TWO FORMS OF REPAIR OF DNA IN MAMMALIAN CELLS FOLLOWING IRRADIATION

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ABSTRACT When Chinese hamster cells are lysed on top of an alkaline sucrose gradient, in time a fairly discrete DNA-containing molecular species is released from an apparently more complex material. Small doses of X-radiation speed the resolution of this complex while large doses degrade the material released from it. Incubation after irradiation reverses both effects.

INTRODUCTION

The lysis of cells on top of an alkaline sucrose gradient leads to the release of molecules of denatured DNA which, for *Escherichia coli*, are an appreciable fraction of the genome (1). This technique, presumably limited to denatured DNA, in principle affords a way of examining radiation or other effects relative to sedimentation properties of molecules considerably larger than those obtainable by other methods. That this procedure is applicable to the DNA from mammalian cells was shown first by Lett and his collaborators (2) and since then by a number of others (3–7). A repair process relative to single-strand breakage by radiation was also demonstrated in these instances, although the X-ray doses used were so large that the relevance of this DNA repair to repair measured functionally (e.g., by colony formation) is questionable. Large doses (5000–10,000 rad or more) were considered to be necessary to reduce the strands to sizes small enough to permit reproducible results with the relatively small tubes which fit the swinging-bucket rotors commonly employed.

We, too, have employed the technique of alkaline sucrose gradient analysis with mammalian cells and report here several new findings. First, under appropriate conditions of cell lysis, *unirradiated* cells release, in time, molecules fairly homogeneous in size whose weights are estimated to be $\sim 2.5 \times 10^8$ daltons. Second, consistent with a unit of this size, the dose at which radiation-induced single-strand breaks become readily demonstrable is $\sim$1400 rad, a dose considerably lower than

those used in earlier studies (1–6). Third, at even smaller doses, we find a dose-dependent effect on sedimentation properties. And last, while single-break repair is readily made evident after doses equal to or greater than ~1400 rad, we have observed another repair process quite different from this after lower doses.

MATERIALS AND METHODS

For these studies we used subline *V79-753B-3* of a line of Chinese hamster cells originally designated by us *V79-1* (8–10). These cells were cultured essentially after the methods of Puck and collaborators (11) in a modified Eagle's medium (12) containing 15% fetal calf serum. Cells double in number in ~9 hr on glass or plastic. DNA was essentially uniformly labeled by growing cells for ~19 hr in thymidine-^{3}H; high specific-activity material (>6 Ci/mM) was added to give a final concentration of ~0.25 μCi/ml, a concentration which had at most a small effect on growth rate and yielded ~1 cpm per cell. After this, cells were incubated in normal medium for 30 min to minimize unincorporated label and replicons containing label but only partially replicated.

While still attached, cells were irradiated at ice temperature with 55 kv X-rays (722 rad/min) (9). Then, they were either suspended immediately or first incubated further to permit repair. Suspension was effected by treating cells for 20 min at ~3°C with a standard trypsin solution (9–10); trypsin action was terminated by adding an equal amount of medium. Thus, cells were kept at, or close to, ice temperature from the beginning of their trypsinization to the time when they were placed on a gradient.

Linear gradients were formed in the 5 ml tubes used in a SW-39 rotor (Beckman Instruments, Inc., Palo Alto, Calif.): 5–20% sucrose in a water solution of 0.1 M NaOH, 0.9 M NaCl, and 0.003 M Na_2EDTA. 0.25 ml of a lysing solution was added on top of 4.8 ml of gradient solution: 0.05 M NaOH, 0.95 M NaCl, and 0.01 M Na_2EDTA (pH 12). On this, 0.025 ml of a cell suspension containing 3000–5000 cells (0.03–0.05 μg DNA) was carefully placed. Following lysis for various periods under ambient conditions (24–28°C), tubes in an SW-39 rotor were loaded into an L2-50HV Spinco ultracentrifuge (Beckman Instruments, Inc., Spinco Div.) and spun initially at 4000 rpm (without evacuation) for 15 min to facilitate cooling to 12–13°C. The rotor speed was then increased to 38,000 rpm for 60 min (with evacuation).

