SATURATION OF DARK REPAIR SYNTHESIS: ACCUMULATION OF STRAND BREAKS

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ABSTRACT Reversal of ultraviolet light damage to DNA by the dark repair system is limited. Experiments utilizing density and radioactive labels demonstrated that repair synthesis is not proportional to dose at doses above 200 ergs/mm^2. In addition, the number of residual excision induced gaps in *Escherichia coli* B/r hcr$^+$ DNA increases with higher UV doses. The extent of repair is apparently limited by saturation of the repair synthesis step.

INTRODUCTION

Certain strains of *Escherichia coli* are able to repair damage to DNA by a "dark repair" system (1) after ultraviolet (UV) light exposure. As the dose is increased, the cell is unable to cope with all the lethal damage induced by the UV radiation (5), and colony-forming ability is lost. This paper describes experiments in which *E. coli* B/r hcr$^+$ cells are used to investigate the consequence of saturation of the repair enzyme system. Analysis of DNA contained in cells at the time of UV exposure and subsequently subjected to repair shows a decrease in size with increasing dose. This suggests that excision, which produces gaps, occurs in excess of the repair synthesis and rejoining steps at doses greater than 100 ergs/mm^2. Using ^{13}C, ^{15}N, and ^{2}H as density labels, it was further determined that repair synthesis is not proportional to dose above 200 ergs/mm^2.

Our results lead to the conclusion that the extent of repair is limited by saturation of the repair synthesis step.

METHODS AND MATERIALS

Ultraviolet Irradiation

Two GE 15 watt germicidal lamps with an effective dose rate of 7.4 ergs/mm^2 per sec at the sample position were used as the source of UV radiation. Cells were suspended in medium C salts without glucose to a titer no greater than 2×10^8. The cell suspension, with a depth no greater than 5 mm, was stirred continuously during irradiation at ice temperature.

Density Gradient Studies

Details of bacterial growth and isopycnic analysis have been published (4), and only an outline of the procedures follows. *E. coli* B/r (hcr^+) thy_2^- $tryp^-$ were grown for at least four generations in a minimal salts medium, called "heavy medium," with the following substitutions: H_2O replaced by ^2H_2O, $^{14}NH_4Cl$ replaced by $^{15}NH_4Cl$, and glucose-^{12}C replaced by 40% substituted glucose-^{13}C (Merck, Sharpe, and Dohme of Canada, Ltd., Montreal). The heavy medium was supplemented with thymine-^{14}C as a label for the DNA. Growth for 30 min immediately prior to irradiation in light medium without radioactive label served as a chase. Following UV irradiation, cells were grown in medium without density label, called "light medium," containing thymine-^3H to identify DNA synthesized after irradiation. Repair synthesis was quantitated by measurement of the amount of ^3H-label incorporated into the single-stranded heavy parental DNA.

Alkaline Sucrose Gradient Studies

E. coli B/r (hcr^+) was grown for at least four generations in medium C supplemented with thymine-^3H (50 μc/ml, 2 μg/ml) to a concentration of about 10^8 cells/ml, and then irradiated. After irradiation the cells were grown 45 min in complete medium in order to allow repair to take place. 45min was chosen in view of results derived from the repair synthesis studien, which indicate repair synthesis to be essentially over by 40 min. Following 45 min postirradiation incubation, alkaline sucrose gradient analysis of the bacterial DNA was carried out. Sucrose gradient analysis was performed as described earlier (2). The technique developed by McGrath and Williams (7) for the isolation of large pieces of DNA was used to assay discontinuities. Briefly, spheroplasts were prepared by incubation with lysozyme in the presence of sucrose. Approximately 5×10^7 spheroplasts were layered over a preformed 5–20% sucrose gradient, and ^{14}C-labeled T2 phage DNA was added as a reference. The gradient was centrifuged 2 hr at 35,000 rpm in a Beckman type SW 65 rotor (Beckman Instruments Inc.,Palo Alto, Calif.). Drops were collected from the bottom and counted.

