Inhibition of Excision Repair without Influence upon UV-Sensitivity and UV-Mutability in *Escherichia coli* B/r Hcr +

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(Z. Naturforsch. 30 c, 406—411 [1975]; received August 1/November 22, 1974)

DNA-Repair, UV-Sensitivity, UV-Mutagenesis, *Escherichia coli*, UV-Radiation

Pre-irradiation starvation of exponentially growing *Escherichia coli* B/r Hcr + thy~ trp~ strain for thymine and tryptophan causes inhibition of pyrimidine dimer excision from ultraviolet damaged cells DNA. This inhibition of excision repair has not resulted in increasing ultraviolet sensitivity nor in increasing frequency of ultraviolet induced tryptophan revertants. The possible mechanisms of the non-excision repair in the prestarved cells, which is at least as accurate and effective as the whole dark repair in exponentially growing cells, are discussed.

Most of the ultraviolet induced pyrimidine dimers in *Escherichia coli* DNA can be repaired by the excision and post-replication repair systems (Howard-Flanders 1). UV-induced mutations occur as errors in these repair systems (Witkin 2, Nishioka and Doudney 3). Inhibition of excision repair by caffeine and acriflavine increase the UV-mutability of wild-type strains (for review see Witkin 2); furthermore the frequency of UV-induced mutations is increased, at equal dose levels, in *uvr~* mutants which are deficient in excision of pyrimidine dimers (Hill 4, Witkin 5). It appears from these results that unexcised, but repaired, pyrimidine dimers are more mutable than excised ones, i.e. excision repair is less erroneous than post-replication repair.

However, this conclusion cannot be generalized — it will be shown here that UV-sensitivity and UV-mutability of *Escherichia coli* B/r Hcr + thy~ trp~ cells are unchanged when excision of pyrimidine dimers is inhibited by pre-irradiation starvation of cells for thymine and tryptophan. The study presented in this report is a continuation of the work of authors from our laboratory who have shown that it is possible to inhibit excision of pyrimidine dimers in excision-proficient *Escherichia coli* strains by pre-irradiation inhibition of DNA and protein syntheses 6–9.

### Materials and Methods

#### Bacteria

The bacterial strains used were *Escherichia coli* B/r Hcr + thy~ trp~, *Escherichia coli* B/r Hcr~ thy~ trp~, and *Escherichia coli* B$_{8-1}$, the kind gift from Dr. D. Billen.

#### Media

The M-medium is glucose-salts minimal medium described by Sedlikáková *et al.* 10. M-THY+ TRP+, M-THY+ TRP$, M$-TdR$+ TRP$, M-TdR$+ TRP$, M-TdR$+ TdR$ and M-THY$+ TdR$ are media prepared from M-medium by adding 2 $\mu$g mL$^{-1}$ T + 28 $\mu$g mL$^{-1}$ DL-trp, 2 $\mu$g mL$^{-1}$ T + 1.5 $\mu$g mL$^{-1}$ DL-trp, 4 $\mu$g mL$^{-1}$ TdR + 28 $\mu$g mL$^{-1}$ DL-trp, 4 $\mu$g mL$^{-1}$ TdR + 1.5 $\mu$g mL$^{-1}$ DL-trp, 4 $\mu$g mL$^{-1}$ TdR and 2 $\mu$g mL$^{-1}$ T, respectively (T = thymine, TdR = thymidine, DL-trp = L-tryptophan). Agar media prepared from liquid media by solidification with 2% agar are marked with identical symbols.

#### Cultivation and irradiation

The bacterial strains were maintained for a prolonged period on nutrient agar slants at 4 °C. From there any given strain was inoculated on to a nutrient agar plate and after 24-hour cultivation at 37 °C cells were transferred from there on to a synthetic M-THY+ TRP$^+$ agar plate. After 24 hours' cultivation a loopful of bacteria was transferred from this plate into 25 ml of liquid M-THY+ TRP$^+$ medium. A cell suspension was cultivated in a shaker at 37 °C until the middle of the exponential phase of growth ($A_{550} = 0.4$), then cooled and maintained at 4 °C. The inoculum thus prepared (not older than 2 days) had prior to the experiment been diluted in a 6:100 ratio in the M-THY$^+$ TRP$^+$ liquid medium and cultivated in a shaker at 37 °C up to the early

