DINITROPHENOL INHIBITS THE REJOINING OF RADIATION-INDUCED DNA BREAKS BY L-CELLS

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ABSTRACT The production and rejoining of X-ray–induced single-stranded DNA breaks was studied using the alkaline sucrose density gradient technique and by measuring the disappearance of both 5′ termini and 3′-OH termini using polynucleotide kinase and DNA polymerase, respectively. All studies were conducted using L-cell suspensions irradiated both in the presence and absence of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. Results show that the induction of single-stranded DNA breaks probably includes a nucleolytic component in addition to indirect free radical effects. A greater number of breaks were produced in the absence of DNP, suggesting that depressed adenosine triphosphate (ATP) levels reduce endogenous nucleolytic activity. The rejoining mechanism is enzymatic and requires an available ATP supply for operation. In the presence of DNP no DNA rejoining was observed following 30 min incubation after 10,000 rad. These results suggest that DNA breaks produced may be characterized by 5′-PO_4–3′-OH termini and are rejoined by DNA ligase.

INTRODUCTION

Ionizing radiation produces breaks in the DNA of microorganisms and mammalian cells (1–11). The repair of radiation-induced DNA damage has been the subject of an increasing number of investigations during the past few years, since DNA represents a target of great biological importance. Initial studies by McGrath and Williams (1), and by Kaplan (2), showed that bacteria rejoin single-stranded DNA breaks during the immediate postirradiation period. Later experiments by Lett, Caldwell, Dean, and Alexander (3), Lohman (4), and Humphrey, Steward, and Sedita (5), showed similar findings for cultured mammalian cells.

The results of studies by Dalrymple, Sanders, Moss, Baker, and Wilkinson (6–9), indicate that at least a portion of the DNA breaks following irradiation are char-

acterized by 5′ termini. The occurrence and the rejoining of DNA breaks characterized by 5′ termini has been demonstrated for cultured mammalian cells and for mouse liver, in vivo (6). Other studies have indicated that the presence of 5′ termini is paralleled by 3′-OH termini, with rejoining of DNA breaks resulting in a decrease in the number of both 5′ and 3′ termini (8). Treatment of L-cells with DNP prevented the rejoining of these breaks (9).

Ultracentrifuge experiments by Humphrey, et al.[1] showed that cyanide is capable of preventing rejoining of DNA breaks produced by X-rays. These findings do not agree with results reported by Swada and Okada (10) who found that DNP did *not* prevent DNA rejoining.

The present study reports experiments in which the influence of DNP on the rejoining of X-ray–induced DNA breaks by L-cells was measured. The results indicate that DNP prevents the rejoining of the DNA breaks.

METHODS

Cells

In all experiments Sanford's L-929 strain (12) of cultured cells was used. They were grown in Eagle's minimum essential medium (MEM) (13) supplemented with "nonessential" amino acids, antibiotics, and 10% calf serum. The culture bottles were seeded on the first day of the experiment and the cells were allowed to grow 48 hr at 37°C. If radioactive labels were used, the monolayers were labeled 24 hr prior to irradiation by the addition of tritiated thymidine (thymidine, methyl-T, 21.9 Ci/mM, Amersham/Searle Corp., Arlington Heights, Ill.) to each bottle to a final concentration of 0.2 μCi/ml of medium. Monolayers were maintained at 37°C after the addition of the label. The cells were in log growth at the time of irradiation.

The monolayers were washed with Hanks' balanced salts solution (HBSS) and then detached with dilute trypsin solution. The cells were pooled, washed two additional times with HBSS, then resuspended in either HBSS or HBSS which contained DNP at a concentration of 10^{-4} M. In all experiments the HBSS was glucose-free.

Irradiations

The cells were irradiated with 250 kvp X-rays (0.5 mm Cu HVL). The irradiations were made under conditions of full backscatter. The dose rate was 1000 rad/min.

Ultracentrifuge Method

After irradiation, cell lysates were prepared (at room temperature) by the addition of an equal volume of lysing solution to each cell suspension sample. The cells were suspended at a concentration of 10^6/ml. This lysing solution contained 0.05 M EDTA in addition to 2% triisopropyl-naphthalenesulfonic acid and 1% *p*-aminosalicylic acid in 6% secondary butanol in water. The solution was adjusted to a final pH of 12.5 by the addition of sodium hydroxide.

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[1] Humphrey, R. M., D. L. Steward, and B. A. Sedita. 1970. Genetic Concepts and Neoplasia. Williams & Wilkins Co., Baltimore 570–592.

This solution (without the EDTA) was described by Humphrey, et al. (5). The cells were lysed for 60 min before being placed on the gradients.

In all molecular weight determinations, isokinetic alkaline sucrose density gradients (5–23%) were prepared using the technique described by Noll (14). The sucrose solutions contained 0.05 M EDTA; the pH was adjusted to 12.5 with NaOH. Samples were prepared by gently layering 25 μl of T_2 bacteriophage lysate[2] and 50 μl of L-cell lysate onto 6 ml gradients. The phage DNA was labeled with ^{14}C. The total amount of DNA added to the gradient (L-cell and phage) was less than 0.5 μg. Samples were centrifuged using an SW 25.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 4 hr at 24,000 rpm at 20°C. Approximately 24 10-drop fractions were collected from each tube into liquid scintillation counting vials. Both ^3H and ^{14}C activity were measured with a Beckman LS 200 liquid scintillation counter (Beckman Instruments, Inc.). The weight average molecular weight was estimated by the relationship described by Burgi and Hershey (15). This is expressed by

$$\frac{S_2}{S_1} = \left(\frac{M_2}{M_1}\right)^K, \tag{1}$$

where $K = 0.352$ and S_1 and S_2 are the sedimentation coefficients for two different DNA samples with molecular weights M_1 and M_2, respectively. The distances that the DNA samples sediment in a concentration gradient of sucrose, D_1 and D_2 may be substituted for S_1 and S_2 (15):

$$\frac{D_2}{D_1} = \left(\frac{M_2}{M_1}\right)^K. \tag{2}$$

For the estimation of the unknown DNA molecular weight, the sedimentation distance of the unknown was compared to the sedimentation distance of the ^{14}C-labeled T_2 phage.

The centroids of the sedimentation patterns (unknown and T_2 phage) were used as the estimates of the distances sedimented (2). The centroids $\bar{x}$ were calculated by

$$\bar{x} = \frac{\sum x_i y_i}{\sum y_i}, \tag{3}$$

where x_i is the fraction number and y_i is the activity in the ith fraction. The summation is carried over all of the fractions in the gradient.

