DEPENDENCE OF SINGLE-STRANDED DNA LENGTH ON STAGE OF GROWTH CYCLE IN *ESCHERICHIA COLI* B/r AND EFFECT OF IRRADIATION

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ABSTRACT Alkaline sucrose density gradient profiles of DNA from log phase *Escherichia coli* B/r (CSH) show a main peak with sedimentation coefficient at approximately 130S and a shoulder or second peak at approximately 90S. Incorporation of radioactive precursors into the 90S peak precedes their appearance in the main peak. The size of the second peak appears to be directly related to the rate of replication and it is not present in profiles of nondividing stationary phase cultures. The decrease in weight average molecular weight (Mw) of DNA produced by X-rays is also directly related to the rate of replication. It is greatest in log phase *E. coli* B/r and least in stationary phase cells, because of the efficiency of rejoining of radiation-induced single strand breaks in DNA of the latter cells.

INTRODUCTION

Variations in bacterial radiosensitivity during progression through the growth cycle were first described by Stapleton (1955), who found stationary phase cells more radioresistant than log phase cells. A similar result was obtained by Trgovčević and Kućan (1969) who related changes in radiosensitivity to the ability of cells to break down DNA during postirradiation incubation. DNA degradation is greater in irradiated log phase cells than in irradiated stationary phase cells, and this may be related to levels of nucleases which are higher in log phase than in stationary phase cells (Shortman and Lehman, 1964).

In a previous communication (Lehnert and Moroson, 1971), changes, after X-irradiation, in Mw of DNA of log phase *E. coli* B/r (CSH) were described. During that investigation it was observed that the shape of the alkaline sucrose density gradient profile of DNA was different at different stages in the growth cycle, and

further, that the amount of damage to DNA produced by a given dose of X-rays was not constant throughout the cycle. These observations prompted a detailed analysis of the sedimentation behavior in alkaline sucrose density gradients of DNA from bacterial cells at different stages in the growth cycle, the results of which are presented here. Changes in the molecular weight of DNA produced by the irradiation of cells at different stages in the growth cycle were also determined to attempt to correlate variations in DNA damage with known changes in radiosensitivity during the cell cycle.

MATERIALS AND METHODS

Procedures for irradiation and determination of survival of irradiated bacteria have been previously described (Moroson and Tenney, 1968). For labeling of *E. coli* DNA with thymidine-^{3}H an aliquot containing approximately 5×10^8 cells was inoculated into 20 ml of medium containing 5 μCi/ml thymidine-^{3}H (specific activity 17 Ci/mmole) and incubated at 37°C with aeration. Cells were harvested at times ranging from 1 hr (early log phase) to 5 hr (stationary phase). Cells were grown in M-9 medium (Adams, 1952) or M-9 containing 2.5 g/liter Difco Casamino acids (EM-9, Difco Laboratories, Detroit, Mich.). At the end of the labeling time cells were resuspended in 0.01 M Tris buffer, pH 8.0, and diluted to approximately 5×10^8 cells/ml. Before density gradient centrifugation, 1.0 ml of this suspension was converted to protoplasts (Lehnert and Moroson, 1971). Sucrose gradients (5–20%) were prepared containing 0.9 M NaOH, 0.1 M NaCl, and 0.005 M EDTA. 0.1 ml of 0.5 M NaOH and 0.1 ml of the protoplast suspension were layered on top of the gradient. The protoplasts lysed immediately and the tubes were then centrifuged for 80 min at 16°C at 30,000 rpm in the SW39 rotor of the Spinco model L centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). Fractions of four drops each were collected on filter paper discs which were dried, washed in cold TCA, ethanol, and ether, and counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The counting rate for each fraction was expressed as a percentage of the total counts recovered from the gradient and was plotted against distance from the meniscus to give a density gradient profile. Each sample was centrifuged in triplicate and the gradient profile was fitted by eye to the three sets of points which generally fell on the same continuous curve.