Thirty 10-drop fractions were pumped off from the top of each tube, collected on one-inch diameter glass fiber filters, and dried. In some experiments this was followed by extraction with 5% cold perchloric acid (PCA), ethanol, and acetone. However, aside from some improvement in counting efficiency, this did not change the patterns observed and hence, extraction was discontinued. The filters were placed in standard 20-ml vials and counted in a toluene-based counting solution (13). To determine the total amount of radioactivity placed on each gradient, 0.025-ml aliquots of cell supensions were placed directly on filters to which were added aliquots of gradient solution about equal to the ten drops collected per filter. These were dried and counted. In the figures that follow, "P.Y." stands for the "per cent yield": the per cent of the radioactivity in the cell sample, initially placed on a gradient, which was recovered in the 30 fractions from that gradient.

RESULTS

Effect of Small Doses

In descending order, the left half of Fig. 1 shows the effect of increasing doses of X-rays (no repair). The pattern for no irradiation shows two main peaks. (The

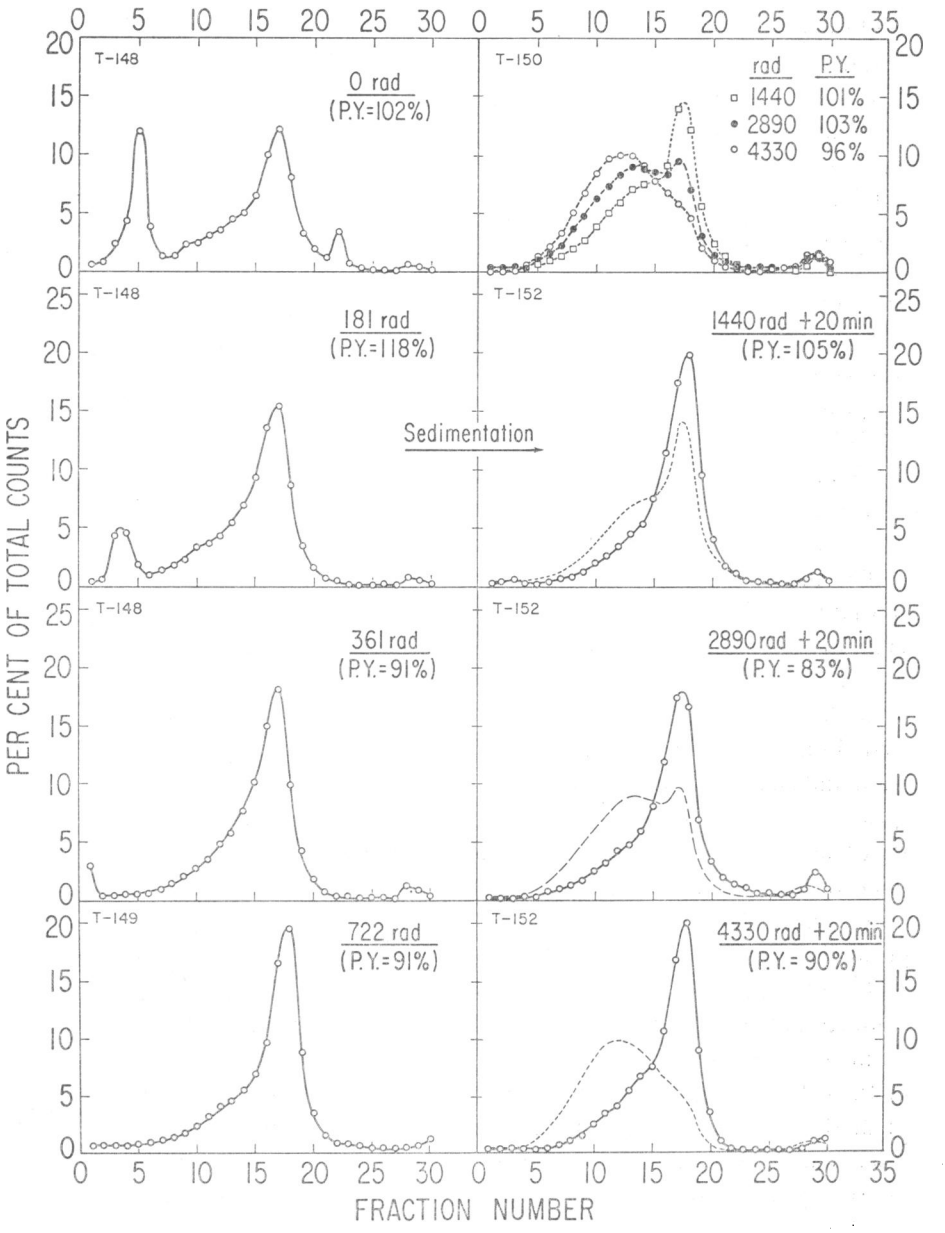

FIGURE 1 Alkaline sucrose gradients of thymidine-^{3}H containing material from Chinese hamster cells irradiated with the doses shown or irradiated and then incubated at 37°C for 20 min for repair. A separate cell suspension was used for each gradient and, in each case, the lysis period was 165 min. Survivals which can be measured by colony formation are: 181 rad, ~60%; 361 rad, ~25%; 722 rad, ~3%; and 1440 rad, ~0.01%. (P.Y. = per cent of the radioactivity in the cell suspension placed on a gradient which was recovered. Sedimentation was from left to right.)

small peak at fraction 22 was only occasionally observed and is not discussed further.) We call the peak whose mode is at fractions 17–18 the "principal" or "main" peak. The peak at fraction 5 depends on the lysis conditions, as we explain presently, and we call this the "complex." (Occasionally, small peaks are observed at fraction 28–30. These, too, we ignore since they may result from a small amount of labeled material displaced from the bottom of the tube by the pump-off solution.)