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exponential phase of growth \((A_{550} = 0.24)\) when cells were harvested by filtration on a membrane filter (Synpor, VChZ Synthesia, Pardubice, Czechoslovakia) on a suction filtration apparatus. On a membrane, cells were washed with 3-fold volume of warm \(37^\circ C\) M-medium and resuspended in the original volume of the warm \(37^\circ C\) M-medium. The resuspended culture was divided into three parts; the first part was, after appropriate dilution in M-medium, spread on to agar plates (= unirradiated control in exponential phase of growth); the second part was UV-irradiated in a Petri dish (thickness of suspension = 2 ± 0.1 mm) with rapid manual stirring and after appropriate dilution in M-medium it was spread on to agar plates (= irradiated control); the third part was cultivated in a shaker at \(37^\circ C\) for 90 min (= THY- TRP- starvation) and subsequently processed in the same manner as the controls. Filtrations and irradiation did not last longer than 5 min. The UV source was a TUV 15 W germicidal lamp (Philips, Eindhoven, Netherlands); incident dose rate was measured with a Latarjet N 90 dose rate meter, recalibrated by Véchet \(^{11}\). Optical density of the cell suspension \((A_{550})\) was measured at 550 nm in a glass cuvette (optical path length = 1 cm) on a Specol spectrophotometer (Zeiss Jena, Germany). All experiments described in the present paper were carried out under yellow light from a NaE spectral lamp (Narva, Berlin, GDR), which does not emit at wavelengths below 568.8 nm.

**Determination of cell survival and frequency of revertants**

Survival \(S\) was determined by the ratio between colony forming units (CFU) per ml of an irradiated and unirradiated cell suspension respectively. Number \(N\) of CFU per ml of suspension was determined on M-THY+ TRP+ or M-TdR+ TRP+ agar plates after 24 hours of plate incubation in the dark at \(37^\circ C\) following spreading of 0.1 ml of the suitably diluted suspension on to single plate; number \(N_A\) of CFU per ml was determined on M-THY+ TRP+ or M-TdR+ TRP+ agar plates after 48-hour incubation of plates in the dark at \(37^\circ C\) following plating of 0.1 ml of the suitably diluted cell suspension. Number \(M\) of Trp+ revertants per ml of cell suspension was scored on M-THY+ TRP+ or M-TdR+ TRP+ plates and determined as the number of cells capable of forming visible revertant colonies (RCFU) per ml suspension after 48-hour incubation of plates in the dark at \(37^\circ C\) following plating of 0.1 to 0.3 ml of the undiluted suspension per single plate. Frequency of Trp+ revertants was determined according to the expression \(F = M : N_A\). In every instance at least 3 agar plates were used as parallels. The averaged values presented in this paper are arithmetical means of experimental data.

**Expression of tryptophan revertants**

The method used was similar to that introduced by Haas and Doudney \(^{12}\). After UV irradiation cells were incubated in the M-TdR+ TRP+ liquid medium and at the indicated time intervals aliquots of the culture were filtered and washed on a membrane filter and resuspended in M-medium. From the resuspended culture aliquots of 0.1 to 0.2 ml were spread on to M-TdR+ TRP+ and M-TdR+ agar plates. The fraction of total number of Trp+ revertants which was expressed in M-TdR+ TRP+ liquid medium was determined as the ratio between RCFU on M-TdR+ agar plates and RCFU on M-TdR+ TRP+ agar plates after 48 hours cultivation of plates following plating in dark at \(37^\circ C\).

**Photoproduct analysis**

The \([^{14}C]\)thymine labelled cells were cultivated in dark at \(37^\circ C\) after UV-irradiation. At the indicated time intervals after irradiation samples were taken into cold trichloroacetic acid (TCA). Thymine dimers were determined in the fraction insoluble in cold 5% TCA. Samples were first extracted with 5% TCA at 0°C, and then with 5% TCA at 90°C; finally TCA was removed with the aid of ethyl-ether extraction. After drying, extracts were hydrolysed in 0.5 ml of 98% HCOOH in sealed evacuated glass tubes for 90 min at 175°C. Hydrolysates were evaporated, dissolved in 0.12 ml of 0.1 n HCl and thymine dimers were separated from thymine by descending chromatography on Whatman N1 paper in a system of n-butanol: glacial acetic acid: H2O = 80: 12: 30 (v: v: v). After drying, chromatograms were cut across into a 0.5 cm wide strips and their background according to the activity of strips adjacent to this region.

**Compounds**

Most of the compounds used in this study was obtained from Lachema, Brno, Czechoslovakia. Radioactive thymine was purchased from the Institute for Research and Production of Radioisotopes, Prague, Czechoslovakia; specific activity of \([2-{^{14}}C]T\) used in this study was 53 mCi·m⁻¹.

