal strains. After confirmation of the cross by fusion analysis, the known heterozygote is sporulated, the spores germinated and the resultant amoebae tested for temperature-sensitivity. ts $F_1$ segregants are then crossed randomly, and the resulting plasmodia fusion-tested. Plasmodia that test as heterozygotes at the fus A locus possess the following genotype: $mt_1/mt_2$, $fusA_1/fusA_2$, $gro^{ts}/gro^{ts}$.

We have constructed five homozygous diploid plasmodia from five ts amoebal strains. All five plsmodia show a rapid and complete inhibition of growth at the restrictive temperature. We have begun to carry out heterokaryon complementation tests among these strains. Surprisingly, two pairs of these

constructs do not complement each other, even though their biochemical characteristics are apparently different. In one case there is a reasonable possibility that the strains were constructed from sibling isolates. The results are being tested by appropriate mating experiments and by further biochemical analyses.

IV. Biochemical Characterization of Temperature-Sensitive Plasmodia (H.H. Evans and T.E. Evans)

The ability of the temperature-sensitive mutants to synthsize RNA, DNA, and protein at the restrictive temperature is initially determined by measuring the incorporation of appropriate radioactive precursors using asynchronous microplasmodial cultures. The incorporaion by the mutant strain is compared to that of the parental wild-type strain at the restrictive and permissive temperatures. If a ts strain demonstrates a specific temperature sensitivity in the incorporation of a precursor into DNA, the ability of synchronous S-phase macroplasmodia of the mutant strain to synthesize DNA at the restrictive temperature is then investigated. Comparison of S-phase macroplasmodia with asynchronous microplasmodia allows the distinction between defects in DNA replication *per se* and a block in G2 or M of the cell-cycle (observable as a decrease in incorporation at the restrictive temperature in the asynchronous cultures only). If a decrease in the incorporation of precursors into DNA is observed in the S-phase plasmodia, the defect is then studied *in vitro*. Homogenates of S-phase plasmodia are incubated with labeled deoxynucleoside triphosphates at permissive and restrictive temperatures according to methods described previously (Brewer, Biochim. Biophys. Acta, 402, 363-371, 1975, COO-78-334). Comparison of *in vivo* and *in vitro* results allows the differentiation between defects in the replication process itself (observable both *in vivo* and *in vitro*) and in the synthesis of the deoxynucleoside triphophate precursors (observable only *in vivo*, where

Table III. Summary of physiological and biochemical characteristics of temperature-sensitive plasmodia

| ts Plasmodium | ts Amoeba | Mutagenesis | Time defect in ts. plasmodial growth observed after shift to 31° (hours)[1] | ts Defect in macromolecular synthesis[2] | | |
|---|---|---|---|---|---|---|
| | | | | Nature | Time observed after shift to 31° | Notes |
| CW 501 x CW502 | MR 24-51 | 4 | 24 | poly $A^+$-RNA | Asynch: 22 hours<br>S-phase: 30 min | |
| 254 - 10 | HE 67-50 | 6 - 4 | 6 | none | - | Temp. sens. growth not complemented in heterokaryon with 255-2 |
| 255 - 2 | HE 68-10 | 6 - 4 | 7 | DNA (in microplamodia) | Asynch: 180 min<br>S-phase: no effect<br>In vitro: no effect | See 254-10<br>Blocked in late G2 at 31° |
| 270 - 4 | SL 10-35 | 7 - 4 | 6 | DNA | Asynch: 180 min<br>S-phase: 90 min<br>In vitro: 60-120 min | ts growth not complemented in heterokaryon with (1 x 23) |
| (1 x 23) | HE 72-46 | 6 - 4 | 20 | RNA, DNA | Asynch: 60-120 min | See 270-4 |

1. The inhibition of growth at 31° was reversible in all 5 strains upon shift to 22°.

2. As measured: 1) In the case of microplasmodia, by the incorporation of $^3$H-nucleosides into RNA and DNA and $^{14}$C-leucine into protein;
2) In the case of S-phase plasmodia, by the incorporation of $^3$H-thymidine into DNA; and,
3) In the case of homogenates, by the incorporation of $^3$H-dATP into DNA.

Incorporation by the mutant strains at 22° and at 31° is compared to the respective levels of incorporation by the wild-type parental strain (648 x 202) at the two temperatures.