The molecular weights computed by this technique represent *weight* average molecular weights (Mw). The *number* average molecular weight (Mn) can be related to the weight average molecular weight by

$$\mathrm{Mn/Mw} = 0.5. \tag{4}$$

This relationship, however, is valid only if the sedimentation patterns are gaussian (the term "random" was used by Charlesby [16]). Since the sedimentation patterns for the present study were gaussian, the number average molecular weight was estimated according to equation 4.

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[2] Equal volumes of bacteriophage suspension (in Tris) and lysing solution were mixed. The 25 μl portion of the lysate (i.e., the mixture of the phage and the lysing solution) contained 2.9×10^9 phage particles.

Biochemical Methods

Polynucleotide Kinase Assay. The methods used for extraction of the enzyme, the preparation of the samples, the steps for purification of the DNA, etc., are all in print (6, 9). Briefly, the following describes the method.

After irradiation, the cells were lysed with 1% sodium dodecyl sulfate, treated with boiled RNase (0.1 mg/ml) for 1 hr, pronase (1.0 mg/ml) for 2 hr, extracted with chloroform-isoamyl alcohol (24:1 v/v) three times, and then dialyzed against 0.01 M Tris-0.05 M NaCl (pH 7.6) for 24 hr at 4°C. We have found this method removes RNA and protein. Cells have been labeled with either TdR-^{3}H, UR-^{3}H, or leucine-^{3}H and then subjected to the procedure described above. Virtually all counts were removed from the UR-^{3}H and the leucine-^{3}H treated cells. Also, all RNA, as measured by the orcinol method (17), and protein, as measured by the Lowry method (18), was removed. The DNA from the TdR-^{3}H–treated cells, on the other hand, had a high count rate after this treatment. Consequently, the method of purifying the DNA described above is sufficient to remove potentially interfering RNA and protein. The removal of RNA is critical because it can also be labeled by the polynucleotide kinase reaction (19). The extraction procedure outlined above removes about 20% (by weight) of the acid-insoluble radioactivity.

The procedures described below were taken, with minimal modification, from methods described by Richardson (19, 20), and Weiss and Richardson (21). After the dialysis step, indicated above, the optical density (OD) (260 nm) was measured and all samples diluted until the OD's were equal. A portion of the dialyzate was also taken for measurement of DNA content by the diphenylamine method (22). In our experience, adjusting the OD's to the same value produces samples which contain equal amounts of DNA.

The next step concerns the removal of 5′-PO_4 groups by *Escherichia coli* alkaline phosphatase. The method for this process is taken from reference 19.

The reaction mixture (3.0 ml) contains: 5 μmoles DNA phosphorous, 0.07 M Tris (pH 8.0), and 0.01 mg *E. coli* alkaline phosphatase (highly purified).

The mixture is incubated at 65°C for 30 min, an additional 0.005 mg of enzyme added, and the incubation continued for another 30 min. The mixture was then dialyzed against 0.01 M Tris-0.05 M NaCl (pH 7.6) for 24 hr at 4°C. At the completion of this step the DNA is 5′-OH terminated. As the work of Richardson indicates (19, 20) the 65°C temperature removes both internal and external 5′-PO_4 termini. We have found that after the treatment outlined above, no additional phosphate is removed. Consequently, we assume that all phosphatase-liable PO_4 groups have been converted to OH groups.

The polynucleotide kinase was prepared and purified through step IV as described by Richardson (20). Although Richardson describes two additional purification steps, V and VI, which greatly reduce the total activity, we have obtained satisfactory (and equal) results using enzyme purified through step IV as contrasted with VI. The enzyme was diluted to a concentration of about 0.2 unit/ml in 0.05 M Tris (pH 7.6), 0.01 M 2-mercaptoethanol, and 0.05 mg/ml BSA.

The polynucleotide kinase assay is performed as follows (21). The reaction mixture, 0.3 ml, contains 20 μmoles Tris (pH 7.6), 3 μmoles $MgCl_2$, 5 μmoles 2-mercaptoethanol, 0.05 μmoles DNA (hydroxyl terminated), 20 μmoles γ-labeled AT^{32}P (prepared by the method of Glynn and Chappel [23]), and 0.05 unit polynucleotide kinase.

The mixture was incubated at 37°C for 30 min; then 0.5 ml of ice-cold 0.5 N perchloric acid (PCA) was added. The precipitate was then collected on a Millipore filter disc (Millipore Corp., Bedford, Mass.) (0.45 μ) and washed with ice-cold 0.3 N PCA (125 ml). Following washing, the discs were dried and counted with a liquid scintillation counter. The results are

expressed as DNA specific activity (cpm/μg DNA). The specific activity, then, is proportional to the number of 5′ termini in the DNA.

For each experimental point duplicate or triplicate tubes are run. The results are expressed as the mean specific activities. The uncertainties of the results, i.e., range of values (in the case of duplicates) and the standard deviations (in the case of triplicates), are of the order of the size of the plotted points.

Because DNA breaks characterized by 3′-OH–5′-PO_4 and 5′-OH–3′-PO_4 would be equally labeled (the phosphatase treatment would yield 3′-OH–5′-OH termini in both cases) we will refer to the breaks labeled by polynucleotide kinase as "characterized by 5′ termini."

DNA Polymerase Assay. The method used for the DNA polymerase assay has been described before (8). For the present study, the ability of the L-cell DNA to prime in the DNA polymerase reaction is used as a measure of the number of 3′-OH termini within the DNA molecule (8, 24). The reaction mixture 1.0 ml, contained 15 μg DNA, 0.1 unit DNA polymerase activity (extracted from T_2 phage infected *E. coli*) (25), $MgCl_2$ (2.4 mM), Tris (30.8 mM), mercaptoethanol (1.2 mM), dCTP, dATP, dGTP, and dTTP (8.6 mM), and 0.25 μCi of TTP-^{3}H (20 mCi/mg [New England Nuclear, Boston, Mass.]).[3] The DNA was in the double-stranded form. The mixture was incubated at 37°C for 30 min; the reaction was terminated by adding 0.05 ml of 10 N PCA. After 30 min in the cold, the precipitates were collected on Millipore filter discs and washed with ice-cold 0.5 N PCA, dried, and counted. All assays were conducted in triplicate.

ATP Assay. The ATP content of the acid-soluble fraction was determined by the firefly luciferase method (26) using a Packard LS 3003 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

RESULTS

A pivotal question of the study concerned the ability of L-cells to rejoin radiation-induced DNA breaks while in a state of severe energy deprivation. Earlier studies (27) suggested the components of the medium might reduce the effectiveness of DNP in depressing intracellular ATP levels. Since this ATP depression was critical to the interpretation of the results, the following experiment was performed.