**Note**

The results presented in this communication are the data obtained in the experiments in which cell survival, frequency and expression of Trp+ rever-
tants and excision of dimers were followed simultaneously "in the same flask". These results were verified in several more extensive experiments in which the above mentioned characteristics were studied separately.

Results

We had expected an increased yield of UV-induced Trp\(^+\) revertants and a decrease of UV-resistance after starvation of exponentially growing *Escherichia coli* B/r Hcr\(^+\) thy\(^-\) trp\(^-\) cells for thymine and tryptophan, since the above mentioned authors from our department\(^6-9\) have found that THY\(^-\) TRP\(^-\) prestarved cells cultivated after UV-irradiation in M-THY\(^+\) TRP\(^+\) or M-TdR\(^+\) TRP\(^+\) liquid medium up to 120 min had excised only 35% or 5% of the induced thymine dimers, respectively, whereas a control culture irradiated in the exponential phase of growth and cultivated under similar conditions after irradiation had excised all of the detectable dimers within 90 – 120 min. However, as appears from Table I, neither UV-sensitivity nor the frequency of UV-induced Trp\(^+\) revertants in the THY\(^-\) TRP\(^-\) prestarved culture was higher than in the control culture. Because the frequency of Trp\(^+\) revertants has been determined on the synthetic selective agar plates with thymine or thymidine and a reduced level of tryptophan we had followed excision of thymine dimers in the same media as the revertants were scored. Experiments have shown that following THY\(^-\) TRP\(^-\) starvation excision of dimers becomes reduced for a long period (at least 8 hours) when, after irradiation, cells are cultivated in a liquid medium containing thymidine and a reduced level of tryptophan, whereas a control culture irradiated in the exponential phase of growth in the same medium will excise practically all (91.8\%) of the dimers within 4 hours after irradiation (see Table II).

Table I. Effect of thymine and tryptophan starvation on UV-sensitivity and UV-mutability of *Escherichia coli* B/r Hcr\(^+\) thy\(^-\) trp\(^-\) strain. Cells were cultivated and irradiated as described under Materials and Methods. Number of colony forming units per ml of suspension \(N\) was determined on M-THY\(^+\) TRP\(^+\) (part A) or M-TdR\(^+\) TRP\(^+\) (part B) agar plates; number of CFU per ml of suspension \(N_A\) was determined on M-THY\(^+\) TRP\(^+\) (part A) or M-TdR\(^+\) TRP\(^+\) (part B) agar plates; frequency of revertants \(F\) was calculated according to expression \(F = M : N_A\).

<table>
<thead>
<tr>
<th>Part</th>
<th>Starvation [min]</th>
<th>UV dose [erg/mm(^2)]</th>
<th>Number of colony forming units per ml</th>
<th>Number of Trp(^+) revertants per ml</th>
<th>Frequency of Trp(^+) revertants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(D)</td>
<td>(N)</td>
<td>(N_A)</td>
<td>(M)</td>
<td>(F)</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>0.9 \times 10^8</td>
<td>1.7 \times 10^8</td>
<td>4.4 \times 10^4</td>
<td>2.6 \times 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>survival:</td>
<td>9.4 \times 10^{-4}</td>
<td>2.1 \times 10^{-4}</td>
<td>17.4 \times 10^{-4}</td>
<td>8.3 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.3 \times 10^5</td>
<td>2.8 \times 10^5</td>
<td>16.5 \times 10^4</td>
<td>5.9 \times 10^{-7}</td>
</tr>
<tr>
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<td>survival:</td>
<td>4.9 \times 10^{-3}</td>
<td>2.1 \times 10^{-8}</td>
<td>20.4 \times 10^{-4}</td>
<td>3.5 \times 10^{-4}</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0.2 \times 10^7</td>
<td>2.0 \times 10^3</td>
<td>12.3 \times 10^4</td>
<td>6.2 \times 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>survival:</td>
<td>5.2 \times 10^{-3}</td>
<td>7.4 \times 10^{-3}</td>
<td>7.5 \times 10^{-4}</td>
<td>5.1 \times 10^{-4}</td>
</tr>
</tbody>
</table>

