Conversion of psoralen DNA monoadducts in E. coli to interstrand DNA cross links by near UV light (320–360 nm): Inability of angelicin to form cross links, in vivo

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Summary. Both angelicin and psoralen monoadducts formed in vivo in E. coli by near UV light produce lethal and mutagenic effects. However psoralen monoadducts are converted to cross links by higher doses of UV; angelicin monoadducts are not. The relevance of these results to psoralen photosensitization is discussed.

Furocoumarins[2] such as psoralen, 8-methoxypsoralen and trimethylpsoralen photosensitize deoxyribonucleic acid in the presence of near UV light (320–360 nm, NUV) producing lethal and mutagenic effects in a wide range of viruses, bacteria, and eukaryotic cells (see table, b for references). DNA interstrand cross links between psoralens and pyrimidine bases in opposite strands of the DNA duplex have been implicated as the major factor contributing to the sensitizing actions of the psoralens[3,6,24]. Excision and genetic recombination, controlled by the uvr and recA genes in E. coli, are responsible for recovery from cross linking damage[3,5]. It has been reported by Reid and Walker[6] and by Ben-Hur and Elkind[7] that mammalian cells possess an efficient mechanism for the repair of monoadducts and cross links in their own DNA. Cells from patients with Fanconi's anaemia (FA) appear defective in the ability to repair DNA cross links[8]. Human fibroblasts from normal and FA subjects have the capacity to repair only monoadducts in the DNA of irradiated, infecting adenovirus; fibroblasts cultured from patients with Xeroderma pigmentosum cannot repair monoadducts in photosensitized adenoviruses[9]. These repair deficiences have been demonstrated by increased sensitivity to kill and high susceptibility to chromosome damage. Many of the known prerequisites for and consequences of psoralen photosensitization in these and other biological systems are presented in the table.

The following mechanism[24,7,10] has been postulated to account for psoralen photosensitization: 1. DNA + psoralen $\rightleftarrows$ intercalated complex of psoralen with DNA. 2. Intercalated complex + NUV $\rightarrow$ psoralen-DNA monoadducts. 3. Psoralen-DNA monoadducts + NUV $\rightarrow$ interstrand DNA cross links. Various values have been suggested for the percentage of total bound psoralen forming cross links[7,10] and a direct relationship between cell survival and the amount of non-cross linked DNA has been reported[9,11]. However, several questions and experiments are generated from the 3 mechanistic postulates as stated. These concern the relative significance of each of the 3 steps in the photosensitizing action of the psoralens. We have attempted to clarify some of the implications of these postulates.

Firstly, how strong is the 'intercalated' complex of psoralen with DNA? In vitro, Musajo and Rodighiero[12] reported weak bond interactions between furocoumarins and aqueous solutions of DNA in the absence of irradiation. If intercalation, in the normally accepted sense, is a necessary prerequisite under step 1 for the operation of step 2, then treatment in the dark of bacterial cells with psoralen, followed by the removal of non-complexed psoralen prior to irradiation, should result in photosensitization as monoadducts and cross links would be formed from the intercalated complexes. This expectation is unfulfilled (figure 1); a finding essentially similar to that observed by Ben-Hur and Elkind in mammalian cells[24]. Recent evidence obtained from bacterial DNA (in vitro) failed to show the changes in DNA melting points or sedimentation characteristics expected if psoralens were to intercalate with DNA (Lown, personal comm.). Thus intercalation is not a prerequisite for photosensitization.

Secondly, the actual role of monoadducts in cell lethality is generally considered to be comparatively small[10,24]. Removal of psoralen after sufficient irradiation to give exponential kill (figure 2, psoralen removed after 97.5% kill) did not affect the slope of the survival curve. This compares well with the data of Ben-Hur and Elkind[24] for mammalian cells in tissue culture. They observed a very slight change in slope and thus suggested that monoadducts largely constituted sublethal damage. However, if the initial radiation dose in the presence of psoralen is decreased (30% kill), then it follows that the number of monoadducts formed, convertible to cross links by continued irradiation, will be fewer. Limited monoadduct