Calculations of sedimentation coefficient and Mw were done as previously described (Lehnert and Moroson, 1971) using the relationships:

$$s_{20,w} = \frac{(7.1 \times 10^{10})d}{\omega^2 t}, \qquad \text{(McGrath and Williams, 1966)}$$

and

$$s_{20,w} = 0.0528\ M^{0.4}. \qquad \text{(Studier, 1965)}$$

The extent of thymidine incorporation into DNA of cells at different stages in the growth cycle was measured by exposing the cells to a 15 sec pulse of thymidine-^{3}H, collecting the cells on Millipore filters (Millipore Corp., Bedford, Mass.), and measuring the radioactivity of the fraction insoluble in cold 5% TCA. When cells were pulse labeled, incorporation was terminated by rapidly pouring the cell suspension onto partially frozen 0.01 M Tris buffer containing KCN, to a final concentration 0.02 M KCN (Okazaki et al., 1968).

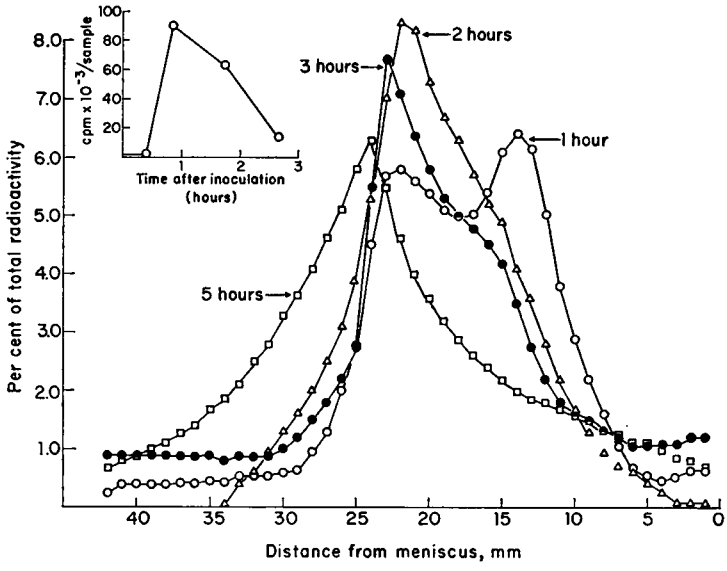

FIGURE 1 Alkaline sucrose density gradient profiles of *E. coli* B/r (CSH) DNA. Samples were taken at 1, 2, 3, and 5 hr after inoculation of an aliquot of late log cells into fresh medium containing radioactive thymidine. *Inset:* Incorporation of thymidine-^{3}H into the TCA-insoluble fraction during a 15 sec pulse at various times after inoculation.

RESULTS

Unirradiated Cells

Alkaline sucrose density gradient profiles were prepared from cells at various times after inoculation of an aliquot of late log phase cells into fresh media containing radioactive thymidine. Sedimentation profiles of DNA from log phase *E. coli* B/r (1–2 hr after inoculation) are observed to have a main peak occurring at a position on the gradient corresponding to a sedimentation coefficient of approximately 130S and a less rapidly sedimenting component, appearing either as a shoulder on the main peak, or as a second peak having a sedimentation coefficient of about 90S (Fig. 1). With increasing time after inoculation the relative amount of radioactivity in the 90S component declines, until 5 hr after inoculation it is no longer present and a symmetrical profile results. Disappearance of the 90S component is accompanied by a slight shift of the main peak to a higher sedimentation value. The result of these two processes, disappearance of the light peak and shift of the main peak, is to increase the calculated Mw of the DNA from 3.5×10^8 to 4.3×10^8 daltons.

The 90S component is most prominent in DNA from early log phase cultures and is absent in stationary phase cultures; thus it appears to be related to the replication rate. To determine the rate of DNA synthesis the incorporation of thymidine-^{3}H into DNA during a 15 sec pulse was measured at various times after inoculation. As seen in the inset of Fig. 1, incorporation of label is at a maximum about 1 hr after

inoculation. Cultures growing for 3 hr incorporate 20% of the total activity observed with 1 hr growth. In these cultures which double approximately every 25 min, the stationary level of 1–3 × 10^9 cells/ml is reached within 2–3 hr; however, the rate of DNA synthesis declines well before the maximum cell number is reached. The time of maximum DNA synthesis (1 hr) corresponds to the time at which the ratio of 90S to 130S peaks in the alkaline sucrose density gradient profile is at a maximum, suggesting that the 90S component is related to the rate of replication. This conclusion is also supported by the results shown in Fig. 2 *a*. Profiles of DNA from cells

