Cyclic Labeling of Mammalian Cell DNA after X-Irradiation: Labeling with Specific Precursors (34215)

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Recently, we reported that relatively low X-ray doses produce a cyclic ^{32}P labeling of DNA in mouse L cells (1). This response appeared for cells which had been starved in glucose-free salt solution or treated with 2,-4-dinitrophenol (DNP), but not for cells irradiated under growth conditions. As a continuation of this work, we have studied the uptake characteristics of precursors which specifically label nucleic acids.

Our results suggest that only precursors involved in the immediate formation of adenosine triphosphate show a cyclic labeling. This finding agrees with the previous contention that cyclic ^{32}P labeling reflects the action of DNA ligase, an enzyme which rejoins single-strand breaks in the DNA molecule. This enzyme requires a phosphylated cofactor.

Cell culture. The L-929 mouse fibroblast was used throughout these studies. Details for the growth and maintenance of these cells have been described elsewhere (2). A cell suspension was used for the labeling experiments because it represented a homogeneous population from which control and irradiated cells could be sampled. The cell density in the suspension was $1.5–3.0 \times 10^6$ cells/ml. A water bath maintained the temperature at 37°. In this system the cells were continuously labeled by adding the precursor directly to the suspension. This procedure permitted continuous labeling over the course of the experiment. At predetermined times, 2.0-ml samples were collected from the suspension and the labeling was stopped with cold 0.3 *N* perchloric acid.

Biochemical analysis. The cellular fractions of interest were separated by a modified Schmidt-Thannhauser method (3). When labeled specific precursors were used, the total acid-insoluble fraction was analyzed. Since each sample contained a constant number of cells, the uptakes are expressed as cpm/10^6 cells. The acid-insoluble fraction was digested in hyamine hydroxide (Packard Co., Downer Grove, Illinois). The radioactivity was measured in a Packard Model 3003 Liquid Scintillation Counter. The radioactive compounds used in these studies and their suppliers are listed below: Sodium phosphate–^{32}P, 25–30 mCi/mg-P, Abbott Laboratories, North Chicago, Ill.; adenosine-8-^{14}C, 32.2 mCi/m*M*, Schwarz Bioresearch, Inc., Orangeburg, N.Y.; deoxyadenosine–$^{3}H(G)$, 570 mCi/m*M*, deoxythymidine–^{3}H(methyl), 20,600 mCi/m*M*, deoxycytidine-5-^{3}H, 14,700 mCi/m*M*, Nuclear-Chicago Corp., Des Plaines, Ill.

Irradiations. Irradiations were performed with a 250 kVp X-ray machine. This machine had a dose rate of 125 R/min. The beam had an HVL of 0.5 mm Cu. The roentgen to rad conversion factor was 0.95.

Results and Discussion. Figure 1 illustrates the ^{32}P uptake into DNA for both control and irradiated cells. In each case, the cells had been starved in a glucose-free preparation of Hanks Balanced Salt Solution (HBSS). The top panel shows that the cells continue to incorporate ^{32}P into DNA despite starvation.

The center panel shows the effect of 100 rads on ^{32}P uptake in starved cells. After irradiation, the uptake remains essentially unchanged from the control until about 20

min postirradiation. At some time during the interval of 20–40 min postirradiation, a rapid uptake and loss of label occurred in DNA. Since we used continuous labeling, this finding must mean that label enters and then leaves this nucleic acid. The center panel shows replications of the experiment. The period of cyclic labeling usually lasts for less than 6 min. It has not been observed prior to 20 min or after 40 min postirradiation. At higher doses, the cyclic labeling becomes diffused in time, although several cycles may appear (1).