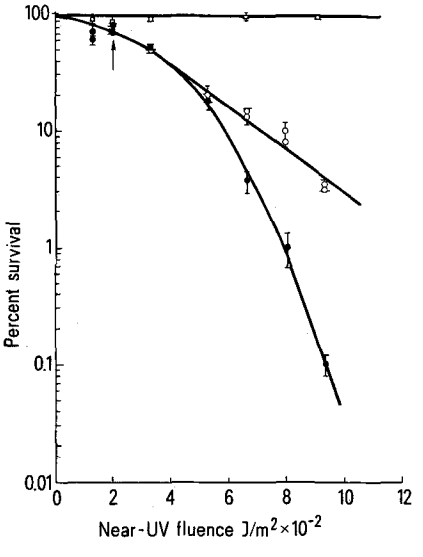


Fig. 1. Effect of near-UV irradiation on survival of E. coli WP2 Try⁻ in the presence of psoralen; compared to the effects of removing the psoralen prior to irradiation and after a short period of irradiation. Cultures grown in brain heart infusion broth to 2×10^8 cells/ml were collected by filtration on 0.22 μm millipore filters, washed and resuspended in 0.07 M PO_4 buffer (pH 7.0). At least 10 min prior to irradiation, psoralen, dissolved in 95% ethanol, was added to the cells at a final concentration of 1.85×10^{-4} M. Cells were protected from light except during irradiation by 2 General Electric Company black lamps (F20T12 BLB) which, at 9 cm distance emitted 1.34×10^1 j/m². Survival figures were obtained by conventional colony counts on nutrient agar after 24 h incubation at 37°C. □ — □ Psoralen was added to the cells as described and after 10 min, in the dark, cells were refiltered, washed and resuspended in buffer without psoralen. Cells were then subjected to irradiation procedures. ● — ● Psoralen was present throughout irradiation. ○ — ○ After a NUV fluence of 2.01×10^2 j/m² (yields 68.3% survival) indicated by the arrow, unbound psoralen was washed away and irradiation continued. Bars indicate ± SD.

formation results in a reduced rate of kill by cross links as shown in figure 1. The survival of E. coli in the presence of angelicin, an angular furocoumarin incapable of forming DNA interstrand cross links[13] was also examined (figure 2). The absence of cross link formation with angelicin was confirmed with evidence obtained from ethidium bromide spectrofluorimetric analysis on bacterial DNA irradiated in vitro (Lown, personal comm.) and by experiments in this laboratory on DNA obtained from irradiated bacteria and analysed by alkaline density gradient ultracentrifugation. At equimolar concentrations, angelicin required 5.5 times more irradiation than psoralen to give 90% kill. Irradiation following removal of unbound angelicin immediately terminated photosensitivity to further NUV; slight recovery of lethal damage was apparent. The covalent angelicin-DNA bonds are not

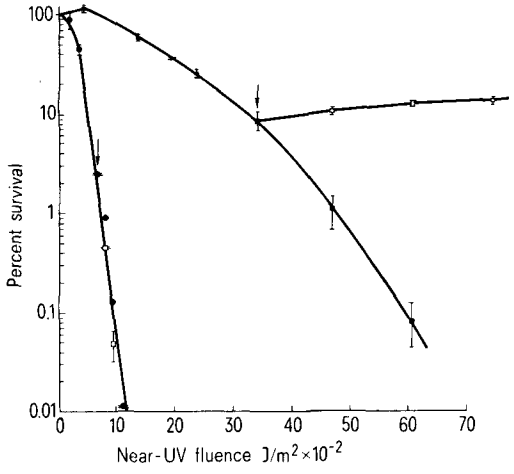


Fig. 2. Sensitivity of E. Coli WP2 Try⁻ to near-UV irradiation in the continuous presence of psoralen or angelicin and following removal of these photosensitizing agents. Cells were grown and treated as described in figure 1 except both psoralen and angelicin (isopsoralen) were examined. ● — ● Irradiation in the presence of psoralen. ■ — ■ Irradiation in the presence of angelicin. Arrows indicate the times at which cells were filtered, washed with buffer to remove unbound psoralen or angelicin, then resuspended in the original volume for contained irradiation in the absence of free photosensitizing agents. Hollow symbols represent post-washing irradiation: □ — □ unbound psoralen removed; ▫ — ▫ unbound angelicin removed. Bars indicate ± SD.