As the dose is increased from zero to 722 rad, the patterns show two things. First, the main peak increases in height and moves slightly in the direction of a larger sedimentation value. Second, the complex gets smaller in size until it is no longer present for doses greater than about 361 rad. Since the amount of radioactivity recovered in each case is essentially equal to the initial charge (P.Y.'s $\simeq$ 100%), the preceding progression means that as DNA is lost from the complex, it appears in the principal peak. And, consistent with other observations that we have made that DNA is not lost from irradiated Chinese hamster cells, there is no indication of DNA breakdown in Fig. 1. No radioactivity appears at the top of the gradients even after doses as high as 4330 rad.

Two points help us to understand this dose-dependent change relative to the complex. The first is that the general features of this change are demonstrable with unirradiated cells at increasing lysis times as well as at a fixed lysis time with increasing doses, as in Fig. 1. We will show elsewhere that after cells are placed on a gradient, with time, the main peak appears and grows in height at the expense of the complex. This is most clearly demonstrated when a single suspension of cells is used. Other things being equal, however, with different cell suspensions the position and shape of the peak we call the complex varies somewhat. Occasionally, the peak is very sharp (i.e., almost all of the radioactivity is in one 10-drop fraction); occasionally, the complex consists of more than one sharp peak and from gradient-to-gradient the complex might vary from an average position by a few fractions. Nevertheless, as radioactivity is lost from the complex with lysis time it appears in the main peak. For present purposes, we base our use of the term "complex" for this material in part on its sedimentation characteristics, which suggest some degree of nonspecific aggregation during lysis, and in part on the fact that with time (or dose, see below), a species of molecules, which account for essentially all of the cell's DNA, is resolved from it. The sedimentation properties of the material in the main peak, in contrast to those of the complex, are predictably reproducible and consistently yield a relatively narrow peak close to fractions 17–18.