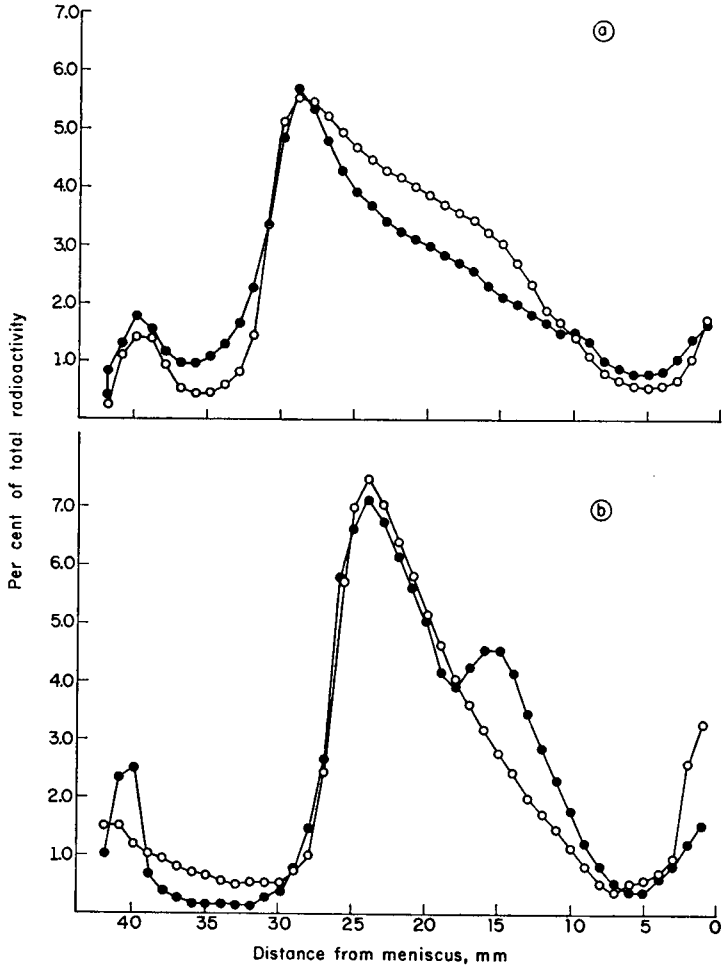

FIGURE 2 (*a*) Alkaline sucrose density gradient profile of DNA, 2.5 hr after inoculation of cells into fresh medium. O—O, cells grown in EM-9 medium, doubling time 25 min; ●—●, cells grown in M-9 medium, doubling time 40 min. (*b*) ●—●, cells exposed to a 15 sec pulse of thymidine-^{3}H 2 hr after inoculation into fresh medium; O—O, cells exposed to 15 sec pulse followed by addition of 100-fold excess of nonradioactive thymidine and incubation for 20 min.

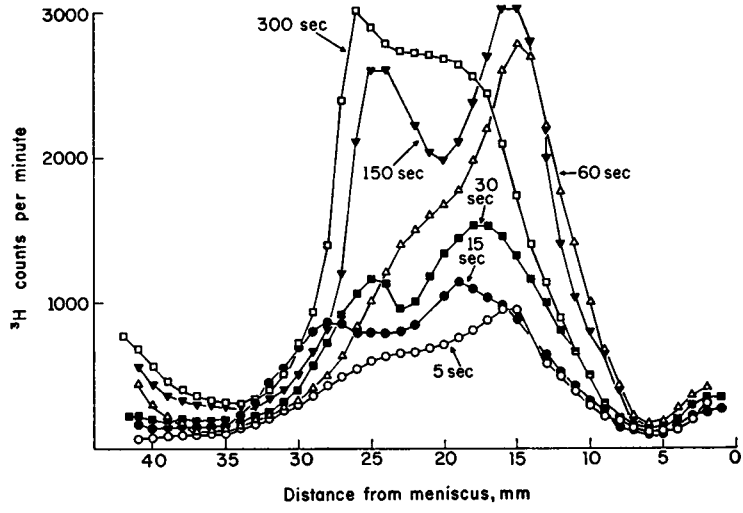

FIGURE 3 Alkaline sucrose density gradient profiles of cells pulse labeled with tritiated thymidine for the indicated time, 2 hr after inoculation into fresh medium.

growing for 2 hr in EM-9 (doubling time 25 min) had a 90S component which was relatively larger than that observed when cells were grown for the same length of time in M-9 (doubling time 40 min).