L-cells (nonlabeled) were suspended at a concentration of 10^6 cells/ml in the following fluids:

Complete medium with 17% calf serum.
Complete medium with 10% calf serum.
Complete medium without serum.
Complete medium + 17% calf serum + DNP.
Complete medium + 10% calf serum + DNP.
Complete medium without serum + DNP.
HBSS without DNP.
HBSS with DNP.

The DNP was at a concentration of 10^{-4} M in all cases; this concentration of DNP

[3] CTP, cytidine triphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; TTP, thymidine triphosphate.

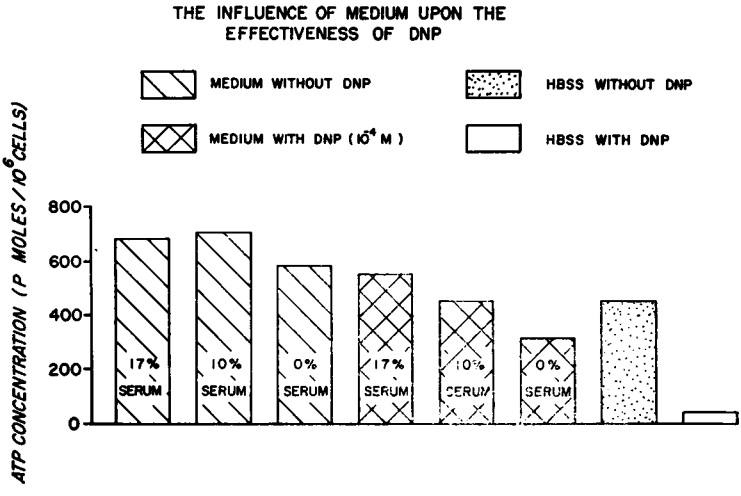

FIGURE 1 A bar graph which shows the effect of both Eagle's growth media and serum on the effectiveness of 10^{-4} M DNP. The ordinate has units of p moles ATP/10^6 cells.

is nontoxic to L-cells (27, 28). The suspensions were incubated at 37°C for 1 hr after which PCA was added to a final concentration of 0.5 N. After a 30 min period in the cold, the tubes were centrifuged. The ATP content of the acid-soluble fraction was measured by the firefly lantern method (26); the results are expressed as p moles ATP/10^6 cells.

Fig. 1 shows that DNP dissolved in HBSS markedly reduces the intracellular ATP concentration when compared with complete growth medium. However, this reduction was not seen when the DNP was dissolved in the growth medium. In fact, the addition of increasing amounts of serum to the growth medium further reduced DNP effectiveness. Consequently, these results show that the components of the medium do inhibit the ability of DNP to reduce ATP levels. When DNP is added to the medium the ATP levels are not reduced below the level for cells suspended in HBSS (which, in this case, represents our "control").

Fig. 2 shows the effect of increasing radiation dose on the sedimentation of single-stranded L-cell DNA. The cells were labeled (TdR-^{3}H), suspended in HBSS, and irradiated with doses from 0–10,000 rad. The cells were lysed with the lysing solution 20 sec after irradiation. For each sample the per cent of total ^{3}H activity applied to the gradient was plotted as a function of the fraction number. All patterns are point-to-point representations plotted using a Hewlett-Packard Model 9100A calculator and digital plotting system (Hewlett-Packard Co., Palo Alto, Cal.). The sedimentation patterns show that the distance sedimented rapidly decreases with increasing dose up to the highest dose used. In each panel, the irradiated DNA is compared with the unirradiated control from the same cell suspension (notice that the control pattern is repeated with the irradiated samples).

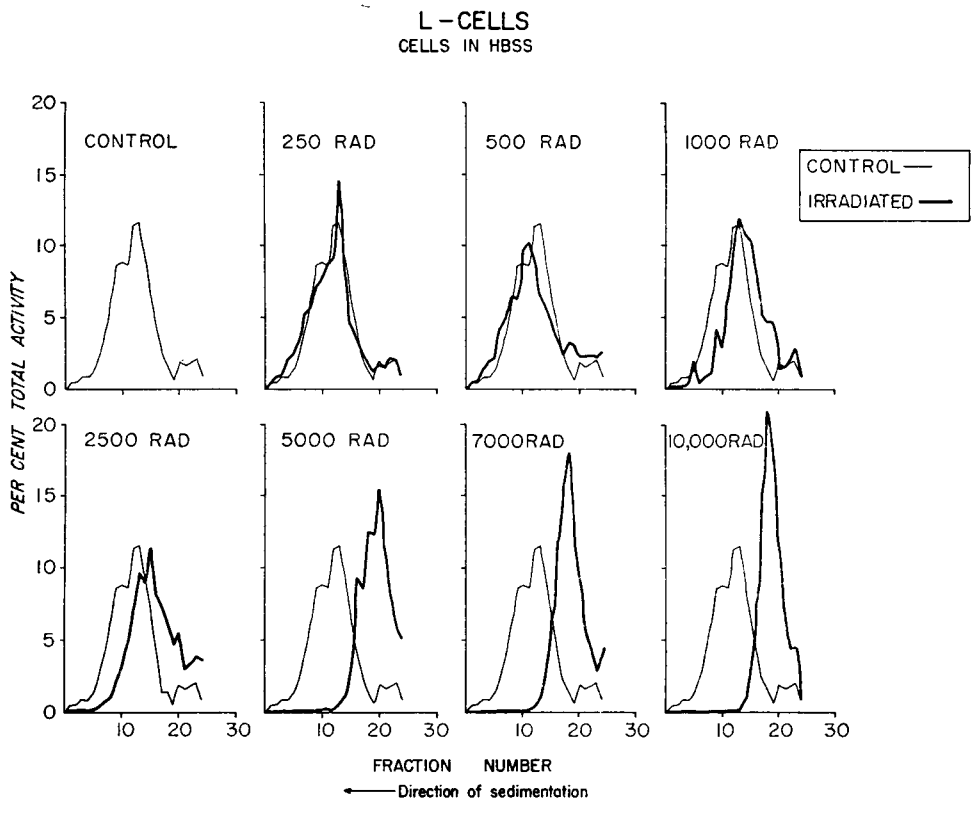

FIGURE 2 Alkaline sucrose sedimentation patterns of L-cells irradiated in HBSS. These patterns show the influence of X-ray dose on the sedimentation properties of single-stranded DNA. All cell suspensions were lysed 0.3 min after irradiation.