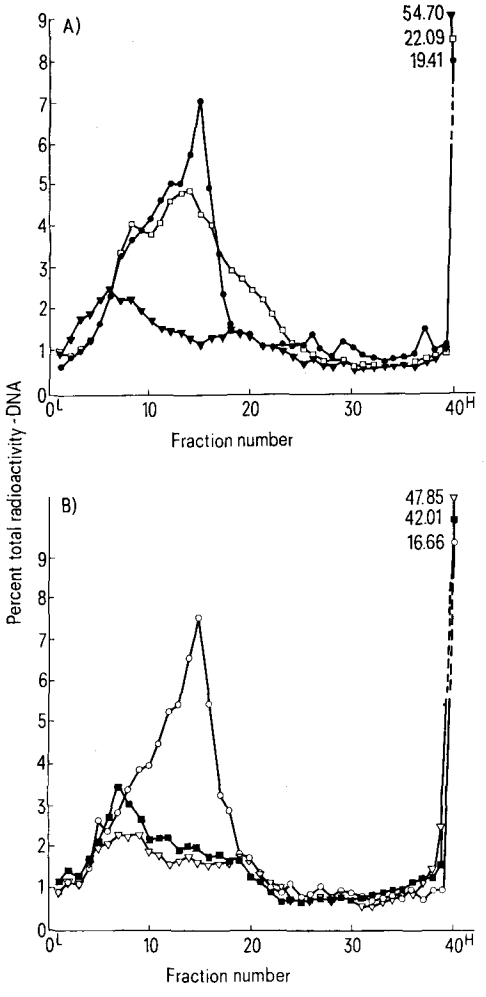


Fig. 3. Changes in the sedimentation characteristics of ^{3}H-labelled E. coli DNA following NUV irradiation in the presence of psoralen and after removal of unbound psoralen. Cultures of E. coli WP2 Try⁻ were grown for 12 h (~ 1×10^8 cells/ml final density) in minimal medium supplemented with 10 μg/ml L-tryptophan and 4 μCi/ml [methyl-^{3}H] thymidine (spec. act. 17 Ci/mmole). Cells were filtered, resuspended, treated with psoralen and irradiated as described in figure 1. Post-irradiation treatment: 0.1 ml of cells were lysed in 0.15 ml 2% sodium dodecyl sulfate containing 0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA and 0.25 N NaOH on the surface of alkaline sucrose gradients (3.25 ml of 5–20% [w/v] sucrose on top of 0.75 ml 60% [w/v] sucrose in 0.2 N NaOH and 10^{-3} M EDTA). Gradients were kept 30 min at room temperature to facilitate lysis and release of DNA prior to centrifugation at 95,000 × g for 60 min at 20 °C in a Beckman SW 56 rotor. 5-drop fractions were collected onto filter paper squares by upward displacement (fraction 1 = 5% sucrose); fractions below No. 40 represent material in the 60% sucrose layer therefore have been represented as single points. Papers were washed sequentially in ice-cold 5% TCA, ethanol and acetone before counting for tritium in toluene-PPO scintillation fluid.

A ● — ● Control plus psoralen, no irradiation.
□ — □ 2.10×10^2 j/m² in the presence of psoralen.
▲ — ▲ 8.04×10^2 j/m² in the presence of psoralen.

B ○ — ○ Control plus psoralen, no irradiation, psoralen, washed away prior to layering onto gradient.
■ — ■ 2.01×10^2 j/m² plus psoralen, unbound psoralen washed away, an additional irradiation of 6.03×10^2 j/m² then onto gradient.
△ — △ 8.04×10^2 j/m² plus psoralen, then unbound psoralen washed away and cells layered onto gradient.

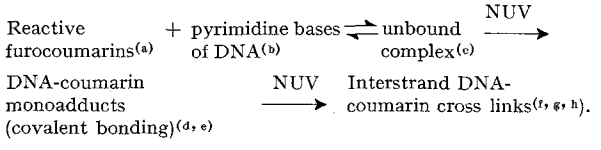
Ordinate = ^{3}H cpm per fraction taken as percentages of the total radioactivity. on the gradients (~ 19,000 cpm). Abscissa = fraction numbers from 1 (5% sucrose) to 40 (the 60% sucrose layer). L = 5% sucrose, H = 60% sucrose.