An experiment in which rapidly dividing log phase cells in EM-9 were pulse labeled for various lengths of time shows that most of the radioactive material is found in the 90S component after short labeling times. With longer pulse times label shifts into the 130S peak, until after 300 sec the profile resembles that obtained with cells which are continuously labeled (Fig. 3). These results suggest that formation of the 90S component precedes that of the 130S component. This conclusion is supported by the results of an experiment in which mid-log cells were labeled for 30 sec followed by a "chase" of 100-fold excess "cold" thymidine. The resulting profile did not show the lighter peak (Fig. 2 *b*). During incubation the unlabeled thymidine had apparently displaced the radioactive label from the lighter to the heavier fraction, demonstrating that the 90S component is labeled earlier than, and is a precursor to, the 130S component. Thus, when alkaline sucrose density gradient profiles are prepared from continuously labeled log phase cells the most recently labeled material will be in a peak with a lower sedimentation coefficient than the main peak.

Irradiated Cells

Investigation of irradiated bacteria was prompted by the observation that the radiation-induced reduction in weight of DNA (Mw) was dependent on the stage in the growth cycle at which cells were irradiated.

In Fig. 4 the change in Mw of DNA produced by irradiation is shown by plotting 1/Mw against dose. Three populations of cells replicating at different rates were

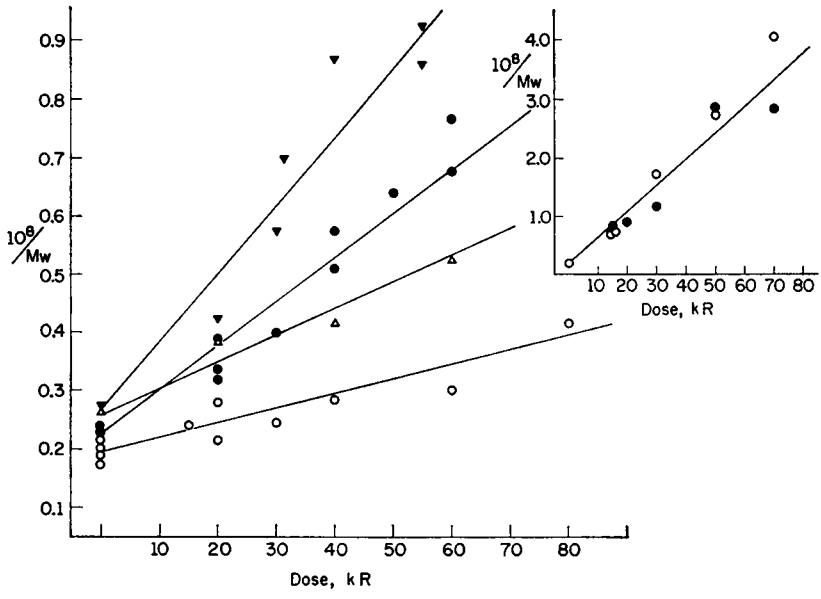

FIGURE 4 Reciprocal of Mw of DNA vs. X-ray dose. O—O, values for DNA of cells inoculated into fresh EM-9 medium and grown 7 hr before irradiation (stationary phase); △—△, cells grown 2 hr in M-9 medium (mid-log phase); ●—●, cells grown 2 hr in EM-9 medium (mid-log phase); filled triangles, cells grown 2 hr in EM-9 medium, 100-fold excess of nonradioactive thymidine added 30 min before end of incubation (only main peak labeled). *Inset:* Cells irradiated in the presence of 0.02 M EDTA. O—O, irradiated after 7 hr growth; ●—●, irradiated after 2 hr growth. Note ordinate scale of Fig. 4 is 10 times that of inset.

studied: rapidly replicating log phase cells in EM-9, less rapidly replicating log phase cells in M-9, and nonreplicating stationary phase cells. The slope of the line for the most rapidly replicating cells is three times greater than that for the nondividing cells, indicating that most damage to DNA is sustained when cells are replicating at maximum rate.

