Effects of Coumarin, Pyronin Y, 6,9-Dimethyl 2-Methylthiopurine and Caffeine on Excision Repair and Recombination Repair in *Escherichia coli*

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SUMMARY

Pyronin Y (0.06 and 0.16 mM), coumarin (2 and 4 mM), 6,9-dimethyl 2-methylthiopurine (2 mM) and caffeine (8 mM), strongly inhibit Uvr repair (presumed excision repair) of u.v. lesions in *Escherichia coli*. With 0.16 mM-pyronin Y or 4 mM-coumarin, one thymine dimer was a lethal event in the K12 strain JC2926 *rec A-13* and one to three dimers in the B/r derivative WP-2 *rec A-13* and the B *exr A* derivative BS-2. Neither these compounds nor 8 mM-caffeine had a significant effect on the survival of irradiated bacteria of the corresponding *uvr* strains WP-2 Hcr⁻, BS-12, and BS-8. I suggest that 8 mM-caffeine, 0.16 mM-pyronin Y, 4 mM-coumarin and 2 mM-6,9-dimethyl 2-methylthiopurine selectively block excision repair without substantially affecting recombination repair.

Caffeine at 12 mM only slightly depressed the viability of a u.v.-irradiated culture of WP-2 Hcr⁻ or BS-12, but at 16 mM there was a much greater effect, particularly at low u.v. doses (<40 ergs/mm²). Since excision repair appeared to be effectively blocked by 8 mM-caffeine, the depression of u.v. survival of the *uvr* mutants by 16 mM-caffeine was presumed to be due to interference with recombination repair (Witkin & Farquharson, 1969).

INTRODUCTION

The ultraviolet (u.v.) photoproducts of principal biological significance in *Escherichia coli* are pyrimidine dimers in DNA which interfere with normal DNA replication, and may lead to cell death (Setlow, 1968).

Escherichia coli can cope with these lesions in the dark in at least two ways. In one, passage of the replicating fork past pyrimidine dimers in a priming strand results in a series of gaps in the freshly replicated strand, presumably because the dimers are unable to direct the insertion of nucleotides into the growing polynucleotide. The existence of discontinuities in the latter is thought to initiate recombination between daughter DNA strands permitting the reconstruction of the strands (Rupp & Howard-Flanders, 1968). This is referred to as 'recombination repair'. Two genes with alleles giving an impaired recombination repair effectiveness are *rec A-13* and *exr A*.

The other dark repair system involves the excision of pyrimidine dimers from the DNA and is termed excision repair. *uvr* Mutants are defective in this function.

Some years ago caffeine (5 to 10 mM) was shown to inhibit dark repair (Witkin, 1958), and more recently excision repair (Setlow & Carrier, 1968). *In vitro* it neither inhibits DNA polymerase I activity (at a concentration of up to 100 mM, Grigg, 1968) nor the excision endonuclease isolated by Carrier & Setlow (1970). Excision repair *in vivo* is inhibited by

caffeine, however, so either different enzymes are involved *in vivo* or caffeine inhibits the endonuclease or exonuclease activity of Kornberg's enzyme *in vivo* (Kelly, Atkinson, Huberman & Kornberg, 1969). A number of factors other than caffeine interfere with dimer excision. These include crystal violet, acriflavine (Feiner & Hill, 1963; Setlow, 1964) cyanide, and starvation (Setlow & Carrier, 1968).

The exact effects of caffeine on recombination repair are less obvious. Caffeine ≤ 10.7 mM had very little effect on the u.v. survival of the *uvr A* mutant WP-2 Hcr⁻, which implies that the independent recombination-repair pathway was unaffected (Sideropoulos & Shankel, 1968; Witkin & Farquharson, 1969), although 10.7 mM-caffeine seems to depress normal recombination frequency in *Escherichia coli* (Witkin & Farquharson, 1969). Harm (1967) reported that 4 mM-caffeine depressed the survival of u.v.-irradiated *B syn* (*uvr C*, Strauss, 1968) cells. Survival of u.v.-irradiated bacteria of the *uvr B* (and *exr A*) strain BS-1 was aided by the presence of 10.7 mM-caffeine in the post-irradiation incubation medium when the u.v. dose was < 18 ergs/mm². Above this dose caffeine inhibited recovery, and Harm suggested that the *uvr* strains he studied exhibited residual *uvr*⁺ activity.

In this paper several compounds of low toxicity are described which selectively inhibit Uvr repair (presumed excision repair) and the effects of caffeine on u.v. survival of several *uvr* mutants are re-examined. The active compounds share the common characteristic that they bind selectively to denatured DNA.