Table II. Effect of thymine and tryptophan starvation on thymine dimer excision in UV-irradiated *Escherichia coli* B/r Hcr\(^+\) thy\(^-\) trp\(^-\) cells. \(T<>T\) — thymine — thymine dimer; \(T\) — thymine; UV dose — 756 erg/mm\(^2\). After UV-irradiation cells were incubated in the M-TdR\(^+\) TRP\(^+\) liquid medium and at the indicated time intervals post-UV concentration of thymine dimers was determined.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Starvation [min]</th>
<th>Time post-UV [hours]</th>
<th>Activity in (T&lt;&gt;T) region [cpm]</th>
<th>Activity in (T) region [cpm]</th>
<th>Fraction of (T) in (T&lt;&gt;T) [%]</th>
<th>Excised (T&lt;&gt;T) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
<td>781.4</td>
<td>458 320.0</td>
<td>1.70 \times 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>4</td>
<td>43.6</td>
<td>311 409.0</td>
<td>0.14 \times 10^{-3}</td>
<td>91.8</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>8</td>
<td>379.8</td>
<td>498 941.0</td>
<td>1.60 \times 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>6</td>
<td>321.9</td>
<td>348 738.0</td>
<td>0.92 \times 10^{-3}</td>
<td>42.5</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>6</td>
<td>542.8</td>
<td>351 084.0</td>
<td>1.55 \times 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>6</td>
<td>396.9</td>
<td>417 389.1</td>
<td>0.95 \times 10^{-3}</td>
<td>38.5</td>
</tr>
</tbody>
</table>
Since it cannot be excluded that reconstruction of genetic material takes place in a liquid medium in a different way than on the same medium solidified with agar we studied the expression of tryptophan revertants in a liquid medium with thymidine and a reduced level of tryptophan. It is evident from the results presented in Fig. 1 that expression of Trp$^+$ revertants take its course in the control culture irradiated in the exponential phase of growth within 4 hours after irradiation, i.e. at a time interval when cells will have excised all the induced dimers, whereas in the THY$^-$ TRP$^-$ pre-starved culture expression will take place more slowly, but within the time period of inhibition of dimer excision (compare with Table II).

All plates with membranes were further incubated for 48 hours in the dark at 37 °C after which the number of Trp$^+$ revertants was read on each plate. The number of Trp$^+$ revertants per one ml on single plates without tryptophan was 93, 132, 123 and 95 respectively, while on selective agar plates the corresponding numbers were 163, 222, 137, 117 and 154. It follows from this that after 12 hours cultivation on selective agar plates approximately 70% of UV-induced Trp$^+$ revertants had phenotypically been expressed. During this period of cultivation cells had excised less than 10% UV-induced dimers (see Fig. 2).

For purposes of comparison we also state the data characterizing UV-sensitivity of the excision repair deficient $E. coli$ B/r Hcr$^+$ thy$^-$ trp$^-$ cells, dark repair deficient $E. coli$ B$_{s-1}$ strain and cells of $E. coli$ B/r Hcr$^+$ thy$^-$ trp$^-$ strain after THY$^-$ TRP$^+$ starvation (Table III). When comparing the UV dose $D_s$, after which in the dark repair-deficient $E. coli$ B$_{s-1}$ strain
Table III. Comparison of UV doses after which in the indicated strains of *Escherichia coli* survive the same fraction of cells (1.6 x 10⁻³).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Surviving fraction</th>
<th>UV dose [erg·mm⁻²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₆₋₁</td>
<td>1.6 x 10⁻³</td>
<td>12.5</td>
</tr>
<tr>
<td>B/r Hcr⁻</td>
<td>1.6 x 10⁻³</td>
<td>151.0</td>
</tr>
<tr>
<td>B/r Hcr⁺</td>
<td>1.6 x 10⁻³</td>
<td>756.0</td>
</tr>
</tbody>
</table>

the same fraction of cells will survive as in the THY⁻ TRP⁻ pre-starved *E. coli* B/r Hcr⁺ strain after the dose of D₄, we can see that the “repaired sector” defined as [1 – Dₛ/D₄]·100% (i.e. the fraction of the dose that is repaired in the pre-starved B/r Hcr⁺ strain) is about 98.4%. Similarly, repair process in the pre-starved B/r Hcr⁺ strain will eliminate 80% of lesions, which have proved lethal to the B/r Hcr⁻ strain.

**Discussion**

A critical point in our experiments has proved to be the question of inhibition of dimer excision. The possibility that dimers might perhaps have been excised in longer pieces from DNA, which are insoluble in cold 5% trichloroacetic acid and thus indistinguishable from high-molecular repaired DNA with the aid of our method of dimer determination, was excluded experimentally by Masek et al. Another possibility — application of too high a dose and in consequence thereof a saturation of excision — appears to be excluded by the fact that with an equally high dose and equal survival the control culture will excise dimers. Expression of UV-induced Trp⁺ revertants in the pre-starved culture indicates that the repair process is finished and first round of DNA replication is completed during the period of inhibition of dimer excision.