Table IV. Incorporation of labeled precursors by wild-type (648 x 202) and temperature-sensitive plasmodial strains

| Exp. | Plasmodia | | Time of incorporation | Specific activity at the restrictive temperature / Specific activity at the permissive temperature | | |
|---|---|---|---|---|---|---|
| | Strain | Type | (min after shift to 31°) | RNA | DNA | Protein |
| 1. | 648 x 202 | Asynchronous | 150 - 180 | 5.75 | 0.89 | 1.73 |
| | 270-4 (ts) | " | " | 1.73 | 0.33 | 1.41 |
| 2. | 648 x 202 | S-phase | 60 - 90 | | 0.97 | |
| | 270-4 | " | " | | 0.67 | |
| 3. | 648 x 202 | Homogenate | 0 - 120 | | 1.10 | |
| | 270-4 | " | " | | 0.57 | |
| 4. | 648 x 202 | Asynchronous | 0 - 180 | 1.15 | 1.40 | 1.85 |
| | 255-2 (ts) | " | " | 1.23 | 0.27 | 1.79 |
| 5. | 255-2 | S-phase | 5 - 265 | | 0.98 | |
| 6. | 648 x 202 | Homogenate | 0 - 60 | | 0.64 | |
| | 255-2 | " | 0 - 60 | | 0.71 | |

Specific activities were determined as described in the legend to Table III. Permissive or restrictive temperatures were 22° and 31°, respectively, for in vivo experiments and 35° and 42°, respectively, for in vitro experiments.

nucleosides are used as the labeled precursors).

Table III shows the characteristics of the five temperature-sensitive plasmodia studied to date. Incorporation data (Table IV) indicate that two of the strains have a specific defect in DNA synthesis when tested as micro-plasmodia at the restrictive temperature. One of these strains (270-4) is defective as an S-phase plasmodium and in the in vitro system, whereas the other strain (255-2) exhibits no decrease in the incorporation of precursors at the restrictive temperature when S-phase plasmodia or homogenates are tested. Strain 255-2 appears to be blocked in late G-2 phase at the restrictive temperature. Further characterization of both strains is in progress.

V. Radiation Sensitivity of Amoebae (P.M. Mulleavy and T.E. Evans)

The long-term objective of this project is to isolate UV-sensitive amoebae of Physarum polycephalum and to use these strains to study the relationship of DNA repair to mutagenesis in this eukaryote. Initial experiments were carried out in order to determine the survival response following UV or ionizing radiation. Since studies by other investigators of the relationship of ploidy to radiation response have yielded contradictory results (perhaps because the ploidal series were not isogenic), we decided to carry out our work using an isogenic ploidal series (haploid, diploid and tetraploid). The series was constructed by isolating single cells representing the different ploidal subpopulations within the original strain. Ploidy was established by determining the DNA content of single cells using Feulgen-DNA cytophotometry.

Following treatment with UV-radiation, the response in terms of survival differed markedly with ploidy: The $D_q$ was directly related to ploidy, as might be predicted from the multi-target theory. The $D_o$ was inversely related to the ploidy of the cells; the possibility that the increase in resistance with ploidy is primiarily due to an increase in repair capacity is now under

investigation. In contrast to the UV response, the $D_o$ and $D_q$ did not appear to change significantly as a function of ploidy following treatment with ionizing radiation. Further studies of this phenomenon are in progress.

## APPENDICES

A. Brewer, E.N., "Isolation of a Stimulatory Factor for Nuclear DNA Replication", Biochim. Biophys. Acta, 564, 154-161 (1979).

B. Brewer, E.N. and Busacca, P.A., "DNA Synthesis in a Sub-Nuclear Preparation Obtained from Isolated Nuclei of Physarum", Biochem. Biophys. Res. Commun., 91, 1352-1357 (1979).

C. Brewer, E.N., "Repair of Radiation-Induced DNA Double-Strand Breaks in Isolated Nuclei of Physarum", Radiat. Res., 79, 368-376 (1979).

D. Kuncio, G.S. and Brewer, E.N., "DNA Strand Breaks and Cell Cycle-Dependent Repair Induced by 4-nitroquinoline-1-oxide in Physarum", submitted for publication.

## STAFFING

| | |
|---|---|
| Eugene N. Brewer, Ph.D., Principal Investigator<br>Time devoted to project during current term of agreement: | 56 %, 11 mo. |
| Thomas E. Evans, Ph.D., Co-principal Investigator<br>Time devoted to project during current term of agreement: | 63 %, 7 mo. |
| Oddvar F. Nygaard, Ph.D., Co-investigator<br>Time devoted to project during current term of agreement: | 5 %, 12 mo. |
| Gerald S. Kuncio, Ph.D., Research Associate<br>Time devoted to project during current term of agreement: | 100%, 6 wk. |