METHODS

Bacteria. The bacteria used and their characteristics are shown in Table 1.

Culture media. G.T. – a modified Gray and Tatum glucose salts medium (for composition see Grigg, 1969). BAB – Oxoid Blood Agar Base – a broth agar plating medium. TNM buffer – tris, 0.1 M; NaCl, 0.15 M; MgSO₄·7H₂O, 0.01 M; adjusted to pH 7.1. T1 diluent –

Table 1.

Strain	Genotype					Dimer excision status	Recom- bination repair status	Reference/origin	Source
	<i>lon</i>	<i>rad</i>	<i>uvr</i>	<i>rec</i> <i>A-13</i>	<i>exr A</i>				
WP-2	—	—	+	+	+	+++	+++	Witkin, 1947; Donch, Chung & Greenberg, 1968; Donch, Green & Greenberg, 1968	Ruth Hill, ex E. M. Witkin
WP-2 Hcr ⁻	—	—	-A	+	+	O	+++	Donch & Greenberg, 1969	Ruth Hill
WP-2 <i>rec A</i>	—	—	+	—	+	+++	O	Conjugation of <i>rec A</i> into wp-2	B. A. Bridges and Ann Rothwell
B	—	+	+	+	+	+++	+++	Donch & Greenberg, 1968	Ruth Hill
BS-8	—	—	-B	+	+	O	+++	Donch & Greenberg, 1968	Ruth Hill
BS-12	—	+	-12	+	+	O	+++	Donch & Greenberg, 1968; Hill & Feiner, 1964	Ruth Hill
BS-2	—	+	+	+	—	+++	+	Donch, Green & Greenberg, 1968	Ruth Hill
JC2926	+	U	+	—	+	U	O		A. J. Clark

U, unknown; O, deficient.

nutrient broth (Difco), 0.8 g; NaCl, 0.58 g; H₂O, 1 l. T1 agar – tryptone (Difco), 10 g; agar (Difco), 11 g; NaCl, 0.58 g; H₂O, 1 l. T1 soft agar – nutrient broth (Difco), 8 g; agar (Difco), 7 g; NaCl, 0.58 g; H₂O, 1 l.

Cultural methods

An overnight culture was grown up in G.T. to exhaustion of the glucose in the medium. The resultant stationary phase culture was washed with 0.9 % saline and resuspended in either saline or TNM buffer at a concentration of about 3×10^7 bacteria/ml. Portions (3 ml) were irradiated in a 5 cm diameter Petri dish with a 15 W cold cathode u.v. tube (Oliphant, Sydney, Australia) at a dose rate of 157, 8 or 1 ergs/mm²/s in a small cabinet with a camera shutter (Shanel 5A) installed in a septum between the u.v. lamp and the bacterial suspension. After irradiation the bacterial suspension was immediately diluted in saline, 0.2 ml portions were spread on BAB plates and then incubated at 37°.

In host cell reactivation experiments a suspension of T1 phage in TNM buffer (about 4×10^7 p.f.u./ml) was irradiated at a dose rate of 157 ergs/mm²/s for 10, 20, 40 or 60 s. Irradiated and unirradiated samples were diluted in T1 phage diluent and samples of appropriate dilutions added to 3 ml of T1 soft agar containing the compound being tested and about 10^8 log phase WP-2 bacteria. The soft agar suspension was then poured over the surface of T1 agar, also containing the compound being tested, in a Petri dish. The plates were incubated at 37° and the resulting T1 plaques scored.

Chemicals. The compounds tested for their biological activity were pyronin Y (G. Gurr, London), coumarin (E. Merk, Ag., Darmstadt, W. Germany), caffeine (Evans Medical Ltd, Liverpool) and 6,9-dimethyl 2-methylthiopurine (a gift from Dr D. Brown, Department of Medical Chemistry, Australian National University).

RESULTS

Effects of coumarin, pyronin Y, 6,9-dimethyl 2-methylthiopurine

The lethal effect of u.v. irradiation on *Escherichia coli* WP-2 was enhanced when the irradiated bacteria were incubated on BAB medium containing coumarin (2 mM or 4 mM), 8 mM-caffeine or 0.16 mM-pyronin Y (Fig. 1). None of these substances depressed the survival of unirradiated control bacteria, but the growth rate was slightly lower on the coumarin medium.