A similar conclusion is reached by comparing profiles of DNA from cells receiving 20 kR at various times during the growth cycle. From the inset of Fig. 1, the rate of DNA synthesis may be seen to be greatest 1 hr after inoculation, the same time at which radiation-induced fall in Mw is greatest (Fig. 5 *a*). Subsequently the rate of DNA synthesis declines as does the fall in Mw produced by irradiation (Figs. 5 *b* and *c*). In stationary phase cells no DNA synthesis is taking place and Mw changes produced by irradiation are least (Fig. 5 *d*).

Rapid rejoining of single strand breaks in bacterial DNA takes place at low temperature (Alexander et al., 1970; Lehnert and Moroson, 1971). This type of rejoining is inhibited by EDTA. In the inset of Fig. 4, 1/Mw is plotted against dose for cells irradiated in 0.02 M EDTA, and the slope is steeper than for cells irradiated in buffer alone (Fig. 4); moreover, when EDTA is present during irradiation, points from

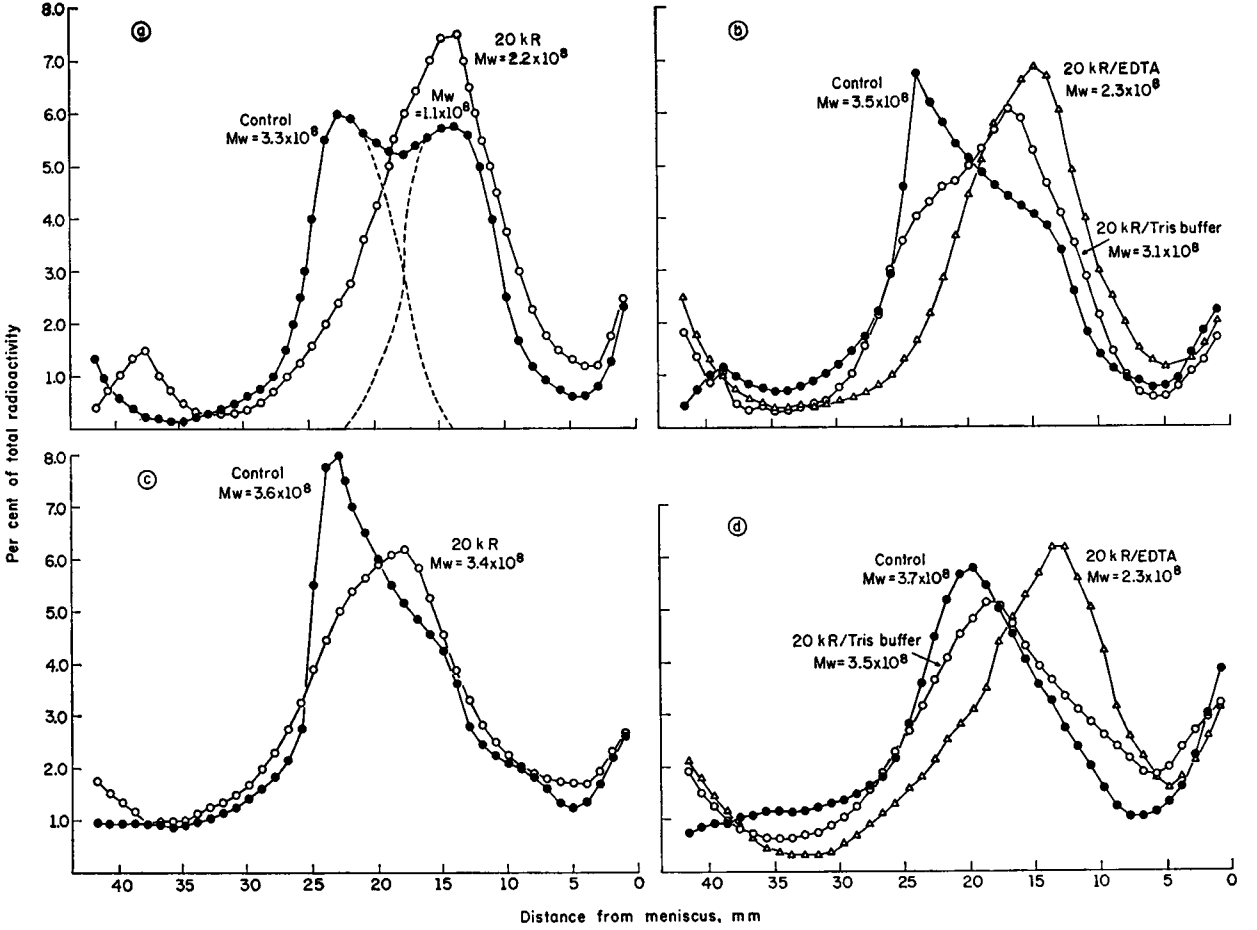

FIGURE 5 Alkaline sucrose density gradient profiles of DNA from cells analyzed 1 hr (*a*), 2 hr (*b*), 3 hr (*c*), and 7 hr (*d*) after inoculation into fresh medium. ●—●, control cells; ○—○, cells suspended in 0.01 M Tris, pH 8.0, for irradiation; △—△, cells suspended in 0.01 M Tris, pH 8.0, 0.02 M EDTA for irradiation. The survival of these cells after irradiation was: (*a*) 10%, (*b*) 8%, (*c*) 9%, (*d*) 19%. In Fig. 5 *a* control, the 90S and 130S components were drawn in separately by assuming each was distributed symmetrically. Mw for each component was calculated from these hypothetical curves.

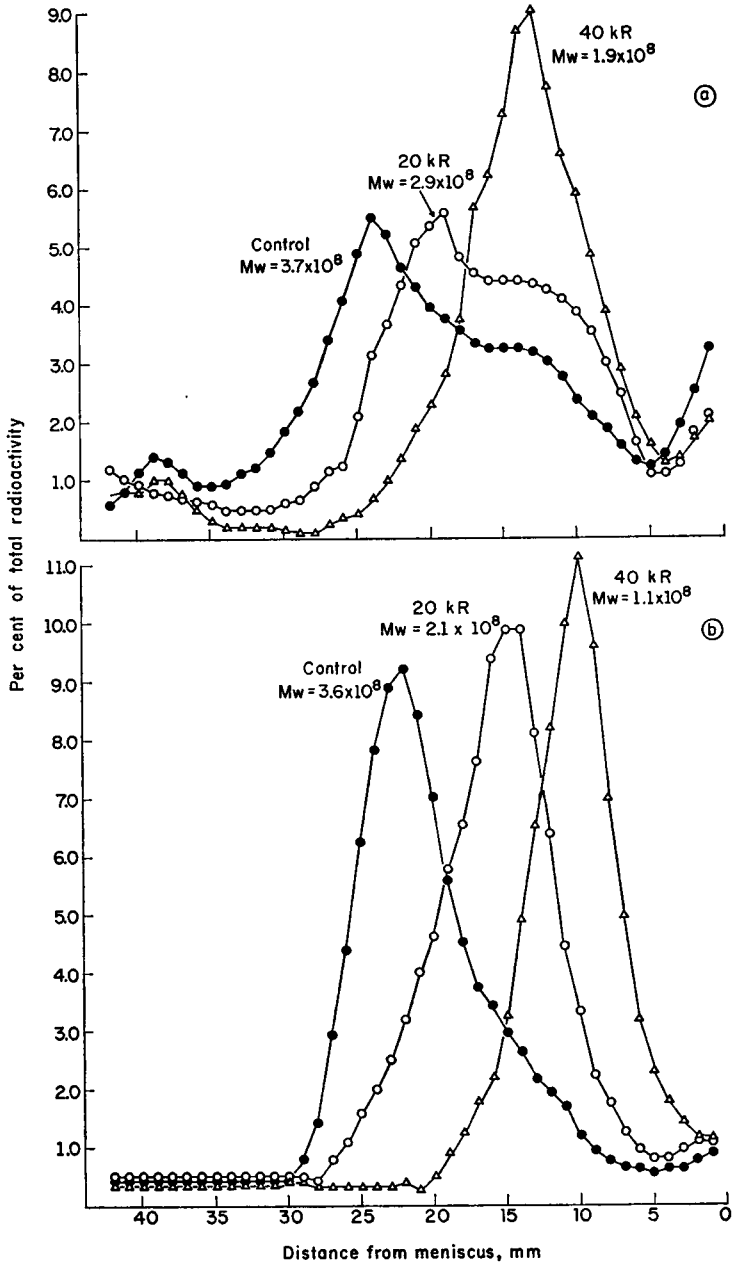

FIGURE 6 (*a*) Alkaline sucrose density gradient profiles of DNA of cells 2 hr after inoculation into fresh medium, and (*b*) 2 hr after inoculation into fresh medium, 100-fold excess nonradioactive thymidine added 30 min before the end of the incubation. ●—●, control; ○—○, 20 kR; △—△ 40 kR.