Since in our experiments inhibition of dimer excision has not resulted in increasing UV-sensitivity nor in increasing UV-mutability, we feel inclined to believe that non-excision repair, which is at least as accurate and effective as excision repair, must necessarily have been stimulated in *E. coli* B/r Hcr⁺ cells. Inaccuracy of a repair process depends on the presence of the *exr⁺/lex⁺* genes products. The same genes are known to have influence also on resistance of *E. coli* cells to ionizing radiation. Billen has observed in the wild-type *E. coli* 15 T⁻ A⁻ U⁻ strain a decrease of resistance to X-irradiation after starvation of exponentially growing culture for thymine, arginine and uracil, i.e. after a starvation similar to that applied by us. If we extrapolate his results to our case, we come to the conclusion that THY⁻ TRP⁻ starvation of wild-type *E. coli* cells will lead to a stimulation of repair different from excision repair and will depress at the same time the error-prone (mutagenic) steps in the repair process that are controlled by the *exr⁺/lex⁺* genes.

This non-excision repair differs from postreplication repair which takes place in the excision-deficient *E. coli* B/r Hcr⁻ strain (otherwise isogenic with the *E. coli* B/r Hcr⁺ strain) at least in that, that it will eliminate 80% of lesions, which have proved lethal to the Hcr⁻ strain. In excision-deficient strains during replication of DNA with unexcised dimers there will occur gaps in daughter strands opposite to dimers. According to Rupp et al. these gaps are subsequently filled with the material which is excised from parental strands and transferred into the gaps. It follows from the data of Ley that the average size of excited regions in parental strands which become filled by repair synthesis is approximately 1.5 x 10⁴ nucleotides in length per every unexcised pyrimidine dimer. In view of the fact that in THY⁻ TRP⁻ prestarved *E. coli* B/r Hcr⁺ thy⁻ trp⁻ cells only a negligible part (less than 2% — see comparison with the *E. coli* Bₛ₋₁ strain) is lethal from the applied dose of 756 erg·mm⁻² and more than 50% of the residual amount becomes repaired by non-excision repair, we arrive inevitably at the absurd conclusion that in case the values stated by Ley hold exact, for the transfer and filling of gaps in the daughter strand in the prestarved *E. coli* B/r Hcr⁺ thy⁻ trp⁻ strain there must well-nigh be utilised the whole of the parental strand (supposed size of the chromosome = 10⁷ nucleotides in length (ref. 19); exposure to 756 erg·mm⁻² results in 7.6 x 10³ pyrimidine dimers per chromosome as calculated from the averaged data in Table II and Fig. 2, using the ratio between initial quantum yields of various pyrimidine dimers stated by Eisinger and Lamola). The size of daughter strand gaps and regions excised from parental strands in *E. coli* B/r Hcr⁺ cells after THY⁻ TRP⁻ starvation must therefore be smaller than the size of gaps in *E. coli* B/r Hcr⁻ cells if they become repaired in the manner as supposed by the above quoted authors. Since these two strains differ in mutation in the *uvr B* locus only, the pos-
sibility cannot be excluded that in wild-type cells products of uvr+ genes may participate in the recombination-like post-replication repair. Recently, there have been published data according to which UV-induced recombination in transduction requires uvr A – uvr B nuclease ²¹. However, we cannot exclude the hypothetical alternative of a “copy choice” – like post-replication repair suggested by Sedliaková et al. ⁸. During such repair gaps in daughter strand would become filled by synthesis for which there would served as an undamaged template the other complementary daughter strand.

Irrespective of the mechanisms of non-excision repair in UV-irradiated THY-TRP- prestarved Escherichia coli B/r Hcr+ thy~ trp~ cells it will seem evident that responsibility for the high resistance of wild-type cells cannot be ascribed only to excision repair, on the other hand non-excision repair in these cells need not necessarily be responsible for most of the UV-induced mutations.

We thank Dr. M. Sedliaková for her interest in this work and Miss K. Sillayová for technical assistance. We also thank Drs. E. M. Witkin and C. H. Clarke for suggesting the membrane transfer experiment and Dr. B. A. Bridges for critical reading of the first version of the manuscript.

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