The second point which bears on the dose-dependent pattern change in the left half of Fig. 1 is that ionizing radiation has the property of indiscriminantly being able to break molecular bonds because of the large energies expended per absorption event (14). It is hard to conceive of bond breakage leading to larger sedimentation velocities — ordinarily implying larger molecular weights — unless such breaks result in a change in conformation and/or density. Relative to the main peak, increasing lysis time with no irradiation has approximately the same effect

as increasing irradiation with a fixed lysis time. It seems reasonable to conclude therefore, that radiation-induced bond breakage (in the low dose range) has the main effect of speeding the action of the lysis process by opening up a complex structure or aiding in its dissociation. The DNA present in the peak we call the complex very likely has a molecular weight at least equal to the DNA in the main peak. Still, the material in the complex could sediment a smaller distance if it had a lower density, a greater viscous drag, or both.

Single-Break Production

As noted, for doses up to 722 rad in Fig. 1, no appreciable change results in the main peak except for the fact that it gets taller and narrower as material initially in the complex is transferred to it. However, when larger doses are used, the shape of the main peak is changed in a progressive way as shown in the upper right panel of Fig. 1. With increasing dose, two effects become evident; the height of the main peak progressively decreases as a bulge forms and grows to the left. Clearly, this is what would be expected from single-break formation. Accompanying a reduction in sedimentation rate, there would be a broadening in the distribution of the latter as the number of breaks randomly produced increases with increasing dose. Moreover, a requirement for an apparent threshold dose of $\sim$1400 rad before single breaks become evident, is consistent with the size of the molecule corresponding to the main peak, as can be seen from the following.

From sedimentation measurements made with bacteriophage T_4, and from extrapolating (15, 16) to estimate the molecular weight of the main peak based upon a molecular weight for denatured T_4 of 6.7×10^7 daltons (17), we obtain a value of $\sim 2.5 \times 10^8$. The average DNA content in a cell in a log phase population is $\sim 1 \times 10^{-11}$ g. This corresponds to a gross molecular weight of 6×10^{12} daltons and hence, there are $\sim 2.4 \times 10^4$ molecules of size $\sim 2.5 \times 10^8$ daltons per cell. From physical measurements (14) we may estimate that 60–70 ev are expended per absorption event — an amount of energy more than adequate to break any molecular bond. Since 1 rad is equivalent to the deposition of 625 ev/(1×10^{-11} g), $\sim$2400 rad are required to produce $\sim 2.4 \times 10^4$ bond-breaking events per 2.4×10^4 molecules or per genome. (If some of these absorption events do not lead to single-strand breaks, a somewhat larger dose would be needed to produce about one strand break in each 2.5×10^8 daltons. Alternatively, the presence of bond breakage which does not result in single-strand breaks might be offset by a more efficient break production due to contributions from, for example, indirect effects.) Thus, from Poisson statistics we would expect the evidence of single-strand breakage to start to become apparent in the dose range 1000–2000 rad (66%–43% unbroken molecules, respectively) and that for larger doses we would expect that the average distance of sedimentation would become less as the gradient distributions become relatively broader. At doses appreciably below about 1000–2000 rad, we would not expect

to observe a significant change in the shape of the main peak in view of the limited resolution of this kind of measurement (only a slight bulge seems present to the left of the main peak in the 722 rad gradient).

In contrast to prior reports, the general features of the systematics and internal consistency contained in the foregoing is demonstrated in the first five panels of Fig. 1. Below 1440 rad no appreciable breakage is apparent in the main peak while at this and larger doses, there is a progressive loss of the peak at fractions 17–18 accompanied by a buildup of slower sedimenting material (lower molecular weight). At doses of ~722 rad or less, there is an apparent lack of breakage because these doses, on the average, yield an insufficient number of breaks to produce a detectable change in the sedimentation rate of molecules of size 2.5×10^8 daltons. Relative to the complex, however, doses of 361 rad or less *do* produce a progressive change. This alone suggests that the complex has a size a good deal larger than the material which constitutes the main peak.