The L-cell DNA number average molecular weight ranged from 6.5×10^7 daltons for the unirradiated control to approximately 7.0×10^6 daltons after 10,000 rad.

Using the relationship described by Corry and Cole (29),

$$B = \left(\frac{NA_1}{NA_2} - 1\right) \frac{6.023 \times 10^{23}}{NA_1}, \tag{5}$$

where NA_1 is the number average molecular weight of unirradiated control and NA_2 is the number average molecular weight of the irradiated sample, and the number of single strand DNA breaks B per gram DNA was estimated for each irradiated sample. These results were then submitted to linear regression analysis (least squares). The regression equation was

$$Y = 7.94X - 2.04, \tag{6}$$

where X is the dose in rads and Y has units of single strand breaks/g DNA $\times 10^{15}$.

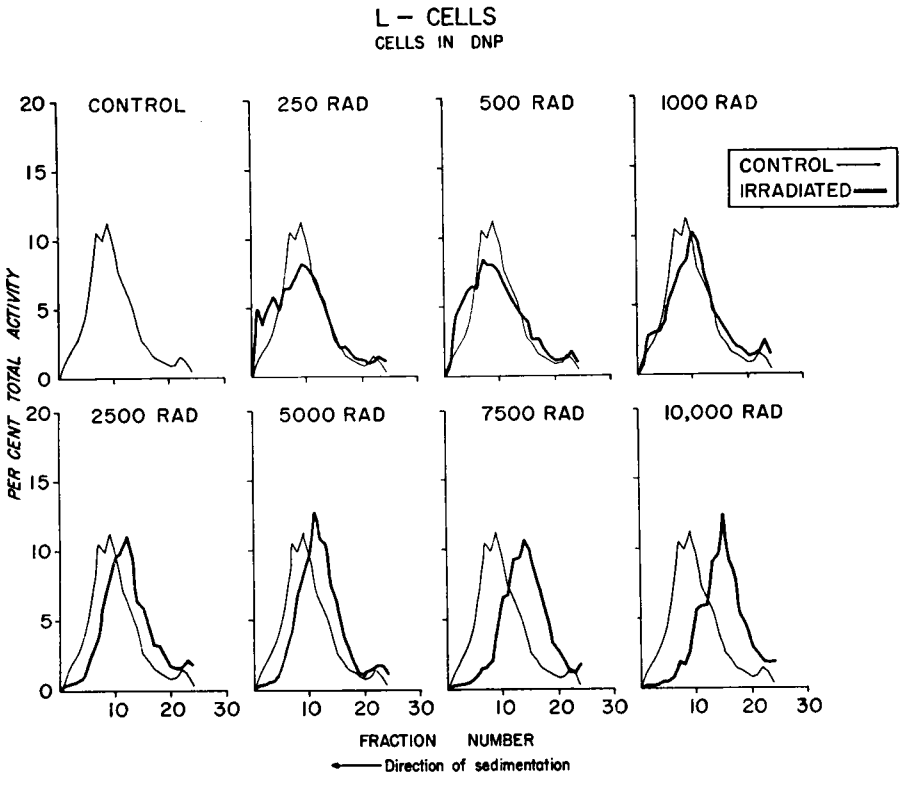

FIGURE 3 Alkaline sucrose sedimentation patterns of L-cells irradiated in 10^{-4} M DNP in HBSS. Cell suspensions were irradiated with doses from 0–10,000 rad. Cell suspensions were lysed 0.3 min after irradiation with various doses.

The correlation coefficient is 0.998. From the slope the rate of production of single strand breaks is 7.94×10^{12} single strand breaks/g DNA per rad.

Fig. 3 shows the result of increasing dose on single-stranded L-cell DNA. The cells were suspended in HBSS which contained 1×10^{-4} M DNP. As was seen in Fig. 2, a decrease in sedimentation is observed with increasing dose and the difference in sedimentation at a given dose is somewhat reduced in DNP, when compared with cells in HBSS. Molecular weights ranged from 7.0×10^7 daltons for the unirradiated control suspension in DNP and approximately 3.2×10^7 daltons after 10,000 rad. As before, using the relationship given by Corry and Cole (29) the number of single strand breaks/g DNA were estimated for cells irradiated in DNP. For cells in DNP the regression equation was

$$Y = 1.19X - 0.159, \tag{7}$$

where X and Y have the same units as equation 6; the correlation coefficient was 0.974. From the slope the rate of production of single strand breaks is 1.19×10^{12}

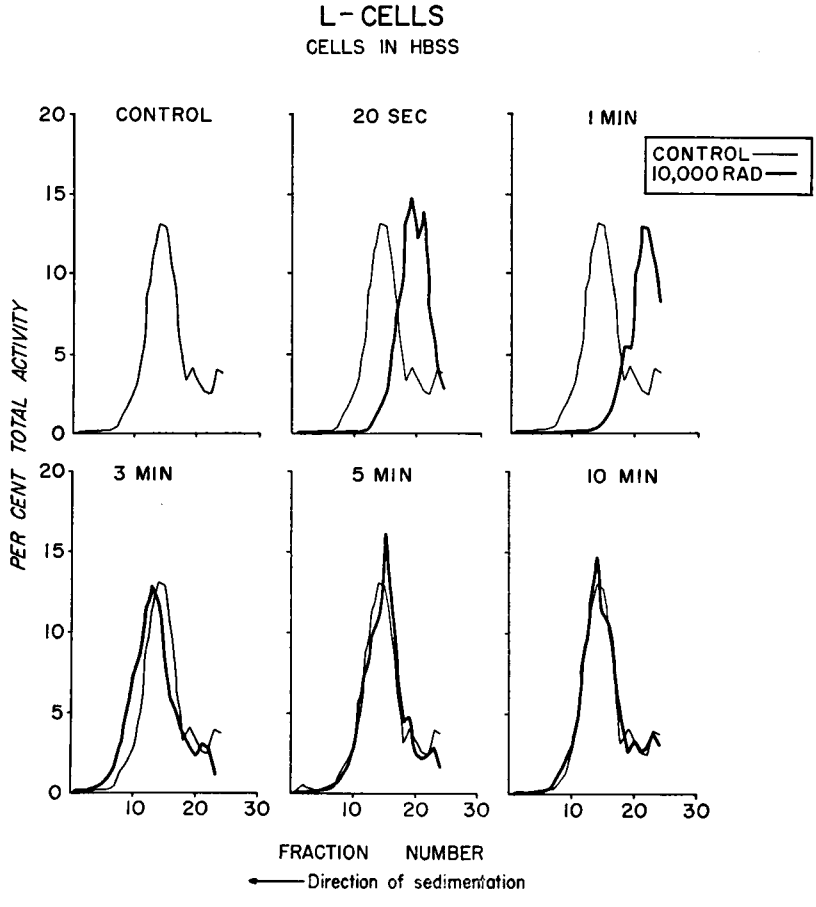

FIGURE 4. Alkaline sucrose sedimentation patterns of single-stranded DNA from L-cells suspended in HBSS. The suspension was given 10,000 rad and aliquots were withdrawn and lysed at the indicated times after irradiation. Rejoining of single strand breaks is complete by 3 min.

single strand breaks/g per rad for cells in DNP. These results show that the presence of DNP reduces the extent of the production of single strand breaks. Therefore, the rate of formation of single strand breaks in DNP is reduced by a factor of 6.67 ($[7.94 \times 10^{12}]/[1.19 \times 10^{12}]$) when compared with cell suspensions irradiated in HBSS.