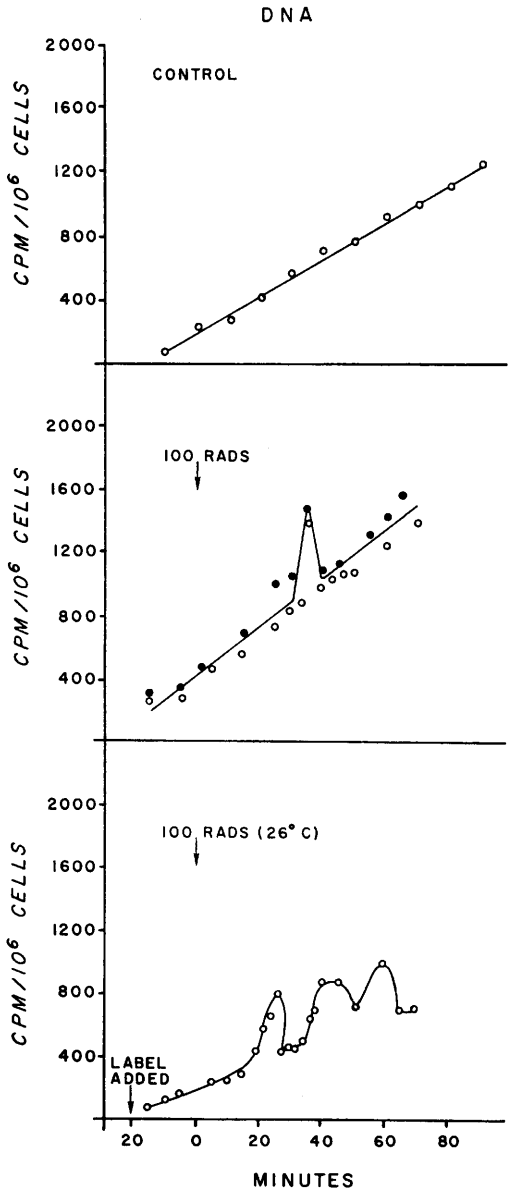

FIG. 1. Cyclic ^{32}P labeling of starved L cells. The cells were placed in glucose-free HBSS 60 min prior to irradiation. Labeling began 20 min before irradiation with the addition of ^{32}P to a final activity of 10 μCi/ml. At time 0, the cells received 100 rads. The open circles and closed circles indicate replications of the same experiment. The bottom panel shows ^{32}P uptake at room temperature (26°).

The bottom panel illustrates the ^{32}P uptake in cells which had been irradiated at room temperature. In this case, 100 rads produced several cycles of labeling which continued until the termination of the experiment. A similar effect also appears in DNP-treated cells (4). Lower temperatures then seem to effect a diffusion of the cyclic labeling. At 4°, neither DNA synthesis nor cyclic labeling could be detected.

We believe the cyclic ^{32}P labeling reflects the action of an enzyme, DNA ligase, which rejoins single-strand breaks in DNA. The rejoining process requires adjacent 3′-hydroxyl and 5′-phosphoryl termini in one strand of a double-stranded molecule. An enzyme–adenylate complex (E-AMP) appears to an intermediate (5). Weiss and Richardson (6) have postulated that ligase adds AMP to the 5′-phosphoryl terminus to form 5′ADP terminus which activates the DNA strand for rejoining. Szybalski (7) discussed the possible significance of this enzyme in the repair of radiation injury. If the AMP were labeled with ^{32}P, label would enter and then leave the DNA fraction. This enzymic mechanism possibly explains the transient labeling we have observed for DNA.

Kapp and Smith (8) have argued that single-strand breaks in the DNA phosphodiester backbone represent only a fraction of the radiation damage; consequently, DNA ligase alone could not effect repair. They feel that DNA degradation and new DNA synthesis are essential features of radiation repair. Such a mechanism does operate in bacterial systems (9). Dalrymple (10) and Painter (11), however, failed to observe DNA degradation except at high radiation doses. Regardless of the mechanism involved,