Repair of Strand Breaks

Each of the gradient patterns in the upper right of Fig. 1 is repeated in the lower right panels. Also shown is the result when, after each of the doses shown, cells were incubated for 20 min for repair. In each case, the bulge due to DNA breakage has essentially completely disappeared, indicating, as demonstrated with other mammalian cells after larger doses (2–6), that these cells rapidly repair single-strand breaks. We note again that after the doses shown and for periods appreciably longer than 20 min, no degradiation of DNA is evident with these cells. The patterns shown, therefore, represent the total amount of DNA present in cells at the time of irradiation.

"Repair" of the Complex

Incubation for repair also leads to changes in sedimentation after doses smaller than those needed to show single breaks. With the exception of the lower right panel, all of the data in Fig. 2 were obtained after 60-min lysis periods (instead of 165 min, Fig. 1). As a result, cells suspended immediately after irradiation with 361 rad show a larger amount of label in the complex than in Fig. 1. This by itself is indicative of the resolution of the complex with time to which we have already referred. But even after only a 60-min lysis period, doses of 722 and 1440 rad each yield a pattern failing to show a significant amount of complex. However, in each instance, a 20-min repair period results in the reappearance of a complex with a concomitant decrease in the main peak. Consistent with the data in Fig. 1, the lower right panel in Fig. 2 shows the effect, on the same cells used in the lower left panel, of a longer lysis time. The complex which had reformed during the repair period, and is event after 60 min of lysis, is resolved after lysis for 165 min.

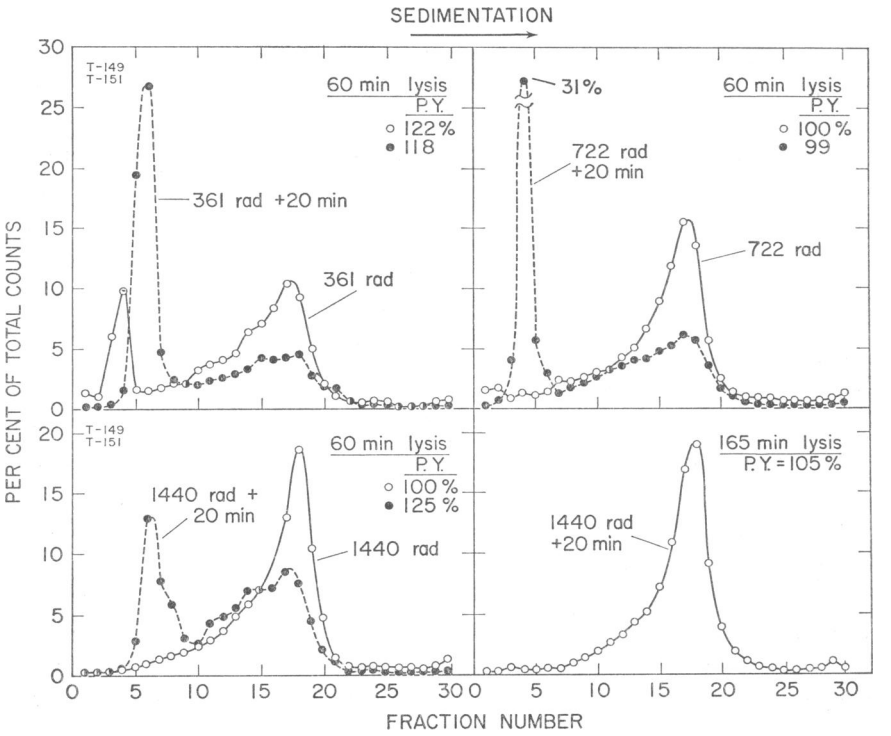

FIGURE 2 Alkaline sucrose gradients of DNA from Chinese hamster cells irradiated with the doses shown or irradiated and then incubated for 20 min. Note the different lysis times. (Sedimentation was from left to right.)