Cells irradiated in both HBSS and DNP were observed for evidence of DNA rejoining. Fig. 4 shows the sedimentation patterns for cells suspended in HBSS and irradiated with a 10,000 rad dose followed by various periods of incubation after irradiation. The initial cell suspension (10^6 cells/ml) was irradiated at 37°C. During the first 10 postirradiation min, aliquot 3 ml samples were lysed with an equal volume of lysing solution. The lysates were handled and centrifuged as de-

scribed before. The results show a marked decrease in the sedimentation of irradiated single-stranded DNA through 1 min following irradiation. At 3 min, the sedimentation pattern returned to the control level indicating complete rejoining. The 5- and 10-min samples parallel the unirradiated control pattern. These data show that in HBSS all detectable single strand breaks have been rejoined by 3 min following a 10,000 rad dose.

Fig. 5 shows sedimentation patterns for cells given a 10,000 rad dose while suspended in 1×10^{-4} M DNP in HBSS. This experiment parallels the rejoining experiment shown in Fig. 4 for cells in HBSS. As can be seen, there is an immediate decrease in DNA sedimentation following irradiation. In contrast to results for cells irradiated in HBSS, however, the 3, 5, 10, and 30 min incubation points show *no* evidence of an increase in sedimentation as was observed with cells irradiated in HBSS. These results show that treatment with 1×10^{-4} M DNP in HBSS prevents the rejoining of the radiation-induced single strand breaks.

Fig. 6 shows the effect of 10,000 rad on L-cells, as measured by the polynucleotide kinase and DNA polymerase assays. The cells were grown as monolayers; at the time of the experiment the cells were detached with dilute trypsin and then washed

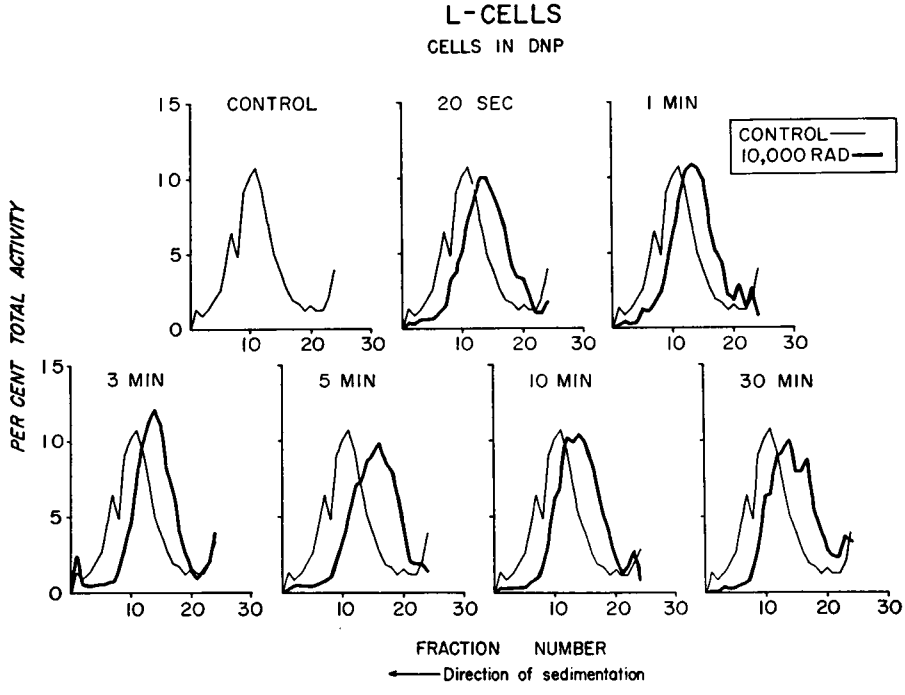

FIGURE 5 Alkaline sucrose sedimentation patterns of single-stranded DNA from L-cell suspensions in 10^{-4} M DNP in HBSS. The suspension was given 10,000 rad and aliquots were withdrawn and lysed at the indicated times after irradiation. In DNP there is no evidence of rejoining.

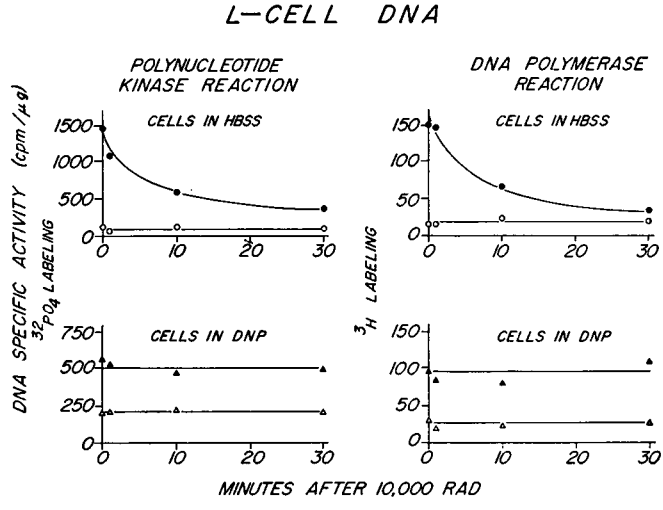

FIGURE 6 The left panels (upper and lower) show the results of the polynucleotide kinase assay. The right panels indicate the effectiveness of DNA to prime in the DNA polymerase reaction. The results are expressed as DNA specific activities; ^{32}P cpm/μg for the polynucleotide kinase assay and ^{3}H cpm/μg for the DNA polymerase reaction. As the lower panels show the DNP prevented the decrease in ^{32}P and ^{3}H specific activity as seen for the cells suspended in HBSS. The closed symbols represent irradiated cells while the open symbols are the nonirradiated controls. Notice that the nonirradiated DNA of cells in DNP contained a moderately increased number of 5′ and 3′-OH termini, as compared to cells in HBSS. At present, we have no explanation for this finding. We have, however, observed this before (7–9).

with HBSS. The cells were *not* prelabeled. Half of the cells were suspended in HBSS while the other cells were suspended in 10^{-4} M DNP in HBSS. They were kept in suspension, 5×10^6/ml, by gentle stirring with a magnetic stirrer; the temperature was 37°C. 3-ml samples were obtained from the HBSS and DNP suspensions, pipetted into test tubes, and then irradiated, at the same time, with 10,000 rad. The cells were maintained at 37°C during irradiation. After irradiation the tubes were incubated at 37°C until the appropriate time when they were quick chilled in dry-ice–acetone and processed as previously described (7–9). As a result of this procedure, the cells in DNP were irradiated and handled in a similar fashion. The nonirradiated controls were also handled in a parallel manner.