RESULTS

Repair Synthesis Studies

To distinguish DNA synthesized postirradiation from parental DNA, density labeling and isopycnic analysis were employed. Density labeling was achieved by growth on medium containing the isotopes ^{15}N, ^{13}C, and ^2H. This produced DNA which was heavy in both strands and had a bouyant density in CsCl of approximately 1.770 in the denatured state. Light medium containing ^{12}C, ^{14}N, and ^1H was used for postirradiation incubation. Thus, DNA synthesized after irradiation was of normal density and banded at approximately 1.725 g/cc in the denatured state. The medium was supplemented with thymine-^{14}C before irradiation and thymine-^3H after irradiation. Under these growth conditions, thymine-^3H incorporation into the heavy denatured fraction was used as a measure of repair DNA synthesis.

The results presented in Fig. 1 show that for times longer than 40 min there is no additional incorporation of ^3H-label into the parental strands; repair synthesis is completed by 40 min. In order to compare quantitatively the amount of repair

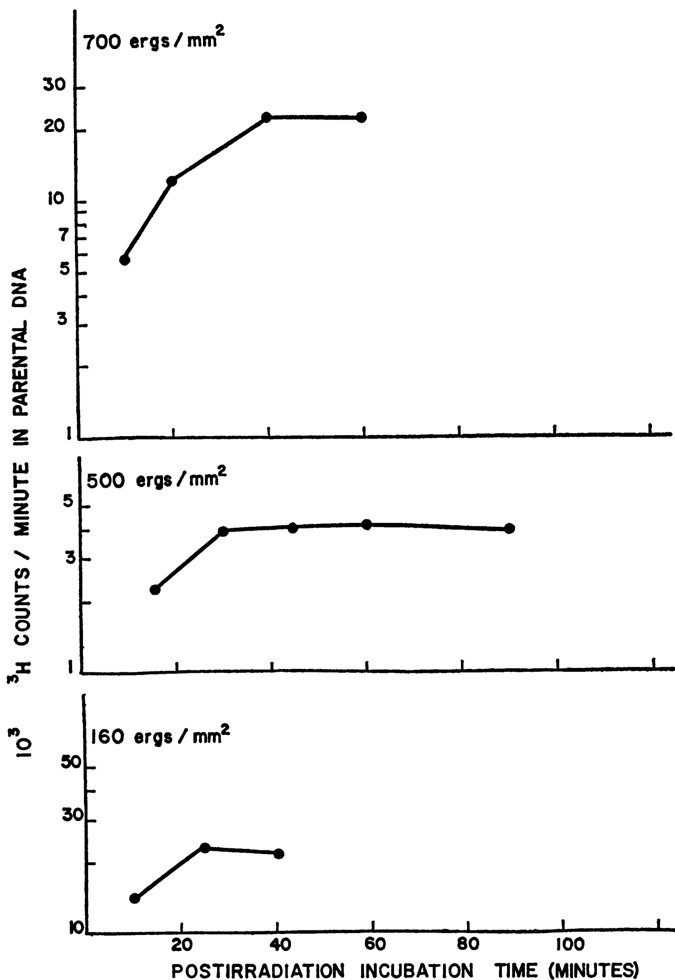

FIGURE 1 Time course of repair synthesis. Cells were grown in heavy medium with thymine-^{14}C. There was a 30 min chase in light medium followed by irradiation and growth with thymine-^{3}H in light medium. Lysates were heated to separate the heavy parental ^{14}C strand from the light ^{3}H strand synthesized after irradiation. The ^{3}H label associated with the heavy parental ^{14}C strand was plotted as repair synthesis vs. time after irradiation. There was no rebanding of the 500 and 700 erg/mm^2 experiments. The 160 erg/mm^2 sample was rebanded to further purify that DNA synthesized in a semiconservative mode away from the DNA synthesized in a nonsemiconservative manner. These were independent experiments.

synthesis in cells receiving varying doses of UV, data were collected from the same culture of cells. Equal portions of the culture were irradiated with several doses of UV, and each was suspended in medium containing the same specific activity thymine-^{3}H for repair synthesis. The amount of repair synthesis as a function of dose is plotted in Fig. 2. The curve which would be obtained if there were a one-to-one correspondence of incremental increase in dose with increase in repair synthesis