Coumarin inhibited host cell reactivation (HCR) of T1 phage by strain WP-2 (Fig. 2). Since this phenomenon seems correlated with excision-repair, experiments were performed on strains defective in excision-repair, WP-2 Hcr⁻ (*uvr A*), or recombination repair, WP-2 *rec A-13*, both to check the ability of coumarin and the other compounds to inhibit Uvr-repair and to test their effect on recombination repair. A lower survival of u.v.-irradiated *uvr*⁻ bacteria on medium containing the test compound, e.g. coumarin, would indicate that the latter inhibited the other dark-repair process, recombination repair, whereas a similar result with a recombination-defective culture would indicate that it inhibited Uvr⁻ (excision) repair, provided that both classes of repair-defective mutants were non-leaky.

Coumarin and pyronin Y depressed the survival of u.v.-irradiated cultures of the WP-2 *rec A* strain, particularly at low u.v. doses (Fig. 1). Unirradiated WP-2 *rec A* cells proved extremely sensitive to 8 mM-caffeine and no useful data were obtained with this additive. Coumarin, pyronin Y and 8 mM-caffeine had little effect on the sensitivity of WP-2 Hcr⁻ bacteria (*uvr A*) to u.v. doses < 6 ergs/mm² although a small effect was apparent at higher

Fig. 1. u.v. Survival of WP-2, WP-2 *Hcr*⁻ (*uvrA*) and WP-2 *recA* bacteria on BAB medium supplemented with nothing (O), 8 mM-caffeine (CAF), 0.16 mM-pyronin Y (PY), 4 mM-coumarin (CU). All bacteria were irradiated in stationary phase of growth.

doses particularly with 8 mM-caffeine (Fig. 1). Similar results were obtained with strains of the *Escherichia coli* B family (Fig. 3). Thus u.v. survival of bacteria of the *uvr-12* strain BS-12 was unaffected by post-irradiation exposure to coumarin (4 mM), 6,9-dimethyl 2-methylthiopurine (2 mM) or caffeine (8 mM). Pyronin Y slightly suppressed survival over the u.v. dose range 3 to 29 ergs/mm². On the other hand, a considerable drop in u.v. survival of the recombination defective (*exrA*) BS-2 bacteria occurred if they were exposed to any of these compounds. That the effect was not limited to cells having a strain B or WP-2

Fig. 2. Host cell reactivation of u.v.-irradiated T1 phage by wp-2 bacteria on medium supplemented with nothing (O) or coumarin (4 mM) (CU). u.v. Dose rate 157 ergs/mm²/sec.

background is indicated by the observation that pyronin Y and coumarin (the only compounds tested) also suppressed u.v. survival of the K12 *rec A-13* strain JC2926 (Fig. 4). In fact, in the presence of these additives, approximately 1 pyrimidine dimer per chromosome appeared lethal to JC2926 bacteria and 1–3 to WP-2 *rec A* and B *exr A* bacteria.

Of the compounds examined for their effects on u.v. survival, on the whole pyronin Y had the highest molar activity, particularly when assayed on strain B *exr A* bacteria (Fig. 3). With the K12 *rec A* strain JC2926 and WP-2 *rec A* 4 mM-coumarin was more effective than 0.16 mM-pyronin Y but both compounds essentially inhibited all repair at low u.v. doses (Fig. 4). The greatest effect of all the substances tested was apparent at the lowest u.v. doses used (Fig. 1, 3, 4), the depression of survival declining progressively with increasing u.v. dose.

The shoulder of the dose-survival curve at low u.v. doses represents the number of DNA lesions, mainly pyrimidine dimers, with which the cell can deal without loss of viability. Double *uvr*, *rec A* mutants lacking both dark repair capabilities require approximately one dimer to induce a lethal event (Howard-Flanders, Theriot & Stedeford, 1969). From the data presented above, coumarin and 0.16 mM-pyronin Y inhibit essentially all excision repair activity and 8 mM-caffeine and 6,9-dimethyl 2-methylthiopurine probably are as effective.

Effects of caffeine

Although ≤ 12 mM-caffeine had minimal effects on any of the *uvr* mutants tested (Fig. 1, 3, 5), 16 mM-caffeine depressed survival very significantly, particularly at low u.v. doses (Fig. 5). This high concentration of caffeine could be used with the *uvr* strains because of their relative resistance to the toxic effects of caffeine (Grigg, 1968). The effects of 16 mM-caffeine on survival after u.v. were reversible for at least 4 h following irradiation. Identical results were obtained with *uvr* mutants having different genetic backgrounds including the *uvr B rad* strain BS-8, the *uvr-12* strain BS-12, and the *uvr A rad* strain WP-12 Hcr⁻.

Fig. 3. u.v. Survival of *Escherichia coli* B, *E. coli* B *uvr* 12 (BS-12), *E. coli* B *exr* A (BS-2) bacteria on BAB medium supplemented with nothing (O), 8 mM-caffeine (CAF), 0.06 or 0.16 mM-pyronin Y (PY 1, PY 11), 2 mM 6,9-dimethyl 2-methylthiopurine (692), 2 or 4 mM-coumarin (CU 2, CU 4). All bacteria were in stationary phase when irradiated.