1 Supported by the National Research Council of Canada.
2 L. Musajo and G. Rodighiero, Experientia *18*, 153 (1962).
3 R. S. Cole, J. Bacteriol. *107*, 846 (1971).
4 L. Musajo, P. Visentini, F. Baccichetti and M. A. Razzi, Experientia *23*, 335 (1967).
5 R. S. Cole, Proc. nat. Acad. Sci. *70*, 1064 (1973).
6 B. D. Reid and I. G. Walker, Biochim. biophys. Acta *179*, 179 (1969).
7 E. Ben-Hur and M. M. Elkind, Biochim. biophys. Acta *331*, 181 (1973).
8 M. S. Sasaki and A. Tonomura, Cancer Res. *33*, 1829 (1973).
9 R. S. Day, A. S. Giuffrida and C. W. Dingman, Mutation Res. *33*, 311 (1975).
10 R. S. Cole, Biochim. biophys. Acta *254*, 30 (1971).
11 M. J. Ashwood-Smith and E. Grant, Cryobiology *11*, 160 (1974).
12 L. Musajo and G. Rodighiero, Photochem. Photobiol. *11*, 27 (1970).
13 F. Dall'Acqua, S. Marciani, L. Ciavatta and G. Rodighiero, Z. Naturforsch. *26*, 561 (1971).

split by reirradiation at near UV wavelengths[14]. Neither psoralen nor angelicin photosensitized kill is modified by enzymatic photoreactivation[6,15]. It would seem, therefore, that this recovery is the result of dark repair mechanisms. These results support the proposed sequential conversion of monoadducts to cross links and show clearly that monoadducts contribute to cell death, albeit less effectively than cross links.

Thirdly, analysis by alkaline density gradient ultracentrifugation of psoralen plus NUV treated bacterial DNA (in vivo) has shown a close correlation between photoinduced kill and the disappearance of normally sedimenting DNA[11]. Figure 3 illustrates the continuation of cross link formation (loss of DNA in the normal peak) following reirradiation in the absence of unbound psoralen after initial sensitization at a low NUV fluence (2.0×10^2 j/m^2). This supports, at the molecular level, the correlations between survival and DNA cross linking shown in figure 2. Angelicin photosensitization (monoadduct formation) did not affect DNA profiles (unpublished results). Continuous irradiation (8.04×10^2 j/m^2) in the presence of psoralen resulted in 20% of the DNA remaining unchanged in sedimentation characteristics. Removal of the unbound psoralen after 2.0×10^2 j/m^2 and then reirradiation to a total of 8.04×10^2 j/m^2 left 25.5% of the DNA unaffected. These results, illustrated in figure 3, indicated a 5.5% difference in cross linked DNA (monoadducts not detected) corresponding to an 8.6% difference in survival (kill by both cross links and monoadducts) under the same irradiation procedures. The difference in these figures represents an inability to detect lethal non-cross linking damage by gradient analysis.

In conclusion, the mechanisms currently postulated to account for the lethal photosensitizing effects of psoralens, excluding non-cross linking coumarins, would appear to be partially correct. Intercalation, as normally defined, is not the necessary prerequisite for psoralen-DNA covalent binding since the 'association' between psoralen and DNA is eliminated by simple washing procedures. Certainly interstrand DNA cross links occur and require unlimited prior formation of psoralen-DNA monoadducts to produce maximum exponential lethality with continuous NUV exposure. However, monoadducts are also responsible for lethality as evidenced by angelicin photosensitization. (As with cross linking coumarins, mutation in bacterial systems and chromosome aberrations in mammalian cells, have been observed following angelicin-NUV treatment[30].



a) For example: psoralen, 8-methoxypsoralen, 4,5′,8-trimethylpsoralen, angelicin (see also ref. 2).

b) The following systems have been studied in vivo:
λ phage[3,10,16,17], T_2[17], T_4[11,18], MS_2*, ΦX 174*, adenovirus 2[9]
Aspergillus conidia[19], E. coli (various strains)[3,5,11,16,20], Saccharomyces cerevisiae*, Salmonella typhimurium*, Sarcina lutea[21], Staphylococcus aureus[22]
sea urchin sperm[15], Drosophila melanogaster[23], Erlich ascites tumor cells[4], mouse leukemia cells[16], Chinese hamster cells[7,24] human amnion cells[25], human fibroblasts – normal and Xeroderma pigmentosum (XP)[26], Fanconi's anaemia (FA) leukocytes[8], mice[27], guinea pigs[28], humans[29].