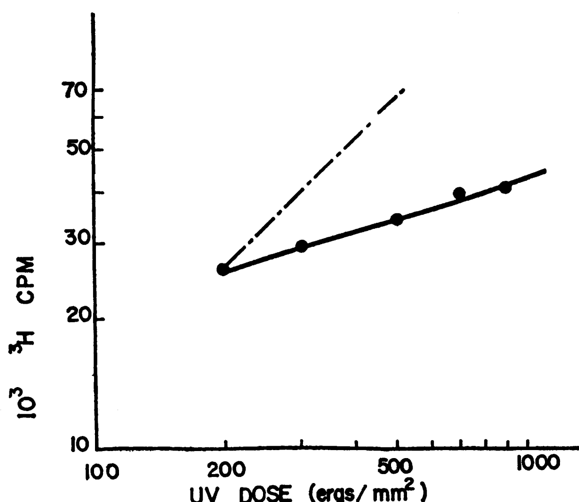

FIGURE 2 All samples from same culture. Cells were harvested after 60 min postirradiation incubation. Density gradient analysis of melted DNA was performed. In order to purify DNA synthesized semiconservatively away from the nonsemiconservatively synthesized DNA, the heavy parental DNA, identified by ^{14}C counts, was rebanded for the 200, 300, and 500 ergs/mm^2 doses. Comparative quantitation of repair synthesis at these doses was achieved by normalizing the number of ^{14}C counts from each sample. Survival was 0.62, 0.29, 0.074, 0.0097, and 0.00029 for dose of 200, 300, 500, 700, and 900 ergs/mm^2, respectively. The amount of repair synthesis, determined by the ^{3}H activity in the heavy denatured band, is plotted against UV dose. Solid line, experimental data; broken line, expected curve if the increase of repair synthesis were proportional to the increase of dose.

is shown for comparison. The data indicate that not all lesions are repaired for doses of UV above 200 ergs/mm^2.

Saturation is not absolute in the sense that for increasing dose there is no additional repair synthesis. It is saturated in terms of a reduced rate of increase in repair synthesis relative to the rate of increase in dose. A doubling of dose is not accompanied by a twofold increase in repair synthesis. The dark repair system is able to cope with only a fractional amount of the damage susceptible to repair at doses above 200 ergs/mm^2, based on the assumption that all damage is repaired at this dose. If indeed not all lethal damage is repaired at 200 ergs/mm^2, which seems to be the case since colony-forming ability is reduced at this dose, then the repair system is saturated at a dose lower than 200 ergs/mm^2. Reliable quantitation of repair synthesis below 200 ergs/mm^2 is difficult due to massive amounts of normal semiconservative synthesis relative to repair.

Measurement of Single Strand Breaks

Cellular DNA was labeled with thymine-^{3}H prior to UV exposure. Incubation for 45 min in nonradioactive medium followed irradiation in order to allow repair.