both log and stationary phase cells fall on the same line indicating that when rejoining of single strand breaks is prevented the decrease in Mw is the same for both groups of cells.

Comparison of the fall in Mw produced by 20 kR at various stages in the growth cycle showed that it was greatest in early log phase and least in the stationary phase (Fig. 5). In contrast the fall in Mw produced by 20 kR in the presence of 0.02 M EDTA is approximately the same for cells irradiated at 2 hr after inoculation (Fig. 5 *b*) and for stationary phase cells (Fig. 5 *d*). This again suggests that, in the absence of EDTA, stationary phase cells may rejoin single strand breaks more rapidly than do replicating cells.

If breaks in the 90S DNA are repaired less efficiently than breaks in 130S DNA the total number of breaks seen would vary with the relative abundance of the 90S component, thus accounting for the greater number of single strand breaks observed in replicating cells for a given dose of radiation. An experiment to test this hypothesis was done using cells in which only the main peak DNA was labeled. If excess nonradioactive thymidine is added during the continuous labeling procedure and incubation is continued for a period exceeding one generation time, the alkaline sucrose density profile shows a single symmetrical peak. Only DNA synthesized before addition of excess thymidine is labeled, and the more recently synthesized, slower sedimenting component is not visualized in the profile. Fig. 6 *a* shows alkaline sucrose density profiles of DNA obtained from continuously labeled cells; after a dose of 40 kR, all the radioactive material is found in a relatively homogeneous band corresponding in position to the 90S component. The same dose of X-rays produces a greater shift of radioactive material towards a lower sedimentation coefficient if only 130S DNA is labeled (Fig. 6 *b*).

When 1/Mw is plotted against dose the slope of the line is greater when main peak DNA alone is labeled than when DNA is continuously labeled (Fig. 4, filled triangles). It can be concluded from the results of these experiments that radiation-induced breaks are not preferentially located in the 90S component but are mainly in DNA of the 130S peak.

DISCUSSION AND CONCLUSIONS

There have been many reports of fractions of bacterial DNA which sediment more slowly than the main peak in alkaline sucrose density gradients. The 8–10S fragments described by Okazaki et al. (1968) and subsequently by other workers (Sugimoto et al., 1968; Sadowski et al., 1968) are not demonstrable by the centrifugation techniques used here. Bacterial DNA which is intermediate in size between the Okazaki fragments and high molecular weight DNA is shown in some alkaline sucrose density gradient profiles (Bird and Lark, 1970; Yudelevich et al., 1968), but has not been commented upon by these workers.

Lehmann and Ormerod (1970) have shown that randomly broken pulse-labeled

DNA appears to sediment more slowly than bulk DNA on an alkaline sucrose density gradient. This is dependent on the different labeling patterns of randomly sheared pieces of bulk and pulse-labeled DNA, the former being labeled throughout the length and the latter only at the ends. The results described here, however, cannot be explained on this basis since the slower sedimenting component is observed with both bulk and pulse-labeled DNA, and very few random breaks are introduced by unavoidable shearing.

A model of the replicating bacterial chromosome with either single or multiple forks (Cairns, 1963; Cooper and Helmstetter, 1968) suggests that, if the two DNA strands are separated by alkaline denaturation, the newly synthesized strands on both sides of the replicating fork would be of variable size, depending upon how far replication of those strands had proceeded, but generally they would be shorter than the nonreplicating strands. Thus, they would form a class of rapidly labeled molecules, lighter than those in the main peak, most numerous in replicating cells, and disappearing when replication ceased. It is possible that the 90S component described here represents these partially completed DNA strands from bacterial chromosomes in which semiconservative replication of DNA is taking place. Alternatively, the newly synthesized molecules or portions of molecules may be more labile and break more readily during the isolation procedure.