c) The association is disrupted by simple washing procedures[24].

d) Lethal damage → death.
1. no O_2 requirement for binding[12]
2. rate limited by DNA and coumarin concentrations[10]
3. temperature dependent, at −196 °C marked reduction in monoadduct induced kill and mutation* (cf. ref. 11)
4. sensitivity dependent on intact excision-recombination repair systems, i.e. exr⁻ and uvr⁻ E. coli extremely sensitive*.

e) Sublethal damage[11] → recovery and/or mutation*.
1. no photoreactivation[13,*]
2. repair of adenovirus 2 by normal and FA cells, not by XP cells[9]
3. Salmonella uvr⁻ strains → base-pair substitutions and +1 frameshift mutations, no −2 frame-shifts*
4. base-substitution and locus mutations but no appreciable suppression mutations or putative frame-shift mutations in S. cerevisiae*.
5. exr⁻ unmutable* – intact exr gene required for mutation.

f) Formation dependent on steric relationships between reactive sites of coumarins and pyrimidine bases[3], reactivity of 4′,5′- and 3,4-double bonds of coumarins[13], and prior monoadduct formation[7].

g) Lethal damage → death.
1. E. coli uvr⁻, rec⁻ and exr⁻ more sensitive than wild type[3,20]
2. temperature dependent – no detectable cross link formation at −196 °C[11]
3. relatively insensitive to pH[22]
4. no O_2 requirement[21,22]
5. inhibition of scheduled DNA synthesis in normal and XP cells[25–27]
6. no RNA or protein synthesis inhibition[11,27].

h) Sublethal damage → recovery and/or mutation[20]
1. no photoreactivation[11,20]
2. excision and genetic recombination → recovery from cross links[3,5]
3. exr⁻, rec⁻ mutants of E. coli non-mutable[20]
4. suppressor + true reversions in E. coli WP2[20]
5. base substitutions, suppressor and locus mutations in S. cerevisiae, negligible frame-shifts*
6. no repair synthesis in XP or FA cells[8,26]
7. no repair of cross linking in adenovirus 2 by normal, FA or XP cells[9]
8. repair synthesis in normal mammalian cells[7], not in XP cells[26]
9. base-pair substitutions (T_4[18] and Salmonella*) and +1 frameshift mutations in Salmonella, no −2 frame-shifts*
10. random non-specific mutation in Aspergillus[19].

* Indicates unpublished results of Ashwood-Smith and Grant.

14 G. Rodighiero, F. Dall'Acqua, S. Marciani, P. Chandra, H. Feller, A. Götz and A. Wacker, Biophysik *8*, 1 (1972).
15 G. Colombo, Exp. Cell Res. *48*, 167 (1967).
16 R. S. Cole, Biochim. biophys. Acta *217*, 30 (1970).
17 R. S. Cole and D. Zusman, Biochim. biophys. Acta *224*, 660 (1970).
18 J. W. Drake and J. McGuire, J. Virol. *1*, 260 (1967).
19 B. R. Scott and T. Alderson, Mutation Res. *12*, 29 (1971).
20 S. Igali, B. A. Bridges, M. J. Ashwood-Smith and B. R. Scott, Mutation Res. *9*, 21 (1970).
21 M. M. Matthews, J. Bacteriol. *85*, 322 (1963).
22 E. L. Oginsky, G. S. Green, D. F. Griffith and W. L. Fowlks, J. Bacteriol. *78*, 821 (1959).
23 B. Nicoletti and G. Trippa, Rc. Accad. naz. Lincei *43*, 259 (1967).
24 E. Ben-Hur and M. M. El Kind, Mutation Res. *18*, 315 (1973).
25 J. E. Trosko and M. Isoun, Int. J. Radiat. Biol. *19*, 87 (1971).
26 H. P. Baden, J. M. Parrington, J. D. A. Delhanty and M. A. Pathak, Biochim. biophys. Acta *262*, 247 (1972).
27 J. H. Epstein and K. Fukuyama, Photochem. Photobiol. *21*, 325 (1975).
28 M. A. Pathak and D. M. Kramer, Biochim. biophys. Acta *195*, 197 (1969).
29 Med. Post *11* (26), 1 (1975).
30 M. J. Ashwood-Smith, E. Grant, Heddle and Friedmann, Mutation Res., in press 1977.