As the upper left panel of Fig. 6 shows, the $^{32}PO_4$ specific activity was greatest immediately after irradiation. Following irradiation, however, the DNA specific activity decreased. We interpret these results to mean that immediately after irradiation the DNA contained a large number of breaks characterized by 5′ termini, but with time these breaks were rapidly rejoined. The left lower panel shows that although breaks with 5′ termini occurred in spite of the presence of DNP, these breaks were not rejoined. In other experiments, we have found that DNP prevents the rejoining of radiation-induced DNA breaks (9).

A portion of the DNA from the original sample was used in the DNA polymerase reaction. The work of Englund, Deutscher, Jovin, Kelly, Cozzarelli, and Kornberg (24) showed that DNA polymerase adds nucleotides to the 3′-OH termini of the DNA molecule. Consequently, the DNA specific activity (^{3}H cpm/μg DNA) is proportional to the number of 3′-OH termini within the sample (8).

As the right upper panel shows, the DNA specific activity (^{3}H label) was greatest immediately after irradiation for cells in HBSS. With time the specific activity returns to control levels. The right lower panel shows that the DNA specific activity for cells in DNP was increased after irradiation. No real change in this value occurred during the 30 min postirradiation period. We interpret these results to indicate that DNP prevents the rejoining of DNA breaks characterized by 3′-OH termini.

The findings given in Fig. 6, then, suggest that the disappearance of 5′ termini is paralleled by the disappearance of 3′-OH termini, and that DNP prevents the disappearance of either 5′ or 3′-OH termini.

We should point out some discrepancies which exist between the ultracentrifugal data and the results of the DNA polymerase and polynucleotide kinase assays. Alkaline sucrose sedimentation analysis indicated that the rate of production of single-stranded DNA breaks was a factor of 6.67 greater for cells in HBSS as compared to DNP. The polynucleotide kinase and DNA polymerase assays, on the other hand, indicated these differences (HBSS vs. DNP) to be factors of 3.0 and 1.5, respectively. At the present, we do not have an adequate explanation for these discrepancies.

Another point concerns the use of the DNA polymerase reaction to measure the number of 3′-OH termini. As the work of Kelly, Cozzavelli, Deutscher, Lehman, and Kornberg (30) showed, double-stranded DNA serves as a template primer for *E. coli* DNA polymerase when the DNA contains a single strand break which has a 3′-OH terminus. Since the reaction involves considerable hydrolysis of the primer strand, it would not be proper to consider the DNA polymerase assay to measure the absolute number of 3′-OH termini unless we could guarantee that all rates of hydrolysis were the same. Since this cannot be done, it is not surprising that differences exist between the alkaline sucrose sedimentation data and the DNA polymerase results.

DISCUSSION

The addition of 1×10^{-4} M DNP to HBSS causes a profound reduction in the intracellular ATP concentration. This marked reduction was not seen when DNP was added to cells suspended in Eagle's growth medium. Furthermore, in the presence of serum the action of DNP is reduced to even a greater degree. Consequently, all experiments of the present study were conducted using HBSS cell suspensions, since a significant concentration of ATP remains in the presence of growth medium or medium containing serum.

The postirradiation rejoining of L-5178Y cells has recently been studied by Swada

and Okada (10). In addition to DNP these investigators used a variety of other metabolic inhibitors. Their results showed that the repair of radiation-induced single strand DNA breaks occurred in the presence of all metabolic inhibitors used, including DNP. For their studies the cells were irradiated with 10,000 rad of 1 Mvp X-rays and incubated after irradiation in Fischer's growth medium; the compounds under investigation were dissolved in the complete medium. Consequently, their finding of the inability of DNP to prevent DNA rejoining may be due to interference by the components of the medium.

In the present study, DNP dissolved in HBSS prevented rejoining of DNA breaks as demonstrated by alkaline sucrose sedimentation, as well as preventing the rejoining of breaks characterized by 5′-PO_4 and 3′-OH termini. Also, in the present study, we found the rate of production of single-stranded DNA breaks by a given dose to be lower when the cells were suspended in HBSS with DNP as compared with HBSS without DNP. This difference may possibly be explained by a decrease in the enzymatic activity of nucleolytic enzymes present in the cell. If a DNA strand at some point undergoes modification by ionizing radiation, either through direct or indirect processes, a possibility exists that some damage may be produced. This may be visualized as base damage, damage to deoxyribose, or a bond alteration in the phosphodiester backbone. This abnormality or radiation lesion perhaps represents a site which is not compatible with the tertiary structure of the remaining macromolecule. As a result, this abnormal site may become liable to attack by nucleolytic enzymes present in the cell and as a consequence, actual single strand breaks are introduced at the primary lesion site.

The existence of nucleolytic activity as part of the repair mechanism has been suggested by several investigators (31–33). Achey and Pollard (34) using bacteria showed that radiation induces DNA breakdown which starts immediately after irradiation. Also, the breakdown is essentially complete within an hour after irradiation if the cultures are maintained at 37°C. Similar results have been reported by others (35–37). Consequently, the decrease in the rate of formation of single-stranded DNA breaks may be the direct result of the depression of available ATP. This view is supported by data from Drakulic and Kos (38) who reported experiments with compounds which uncouple oxidative phosphorylation. These investigators used *E. coli* suspensions containing 5×10^{-4} M DNP and reported an elimination of postirradiation DNA degradation.

For mammalian cells, the issue is less clear. Several investigators (39–41) have indicated the difficulty in finding DNA degradation after doses below 1000 rad. In fact, doses of the order of 10,000 rad or more are needed to cause a measurable degree of DNA degradation above control. Treatment with DNP does not cause any change in the postirradiation response of L-cells, with respect to DNA degradation (41).