The results of irradiation experiments with EDTA in the buffer show that log phase cells are less efficient than stationary phase cells in rejoining single strand breaks. Breaks which are rapidly rejoined at 0°C in buffer are probably terminated by 5′ phosphate and 3′ hydroxyl groups (Alexander et al., 1970) and could be rapidly rejoined by enzymes known to be present in the cell, i.e., polynucleotide ligases (Weiss and Richardson, 1967; Gefter et al., 1967; Olivera and Lehman, 1967; Lindahl and Edelman, 1968). Failure to rejoin these breaks could result from ligase levels being inadequate or inhibited, or by the substrate not being amenable to ligase action. It seems unlikely that ligase levels would be reduced or inhibited in replicating cells, since the enzyme is thought to have a role in DNA replication (Yudelevich et al., 1968; Sadowski et al., 1968; Sugimoto et al., 1968). Postirradiation modification of the single strand breaks seems a more likely possibility. Exonucleases II and III of *E. coli* (Shortman and Lehman, 1964) which attack the 3′ hydroxyl end of the polynucleotide chain and show a preference for native DNA, could delete one or more nucleotides at the site of a single strand break, ensuring that it could not be rejoined by the action of ligase alone. Although 5′-3′, OH breaks are readily rejoined by ligase at low temperature in buffer, these conditions are probably not conducive to the repair of "modified" breaks involving additional enzymic processes (Alexander et al., 1970), and consequently the break would not be rejoined during the period of the irradiation. Exonuclease activity has been shown by Shortman and Lehman (1964) to be 10-fold higher in log phase cells than in stationary phase cells, and this could account for the difference in the number of breaks observed after irradiation of log and stationary phase cells. The postirradiation degradation of bac-

terial DNA has been well documented (Stuy, 1960; Osterrieth, 1963; Grady and Pollard, 1967), and it has been shown that lesions produced by irradiation render bacterial DNA more susceptible to the action of nucleases (Trgovčević and Kućan, 1967). These workers found (1969) that both survival and DNA degradation of irradiated *E. coli* B varied in a similar manner during progression through the cell cycle, the lowest degradation and the lowest radiosensitivity being found in stationary phase cells, while the greatest degradation and highest radiosensitivity is found in cells in the mid-log phase, the time at which exonuclease activity in the cell is also maximal. DNA degradation in irradiated cells is most usually measured during postirradiation incubation at 37°C and under such conditions more than half the DNA of the cell may be broken down after a dose of 15 kR (Stuy, 1960). Cells irradiated and held at 0°C show no similar loss of DNA because of the slowed reaction rate of nucleases at this temperature; however, a very small fraction of the nuclease activity could modify radiation-induced single strand breaks sufficiently to render them nonreparable by the action of ligase alone. Thus, one explanation of the greater degree of X-ray damage to DNA of cells in log phase of growth could be that the higher levels of nucleases found in log phase cells act on radiation-induced single strand breaks in such a way as to render them nonreparable.

In the presence of EDTA it is possible that the single strand breaks introduced by X-rays are not subject to nuclease attack since under assay conditions both exonuclease II and exonuclease III of *E. coli* show a requirement for divalent cations (Lehman and Richardson, 1964). This would not change the number of breaks observed, since in the presence of EDTA no rapid low temperature rejoining of breaks takes place.

In experiments in which only main peak DNA was labeled and the cells then irradiated, density gradient profiles appear to show that main peak DNA sustains more damage from the same dose of radiation than does the 90S DNA. This might be predicted since ionizing radiation introduces breaks at random and initially larger molecules will suffer proportionately more hits; hence, a given dose of radiation will have more effect on large than on small molecules.

Differences in survival after irradiation between stationary and log phase cells have been described by several investigators (Stapleton, 1955; Trgovčević and Kućan, 1969, Town et al., 1970) as well as in this report (see Fig. 5). We observed no correlation, however, between survival and the number of breaks produced at various times during the logarithmic phase. Experiments in which irradiation and postirradiation conditions were varied (Lehnert and Moroson, 1971) also showed that the number of single strand breaks observed is not correlated with survival, and that radiation damage to bacterial DNA is dependent on intracellular as well as extracellular factors.

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