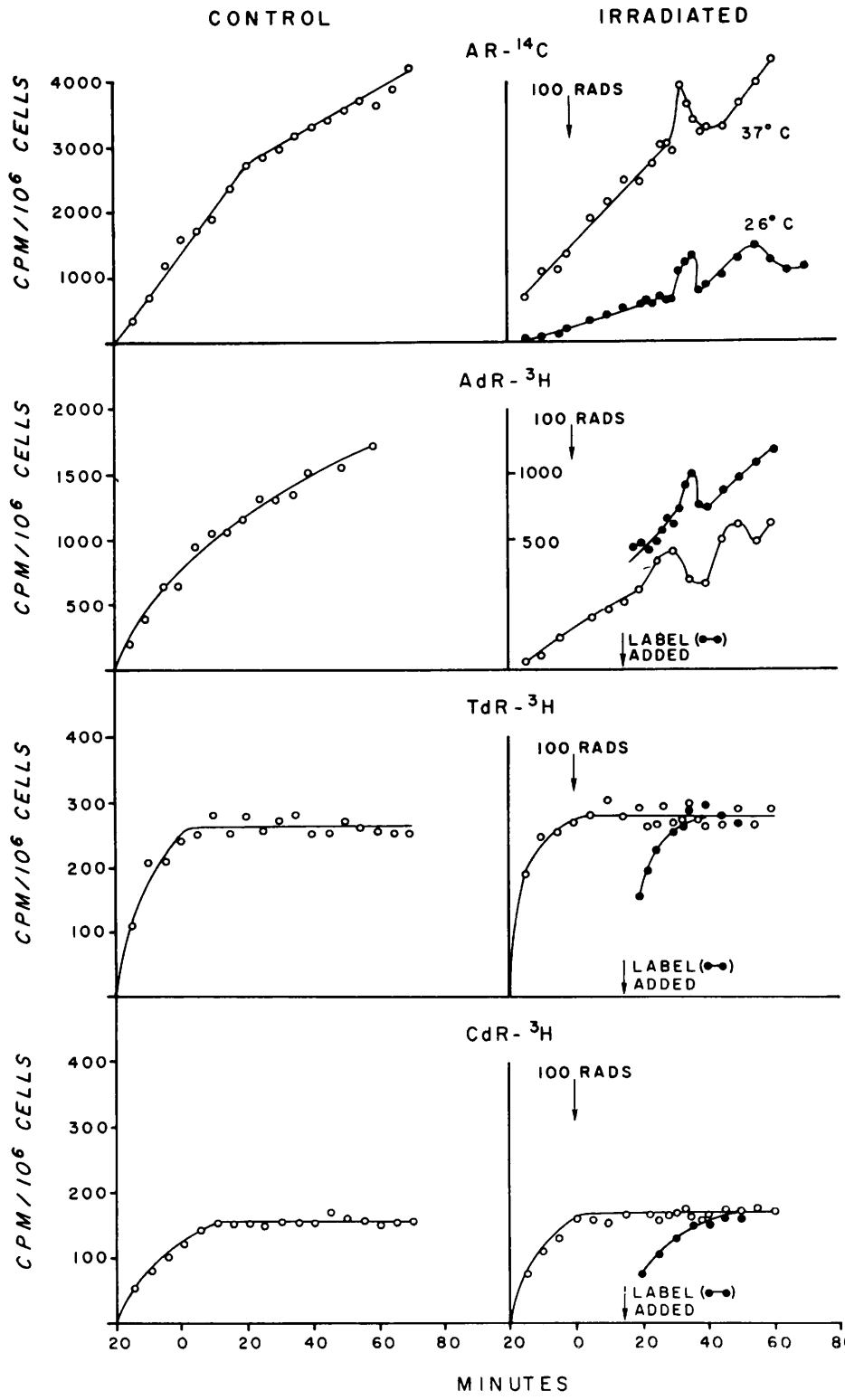
CONTROL
IRRADIATED
AR-^{14}C
100 RADS
37° C
26° C
AdR-^{3}H
100 RADS
LABEL (●—●)
ADDED
TdR-^{3}H
100 RADS
LABEL (●—●)
ADDED
CdR-^{3}H
100 RADS
LABEL (●—●)
ADDED
CPM/10^{6} CELLS
4000
3000
2000
1000
2000
1500
1000
500
1000
500
400
300
200
100
400
300
200
100
20 0 20 40 60 80 20 0 20 40 60 80
MINUTES