Previous studies with L-cells and rat sarcoma cells (7–9) suggest that energy-dependent nucleolytic enzymes are active in the postirradiation period. The addition

of 10^{-4} M DNP to HBSS prevented the appearance of DNA breaks characterized by 5′ termini and 3′-OH termini after 1000 rad.

When the radiation dose is increased to 10,000 rad (as in the present study), the presence of DNP did not prevent the appearance of DNA breaks—as demonstrated by alkaline sucrose sedimentation, the polynucleotide kinase, and DNA polymerase methods. Possibly the ionization, per se, is sufficient to cause many of the breaks. The difference in response between cells in HBSS and cells in DNP would reflect the magnitude of the influence of the energy-dependent nucleolytic processes. As a second possibility, perhaps the small amount of intracellular ATP remaining during DNP treatment is enough to activate the nucleolytic enzyme systems. Regardless of the reason, however, postirradiation DNA breaks do occur when the cells are suspended in DNP. Since the DNP treatment is sufficient to prevent rejoining, we believe that the breaks, most likely, are caused by the ionization, per se. Oishi has recently extracted an ATP-dependent deoxyribonuclease from *E. coli* (42). The absence of ATP virtually obliterated the enzyme activity. Although the properties of the enzyme have not been completely worked out, such an enzyme could be important in the repair of injury to DNA.

The inhibition by DNP of the rejoining of DNA breaks characterized by 5′ termini is reversible (9). In earlier studies L-cells were irradiated in HBSS and then quickly transferred to DNP; the DNA breaks showed no evidence of rejoining during a 2 hr holding period in the DNP. These cells were then transferred back into HBSS; the breaks were rapidly rejoined after the transfer. These findings are in keeping with survival studies with DNP (28). Namely, DNP, at 1×10^{-4} M concentration does not cause biologic injury. Once it is removed, the cell functions normally.

When the cells were suspended in HBSS, rejoining occurred after doses of 1000 rad and 10,000 rad. The present data agree with earlier results that the disappearance of 5′ termini is paralleled by a disappearance of 3′-OH termini. These findings suggest that the breaks are characterized by 5′-PO_4–3′-OH termini and that they are readily repaired. It should be emphasized, however, that the appearance and disappearance of these breaks does not prove that they are rejoined in a simple one-step process. Possibly the rejoining process is a series of steps and the 5′-PO_4–3′-OH termini are the result of prior steps.

The alkaline sucrose sedimentation experiments showed results which quantitatively parallel the polynucleotide kinase and DNA polymerase results. For cells suspended in HBSS there was an immediate depression of the single strand DNA molecular weight during the first postirradiation minute which was followed by a rapid return to the control molecular weight. In the presence of 1×10^{-4} M DNP in HBSS no rejoining activity was observed through 30 postirradiation min. As suggested before, these findings show that the rejoining system in L-cells is quite dependent on an available supply of ATP. The notion is supported by Matsudaira and Furuno who found that ATP is necessary for the rejoining of radiation-induced DNA breaks by Ehrlich ascites tumor cells (43).

Accepting the reservations indicated before, we believe our findings are compatible with the concept that many DNA breaks caused by radiation are characterized by 5′-PO_4–3′-OH termini at some point in their existence and that the enzyme DNA ligase rejoins these breaks. Since DNA ligase requires a high energy cofactor (such as ATP or nicotinamide adenine dinucleotide [21, 44]) for its action, we would anticipate that the effective reduction of intracellular ATP concentration by DNP should prevent the rejoining of DNA breaks. Also, DNA ligase rejoins only single strand breaks; it is unable to rejoin double strand breaks. Since Lindahl and Edelman (45) found DNA ligase in mammalian tissue, the existing evidence is compatible with the notion that DNA ligase is important in the rejoining of single strand DNA breaks by mammalian cells.

Certain (somewhat negative) aspects of the preparation of the DNA for the polynucleotide kinase and the DNA polymerase assays should be pointed out. The several treatments (RNase, pronase, etc.) must be used to provide DNA which can serve as a substrate for the polynucleotide kinase and DNA polymerase reactions. Unfortunately, this handling causes a reduction of the DNA molecular weight by a factor of 10 (Moss and Dalrymple—unpublished observations). Also, as shown by Weiss, Thompson, and Richardson (46), hydrodynamic shear can introduce breaks characterized by 5′ termini. Very likely, 3′-OH termini are also produced. We should emphasize, however, that the irradiated and nonirradiated cells (and DNA) were handled in an identical manner. Also, every irradiated sample was paralleled by a control. As Fig. 6 shows, the control values were very stable. Radiation, on the other hand, produced a marked change in both the $^{32}PO_4$ and ^{3}H specific activities.

We should point out that the rate of rejoining of 5′ and 3′-OH termini seems to be somewhat slower than the rate of rejoining of DNA breaks as measured by alkaline sucrose gradients. Very likely, this difference is a consequence of the limits of resolution of the two types of assays (enzyme vs. ultracentrifuge). Between 0 and 10 min postirradiation the bulk of the 5′ and 3′-OH termini disappear; additional change, although slight, continues through 30 min. Possibly the ultracentrifuge is not able to resolve differences of small magnitude such as may exist between irradiated and control DNA at 10 and 30 min postirradiation. Another possibility concerns the production of additional 5′ and 3′-OH termini as a consequence of the handling of the DNA before the assays. When all of the factors are considered (including the possibility that the 5′ and 3′-OH termini may occur as intermediate steps) DNP prevents the rejoining of DNA breaks identified by three methods—alkaline sucrose gradients, polynucleotide kinase, and DNA polymerase.

For completeness, another, though less likely explanation for the production of some of the DNA breaks should be mentioned. Possibly the DNA molecule (or the nucleoprotein complex) sustains damage from radiation which, under the hydrodynamic shear forces (or enzymatic treatments) used, becomes expressed as breaks characterized by 5′-PO_4–3′-OH termini. Because of the close parallelism between the results of the alkaline sucrose sedimentation, polynucleotide kinase, and DNA

polymerase assays, together with known energy dependence of DNA ligase, we believe that radiation is responsible for many DNA breaks characterized by 5′-PO_4–3′-OH termini and that these breaks are rejoined by DNA ligase.

Perhaps the most difficult problem concerns the actual biologic importance of the rejoining process. Earlier experiments from our laboratory showed that L-cells are able to repair sublethal radiation injury while treated with 1×10^{-4} M DNP in HBSS (27). This would mean, then, that the repair of sublethal injury progresses while DNA rejoining is inhibited. At present, we are not able to rationalize this dichotomy.