DISCUSSION

From autoradiographic measurements (18, 19), and from extrapolations to zero dose of molecular weight changes measured in radiation experiments (2), it has been estimated that the largest DNA molecules obtainable from mammalian cells are $\sim 1 \times 10^{10}$ daltons. We observed a molecular weight about one-fortieth of this, which suggests that there are many regions along the length of a chromatin thread which are labile under the lysis conditions used. It is possible, of course, that these regions bear no relation to repetitive features along the length of a DNA molecule, although the relative sharpness of the main peak that we observed favors the contrary. Further, since we may surmise that points of replication initiation must differ in some way from regions within a replicon (20, 21), it is worth considering the possibility that such points are labile under the alkaline, high salt conditions we used. If this is the case, we would estimate $\sim 2.4 \times 10^4$ natural units per cell, i.e., $\sim 2.5 \times 10^8$ daltons/$(1 \times 10^{-11}$ g) of DNA. Before units of the foregoing size are resolved on an alkaline gradient, larger units no doubt exist, but nonspecific aggregations may prevent reliable determinations of sedimentation properties under the conditions we have used.

It is interesting that a molecular size of about 2.5×10^8 daltons was also observed with unirradiated *E. coli* (1). Although this agreement might be quite fortuitous, it is worth noting the implication that a molecule of this size *might* be a fundamental building block in bacteria as well as mammalian cells.

Concerning the apparently complete repair of single breaks, several implications follow. If this repair is in fact complete, it would mean that two-strand breaks (as well as single-strand breaks) are repaired and, therefore, are unrelated to cell killing. While this might be true, we do not believe that the kinds of measurements presented have enough resolution to permit the conclusion that absolutely *all* breaks are repaired. For example, enough single breaks may remain open to be consistent with a double-break model of cell killing. Further, *mis*repair of single or double breaks would probably not be detected. However, since the gross effect of postirradiation incubation after doses of ~1400 rad or more is a return to the sedimentation pattern of unirradiated cells, then, on the assumption that this represents, in large measure, a true reversal of the effect of irradiation, our data are consistent with one or more of the following: (*a*) these cells have a DNA ligase which lacks substrate specificity since the energies involved in radiation absorption are large enough to break bonds indiscriminately; (*b*) there are many enzymes which have ligase-type activity to account for the array of substrates which might result from radiation absorption; (*c*) molecular energy transfer mechanisms, or indirect effects, lead primarily to the same type of bond breakage and, consequently, the same substrates for ligase action; or (*d*) before single breaks are repaired, nucleases prepare lesions for repair replication followed by ligase action so that specific requirements for substrate specificity are met.

In addition to single-strand rejoining, our results show that cells perform a repair-type process which involves a considerably larger molecular size, judging from the data presented relative to the complex. Since we see clear effects for a dose as low as 181 rad (~60% survival) — one-eighth the dose needed to show the onset of single breaks — we may estimate that this larger target is about ($8 \times \sim 2.5 \times 10^8 =$) $\sim 2 \times 10^9$ daltons provided that only one absorption event is needed to affect the complex. It would seem likely, however, that to produce a change in conformation and/or density, more than one break would be needed and consequently, 2×10^9 daltons is probably a minimum estimate.

Relative to the complex, we have called the change — which results from incubation after irradiation — "repair" because the sedimentation pattern becomes more like that of unirradiated cells. The DNA in eucaryotes consists of structures of nucleoprotein which contain RNA and possibly membranous material as well. Extraction under conditions of neutral pH and low ionic strength initially leads to a gel probably because of the fibrous structure of the large molecules involved and cross-links between them (e.g., see reference 22). A gelatinous material is also obtained when chromatin is extracted under conditions of high pH and ionic strength, although under these conditions the cross-links are labile. It seems likely to us that

the DNA-containing material we call the complex is initially similar to chromatin and that from this, a material containing denatured strands of DNA is released in time. Our results show that small doses of X-rays speed this release probably because the breaks produced in the cross-linked structure make internal bonds more accessible to lytic action.