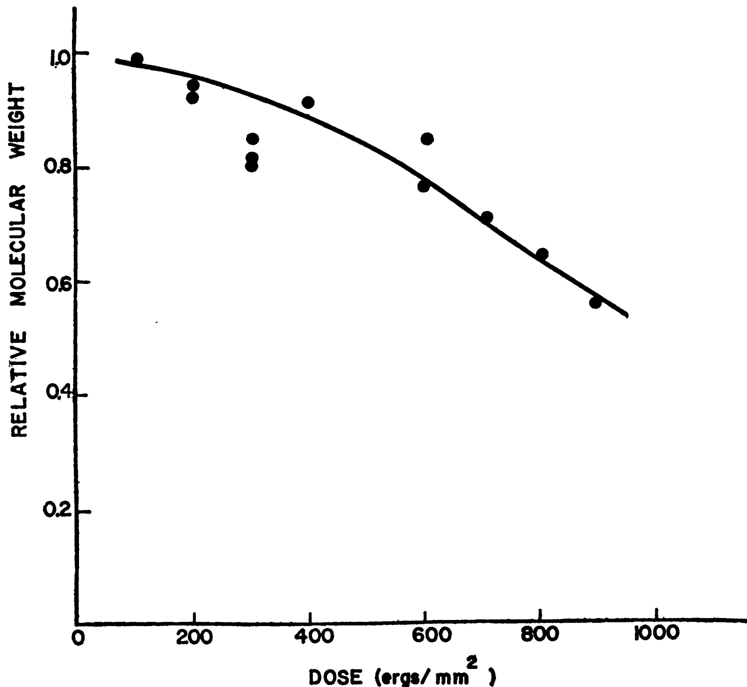

FIGURE 3 Molecular weight of repaired DNA relative to control DNA at various doses. Cells labeled with thymine-^{3}H were exposed to UV, incubated 45 min to allow repair and placed on an alkaline sucrose gradient for molecular weight analysis of the DNA. As the dose increases, the relative molecular weight decreases, thus demonstrating an increase in alkali labile sites on the DNA molecule. These sites are interpreted to represent strand breaks.

Discontinuities in the DNA were measured on alkaline sucrose gradients. The size of UV irradiated DNA which was subjected to repair (called "irradiated" DNA) was compared to the size of UV irradiated DNA which was held in the presence of 0.2% caffeine at ice temperature to inhibit excision (called "control" DNA). Any difference in the relative size of the irradiated DNA and the control DNA is a consequence of the repair process. A marker of ^{14}C-labeled T2 phage DNA was used in the gradients in order to avoid the necessity of absolute determination of molecular weights. Details of the sucrose gradient methodology and calculation of the relative molecular weights along with a sucrose gradient profile have been published (2).

Calculated relative molecular weights of irradiated DNA to control DNA are plotted against dose in Fig. 3. Clearly, there is a reduction in the molecular weight as dose is increased. As dictated by the experiment, the increase in discontinuities (indicated by reduced molecular weight) results from the action of the repair system following UV irradiation. It is recognized that the discontinuities are not necessarily single strand breaks *in situ*, and may represent alkali labile regions in the DNA.

Saturation of either two steps in the repair process could give rise to strand breaks: (1) incomplete repair synthesis of the excised region, or (2) incomplete action of the ligase involved in final rejoining of the repaired region to the intact strand. Observation of reduced levels of repair synthesis at higher doses lends support to the first possibility.

An experiment was done on the related repair deficient strain *E. coli* B/r (hcr^-) thy_2^- $tryp^-$ in order to establish that the increase in strand breaks involves action of the repair system. The hcr^- derivative lacks ability to excise thymine dimers (8), and therefore should not display any breaks in the DNA after postirradiation incubation, if the breaks are related to excision in excess of repair and rejoining. There was no change in the size of DNA harvested from cells receiving 900 ergs/mm^2, which supports the idea that strand discontinuities are a result of incomplete operation of the dark repair system.

DISCUSSION

Survival curves for *E. coli* B/r receiving UV radiation exhibit a "shoulder," that is, a small amount of killing at low doses followed by increased killing response at higher doses. It has been suggested that cells have a finite capacity for dark repair, and that increased killing efficiency is attained at doses for which the repair capacity is exceeded (6). Reduction in repair efficiency at doses above 200 ergs/mm^2, termed "saturation," was demonstrated by two techniques.

It was shown that the repair synthesis step is less active than would be anticipated from the number of dimers produced at doses above 200 ergs/mm^2. Such reduction in repair synthesis cannot be used as evidence to distinguish whether the repair synthesis step per se loses efficiency or whether the preceding step, photoproduct excision, is responsible for reduction of repair synthesis. Additional evidence of incomplete repair was obtained from alkaline sucrose gradient analysis. One would predict that if repair synthesis is saturated with increasing dose and excision is not, the number of observable breaks should increase with dose due to the occurrence of excision in excess of resynthesis and rejoining. Analysis of large molecular weight DNA pieces indicates that this is what happens. Together, the results from density gradient and sucrose gradient analysis indicate that repair synthesis is the limiting step in the dark repair process. Biologically, saturation of the repair system is reflected by reduced colony-forming ability.

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