Another point concerns the notion that radiation produces many types of damage (including breaks) to the DNA molecule. As recent work by Kapp and Smith (47, 48) suggests, breaks characterized by 3′-OH–5′-PO_4 termini do not represent the only type of break present.

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REFERENCES

1. McGrath, R. A., and R. W. Williams. 1966. *Nature (London).* **212**:534.
2. Kaplan, H. S. 1966. *Proc. Nat. Acad. Sci. U.S.A.* **55**:1442.
3. Lett, J. T., I. Caldwell, C. J. Dean, and P. Alexander. 1967. *Nature (London).* **214**:790.
4. Lohman, P. H. M. 1968. *Mutat. Res.* **6**:449.
5. Humphrey, R. M., D. L. Steward, and B. A. Sedita. 1968. *Mutat. Res.* **6**:459.
6. Dalrymple, G. V., J. L. Sanders, A. J. Moss, Jr., M. L. Baker, and K. P. Wilkinson. 1969. *Biochem. Biophys. Res. Commun.* **35**:300.
7. Dalrymple, G. V., J. L. Sanders, A. J. Moss, Jr., M. L. Baker, and K. P. Wilkinson. 1969. *Biochem. Biophys. Res. Commun.* **36**:284.
8. Dalrymple, G. V., J. L. Sanders, A. J. Moss, Jr., and K. P. Wilkinson. 1970. *Biochem. Biophys. Res. Commun.* **39**:538.
9. Dalrymple, G. V., M. L. Baker, J. L. Sanders, A. J. Moss, Jr., J. C. Nash, and K. P. Wilkinson. 1970. *J. Theor. Biol.* **28**:121.
10. Swada, S., and S. Okada. 1970. *Radiat. Res.* **41**:145.
11. Elkind, M. M., and C. Kamper. 1969. *Radiat. Res.* **39**:518.
12. Sanford, K. K., W. R. Earle, and G. D. Likely. 1948. *J. Nat. Cancer Inst.* **9**:229.
13. Eagle, H. 1959. *Science (Washington).* **130**:432.
14. Noll, H. 1967. *Nature (London).* **215**:360.
15. Burgi, E., and A. D. Hershey. 1963. *Biophys. J.* **3**:309.
16. Charlesby, A. 1954. *Proc. Roy. Soc. London Ser. A. Math. Sci.* **224**:120.
17. Merchant, D. J., R. H. Kahn, and W. H. Murphey. 1964. Handbook of Cell and Organ Culture. Burgess Publishing Co., Minneapolis, Minnesota. 165.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193**:265.
19. Richardson, C. C. 1966. *J. Mol. Biol.* **15**:49.
20. Richardson, C. C. 1965. *Proc. Nat. Acad. Sci. U.S.A.* **54**:158.

21. WEISS, B., and C. C. RICHARDSON. 1967. *Proc. Nat. Acad. Sci. U.S.A.* **57**:1021.
22. BURTON, K. 1968. *In* Methods in Enzymology. L. Grossman and K. Moldave, editors. Academic Press, Inc., New York. **12**(Pt. B):263.
23. GLYNN, I. M., and J. B. CHAPPEL. 1964. *Biochem. J.* **90**:147.
24. ENGLUND, P. T., M. P. DEUTSCHER, T. M. JOVIN, R. B. KELLY, N. R. COZZARELLI, and A. KORNBERG. 1969. *Cold Spring Harbor Symp. Quant. Biol.* **23**:1.
25. APOSHIAN, H. V., and A. KORNBERG. 1962. *J. Biol. Chem.* **237**:519.
26. ADDANKI, A. S., J. F. SOTOS, and P. D. REARICK. 1966. *Anal. Biochem.* **14**:261.
27. DALRYMPLE, G. V., J. L. SANDERS, M. L. BAKER, and K. P. WILKINSON. 1969. *Radiat. Res.* **37**:90.
28. DALRYMPLE, G. V., J. L. SANDERS, and M. L. BAKER. 1967. *Nature* (*London*). **216**:708.
29. CORRY, P. M., and A. COLE. 1968. *Radiat. Res.* **36**:528.
30. KELLY, R. B., N. R. COZZARELLI, M. P. DEUTSCHER, I. R. LEHMAN, and A. KORNBERG. 1970. *J. Biol. Chem.* **245**:39.
31. SZYBALSKI, W. 1967. *Radiat. Res.* **7**(Suppl.):147.
32. HAYNES, R. H. 1966. *Radiat. Res.* **6**(Suppl.):1.
33. DALRYMPLE, G. V., J. L. SANDERS, and M. L. BAKER. 1968. *J. Theor. Biol.* **21**:368.
34. ACHEY, P. M., and E. C. POLLARD. 1967. *Radiat. Res.* **31**:47.
35. STUY, J. H. 1961. *Radiat. Res.* **14**:56.
36. FRAMPTON, E. W., and D. BILLEN. 1966. *Radiat. Res.* **28**:109.
37. MCGRATH, R. A., R. W. WILLIAMS, and D. C. SWARTZENDRUBER. 1966. *Biophys. J.* **6**:113.
38. DRAKULIC, M., and E. KOS. 1966. *Radiat. Res.* **27**:2.
39. PAINTER, R. B. 1968. Proceedings of the Monaco Symposium. International Atomic Energy Agency, Vienna. 91.
40. LITTLE, J. B. 1968. *Int. J. Radiat. Biol. Related Stud. Phys. Chem. Med.* **6**:591.
41. DALRYMPLE, G. V., J. L. SANDERS, M. L. BAKER, K. P. WILKINSON, and R. A. WALLS. 1969. *Radiat. Res.* **40**:112.
42. OISHI, M. 1969. *Proc. Nat. Acad. Sci. U.S.A.* **69**:1292.
43. MATSUDAIRA, H., and I. FURUNO. 1970. Fourth International Congress of Radiation Research, Evian, France. Abstr. 541.
44. OLIVERA, B. M., and I. R. LEHMAN. 1967. *Proc. Nat. Acad. Sci. U.S.A.* **57**:1426.
45. LINDAHL, T., and G. M. EDELMAN. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **61**:680.
46. WEISS, B., A. THOMPSON, and C. C. RICHARDSON. 1968. *J. Biol. Chem.* **243**:4556.
47. KAPP, D. S., and K. C. SMITH. 1969. *Int. J. Radiat. Biol. Related Stud. Phys. Chem. Med.* **14**:567.
48. KAPP, D. S., and K. C. SMITH. 1970. *Radiat. Res.* **42**:34.