Although repair of single- or double-strand breaks is easy to visualize in a structure as well known as the double helix of DNA, this does not mean that other post-irradiation effects which constitute a return toward the unirradiated condition are less important with regard to cell proliferation. In mammalian cells the functional integrity of DNA may be dependent on its structural relationship to its molecular environment — a suggestion which we proposed earlier based upon interactive effects between actinomycin D and X-irradiation (13, 23, 24) — as well as upon the linear continuity of the molecule itself.

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REFERENCES

1. McGRATH, R. A., and R. W. WILLIAMS. 1966. *Nature (London).* **212**:534.
2. LETT, J. T., I. CALDWELL, C. J. DEAN, and P. ALEXANDER. 1967. *Nature (London).* **214**:790.
3. HUMPHREY, R. M., D. L. STEWARD, and B. A. SEDITA. 1968. *Mutat. Res.* **6**:459.
4. LOHMAN, P. H. M. 1968. *Mutat. Res.* **6**:449.
5. VEATCH, W., and S. OKADA. 1969. *Biophys. J.* **9**:330.
6. LEHMANN, A. R., and M. G. OMEROD. 1969. *Nature (London).* **221**:1053.
7. TERASIMA, T., and A. TSUBOI. 1969. *Biochim. Biophys. Acta* **174**:309.
8. ELKIND, M. M., and H. SUTTON. 1959. *Nature (London).* **184**:1293.
9. ELKIND, M. M., and H. SUTTON. 1960. *Radiat. Res.* **13**:556.
10. ELKIND, M. M., H. SUTTON, and W. B. MOSES. 1961. *J. Cell. Comp. Physiol.* **58** (Suppl. 1):133.
11. PUCK, T. T., P. I. MARCUS, and S. T. CIECIURA. 1956. *J. Exp. Med.* **103**:273.
12. EAGLE, H. 1955. *Science (Washington).* **122**:501.
13. ELKIND, M. M., W. B. MOSES, and H. SUTTON-GILBERT. 1967. *Radiat. Res.* **31**:156.
14. RAUTH, A. M., and L. A. SIMPSON. 1964. *Radiat. Res.* **22**:643.
15. BURGI, E., and A. D. HERSHEY. 1963. *Biophys. J.* **3**:309.
16. STUDIER, F. W. 1965. *J. Mol. Biol.* **11**:373.
17. RUBENSTEIN, L., C. A. THOMAS, and A. D. HERSHEY. 1961. *Proc. Nat. Acad. Sci. U.S.A.* **47**:1113.
18. SASAKI, M. S., and A. NORMAN. 1966. *Exp. Cell Res.* **44**:642.
19. HUBERMAN, J. A., and A. D. RIGGS. 1966. *Proc. Nat. Acad. Sci. U. S. A.* **55**:599.
20. HUBERMAN, J. A., and A. D. RIGGS. 1968. *J. Mol. Biol.* **32**:327.
21. TAYLOR, J. H. 1968. *J. Mol. Biol.* **31**:579.
22. SWINGLE, K. F., and L. J. COLE. 1968. *In* Current Topics in Radiation Research. M. Ebert and A. Howard, editors. North Holland Publishing Company. Amsterdam. **4**:189.
23. ELKIND, M. M., H. SUTTON-GILBERT, W. B. MOSES, and C. KAMPER. 1967. *Nature (London).* **214**:1088.
24. ELKIND, M. M., C. KAMPER, W. B. MOSES, and H. SUTTON-GILBERT. 1967. *Brookhaven Symp. Biol.* **20**:134.