Fig. 4. u.v. Survival of stationary phase cells of the *rec A-13* strain JC2926 on BAB medium supplemented with nothing (O) 0.16 mM-pyronin Y (PY) or 4 mM-coumarin (CU). The bacteria were grown up to stationary phase in broth. The survival curves for small u.v. doses (< 2 erg/mm.^2) are shown on an expanded dose scale in the insert. The calculated number of thymine dimers (T-T) per chromosome generated by the u.v. dose is also indicated on the dose scale.

DISCUSSION

In the *Escherichia coli* WP-2 and B strains used in this study, coumarin (2 and 4 mM), pyronin Y (0.16 and 0.04 mM), 6,9-dimethyl 2-methylthiopurine (2 mM) and caffeine (8 mM) selectively inhibited the *uvr*- (or excision-) repair process. The results with the K12 *rec A* strain suggested that inhibition of excision repair occurs in this strain also. Although dissimilar chemically these compounds share with caffeine the ability to bind specifically to single-stranded DNA (T'so & Lu, 1964; Grigg, Edwards & Brown, 1971). Domon, Barton, Porte & Rauth (1970) conclude that this property is important in explaining inhibition of excision repair by caffeine. The results reported here suggest that substances which exhibit selectivity of binding for single-stranded DNA may inhibit excision repair. As with caffeine, the mode of action could be by co-operative denaturation of regions of the DNA localized

Fig. 5. u.v. Survival of stationary phase WP-2 Hcr^- (*uvrA*) bacteria on BAB medium containing 0, 12 and 16 mM-caffeine.

around pyrimidine dimers where hydrogen bonding between the complementary strands of DNA was weakened. Since the endonuclease which initiates excision repair is a double-strand specific enzyme, presumably it could not then recognize the substrate. The range of substances which share specific binding to single-stranded DNA is quite large and, apart from the compounds discussed above, includes steroids, naphthalene, phenanthrene (T'so & Lu, 1964) and a number of the thio- and mercaptopurines (Grigg *et al.* 1971). The compounds used in the work reported here also share the ability to amplify the induction of DNA breakdown and cell death by Phleomycin (Grigg, 1970). The order of ranking of the relative efficiencies as amplifying substances and as inhibitors of excision repair is somewhat different. As amplifiers, 0.16 mM-pyronin Y is the most effective with the 2 mM-methylthiopurine second, then 4 mM-coumarin and 8 mM-caffeine a good way behind (Grigg *et al.* 1971), whereas, as inhibitors of excision repair, 0.16 mM-pyronin, 2 mM-coumarin, 2 mM-6,9-dimethyl 2-methylthiopurine and 8 mM-caffeine have comparable efficiencies at low u.v. doses (< 8 ergs/mm²).

Pyronin B, a compound closely related chemically and in its histological staining properties to pyronin Y, does not affect mutation frequency decline (MFD), unlike a number of other basic dyes (Witkin, 1961). MFD is thought to be associated with the excision of thymine dimers from the u.v.-irradiated DNA (Bridges, 1966; Witkin, 1966), and the observation that pyronin Y is an efficient inhibitor of excision repair may seem paradoxical. It would indicate that either pyronin B differs from pyronin Y and does not prevent thymine dimer excision from DNA, or the MFD phenomenon is not related in a simple fashion to the excision of thymine dimers.

Incubation of irradiated cells of *Escherichia coli* B strains in medium containing 8 mM-caffeine effectively suppressed excision repair. Consequently, the ability of a similar or higher concentration of caffeine to depress the survival of excision-defective strains of

Escherichia coli B (Harm, 1967; and see above) may be explained best by the proposition that caffeine inhibits another dark repair process, recombination repair (Witkin & Farquharson, 1969), rather than by the conclusion that these *uvr* mutants are 'leaky', i.e. possess residual excision activity (Harm, 1967). Furthermore, if the excision-defective strains used by Harm are non-leaky then a reinterpretation of his data suggests that acriflavine also inhibits recombination repair.

Coumarin is used in the cosmetic and food industries as a scent and flavouring substance. The effectiveness of another coumarin in inhibiting excision repair has been observed recently by Bridges (1971). Since failure to remove thymine dimers from DNA may lead to the synthesis of antibodies to them (Tan, 1968) which, in laboratory animals at least, cross-react with normal DNA with possible serious side-effects (Cleaver, 1969), the trivial use of such potential or actual u.v.-sensitizing agents should be queried.

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