FIG. 2. Cyclic labeling of starved L cells using specific precursors. The cells had been starved in glucose-free HBSS for 60 min prior to addition of the label. For the control cells (left panel), AR–^{14}C(0.2 μCi/ml) and AdR–^{3}H(0.4 μCi/ml) show either biphasic or curvilinear uptake. TdR–^{3}H(0.2 μCi/ml) and CdR–^{3}H(0.2 μCi/ml) uptake reach an early limiting value. For the irradiated cells (right panel), labeling began 20 min before irradiation. The inserted ordinate for AdR–^{3}H gives the uptake for the closed circles. The ordinate is offset to avoid overlap of the curves.

DNA ligase would still play a central role in repair since its action would be required for joining the newly synthesized polynucleotide to its proper place in DNA (7).

We have attempted to characterize further the cyclic labeling of DNA through the use of precursors which specifically label the nucleic acids. Figure 2 contains results of representative experiments. The left panels show the uptake patterns of adenosine (AR–^{14}C), deoxyadenosine (AdR–^{3}H), deoxythymidine (TdR–^{3}H), and deoxycytidine (CdR–^{3}H) for control cells. The right panels illustrate the uptake patterns for irradiated cells. In each case the cells had been starved in glucose-free HBSS for 60 min prior to irradiation. The dose used was 100 rads since it produces cyclic labeling during a reasonably predictable period of time.

The uptake of AR–^{14}C in control cells appears biphasic. After 100 rads, a cyclic labeling occurs around 30 min postirradiation (open circles). When the cells are irradiated at room temperature (closed circles), several periods of cyclic labeling occur. These results agree qualitatively with our observations on cyclic ^{32}P labeling.

AdR–^{3}H shows a curvilinear incorporation into the acid-insoluble fraction of control cells. An analysis of the various fractions indicated that at least 60% of the label can be found in RNA. This finding means that AdR must be readily converted to AR by the cells. Furthermore, we must assume that AdR uptake reflects AR incorporation for the most part. After 100 rads, the uptake of AdR–^{3}H has at least two periods of cyclic labeling. This particular experiment was one of the few instances in which more than one period of cyclic labeling could be detected after 100 rads. The closed circles represent the AdR–^{3}H uptake when labeling began 15 min postirradiation. A single cycle of increased labeling appears between 30–40 min postirradiation. Again, these results qualitatively agree with our observations on cyclic ^{32}P labeling.

The incorporation of TdR–^{3}H and CdR–^{3}H into the acid-insoluble fraction reached a limiting value early in the uptake period. This response complicated our study of cyclic labeling using these precursors. Cleaver (12) has reported a similar finding, which he attributed to inhibition of DNA synthesis in starved cells. Since we observed a continuous ^{32}P and AR–^{14}C uptake under the same conditions, we feel that inhibition of nucleic acid synthesis does not explain the limited uptake. Why TdR and CdR have a limited uptake, however, remains unclear.

Irradiation with 100 rads did not change the TdR–^{3}H and CdR–^{3}H levels in the acid-insoluble fraction after the limiting value had been reached (open circles). Also, we failed to observe any change from control when uptake began 15 min postirradiation (closed circles) and the cyclic labeling should have appeared before the uptake limit was reached.

Although the labeling with TdR and CdR remains somewhat inconclusive, we feel that the AR and AdR data support the contention that cyclic labeling represents the transient association of labeled AMP and DNA. This labeling could be effected through the action of DNA ligase.

Summary. Starved L cells show a radiation-induced cyclic labeling of the acid-insoluble fraction when adenosine and deoxyadenosine are used as labeled precursors. The response consisted of a rapid uptake and loss of label. A similar effect has also been reported for ^{32}P labeling of DNA.

These findings support the idea that X-irradiation of mammalian cells produces increased action of an enzyme which rejoins single-strand breaks in DNA. The enzyme mechanism involves formation of a transient

DNA–adenylate complex. In such a reaction, labeled adenylate should enter and then leave the DNA fraction. The results reported here agree with such a mechanism.

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