The Effects of Acridine Orange on Deoxyribonucleic Acid in *Escherichia coli*

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1. Acridine Orange inhibits growth of *Escherichia coli* K12 when incubated at pH 7.9, but not at pH 7.4. 2. At a non-permissive temperature for DNA polymerase I, Acridine Orange inhibits growth of a temperature-sensitive strain and also increases the rate of elimination of the F'-Lac plasmid. 3. DNA isolated from cells treated with Acridine Orange under conditions that inhibit growth contains material of low molecular weight, which is absent from DNA isolated from cells treated under conditions in which growth is not impaired. 4. Cells incubated with Acridine Orange at both pH 7.4 and 7.9 suffer degradation of DNA, as shown by loss of labelled DNA from the acid-insoluble fraction, which is not observed with untreated cells at either pH. 5. The results suggest that elimination of the F'-Lac plasmid by Acridine Orange requires inactivation of repair processes.

Aminoacridines exert a number of biological effects on bacterial cells that have been ascribed to interaction with DNA. Albert (1951) discussed the inhibition of bacterial growth by aminoacridines and showed that the effect increases with pH. A similar effect of pH is found in the photodynamic interaction of acridines with bacteriophages (Yamamoto, 1958). In addition to these effects, acridine dyes induce frame-shift mutations (Crick *et al.*, 1961) and stimulate the elimination of F-merogenotes such as the F'-Lac plasmid, which normally occurs at low frequency during bacterial growth. This last effect is also influenced by pH and is most pronounced in the presence of low concentrations of the drug at pH 7.8-7.9 (Hirota, 1960; Bastarrachea & Willetts, 1969).

Albert (1951) ascribed the influence of pH on the antibacterial effect of aminoacridines to their being bound to anionic cellular material, and this is consonant with their interaction with DNA. Quinacrine (Ciak & Hahn, 1967) and nitroakridin 3582 (Wolfe *et al.*, 1971) at bacteriostatic concentrations inhibit DNA synthesis, and these effects have been attributed to interaction with the template (Hurwitz *et al.*, 1962). Acridines are known to bind to DNA *in vitro* and produce free radicals during the process of energy transfer from dye molecules to the DNA backbone, possibly in A+T-rich regions of the duplex (Graslund *et al.*, 1969). The process is catalysed by visible light and results in single-chain

scission (Freifelder *et al.*, 1961). These processes may account for inactivation of bacteriophage by acridines (Yamamoto, 1958).

A different mode of interaction between acridines and DNA involves intercalation between adjacent base-pairs in the double helix (Peacocke & Skerrett, 1956; Lerman, 1963). Such interaction is believed to account for the observed decrease in superhelix density of covalently closed circular DNA (Waring, 1968) and for the production of the frame-shift mutations referred to above (Crick *et al.*, 1961). Southwick *et al.* (1972) have shown that exposure of *Escherichia coli* to sublethal concentrations of Acridine Orange results in intracellular degradation of DNA. In normal growing cultures, this degradation is repaired, but *polA*⁻ mutants, defective in repair processes, are sensitive to concentrations of Acridine Orange that do not inhibit the growth of normal cells.

The present studies are concerned with the elimination of the F'-Lac merogenote from *E. coli* by Acridine Orange. This phenomenon has been ascribed previously to a selective effect of Acridine Orange on extrachromosomal DNA. Such an effect would indeed account for the marked mutational effects of the compound on bacteriophage in comparison with weak effects on the bacterial chromosome (Orgel, 1965). However, our experiments are not in agreement with such an interpretation of the effects of Acridine Orange on the F'-Lac plasmid, and attention has been directed towards the interrelationship of the effects of pH on the antibacterial actions of Acridine Orange and its apparent ability

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to effect elimination of plasmids. Parts of this work have been briefly reported (Barker & Hardman, 1974a,b).

Materials and Methods

Materials

Reagents were A.R. grade (Hopkin and Williams, Chadwell Heath, Essex, U.K.). Acridine Orange, chloramphenicol, rifampicin and enzymes were from Sigma (London) Chemical Co., London S.W.6, U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K.

Growth of bacteria

E. coli strain DF69 (pro^- , arg^- , thy^- , $UvrA^-$, lac^- , str^R), a gift from Dr. D. Freifelder (Brandeis University, Waltham, MA, U.S.A.), was grown with shaking in the Tris/salts medium described by Freifelder & Freifelder (1968). The medium contained 80mM-NaCl, 20mM-KCl, 1mM-KH₂PO₄, 20mM-NH₄Cl, 1mM-MgCl₂, 2mM-CaCl₂, 20mM-Na₂SO₄ and 12mM-Tris/HCl supplemented with 5mg of Casamino acids (Difco, Detroit, MI, U.S.A.)/ml, 20 μ g of L-proline/ml, 20 μ g of L-arginine/ml, 10mg of glycerol/ml and 1 μ g of thymine/ml. The pH of the medium was adjusted by addition of 5M-NaOH or 5M-HCl and was measured at 37°C. This medium is referred to as SM-medium.

E. coli strain DS624 F'-Lac (strain DS624 containing the F'-Lac plasmid) was produced in the following way. *E. coli* DS624 (F^- , rha^- , thy^- , str^R $polA_{ts214}$), a mutant of *E. coli* temperature-sensitive for DNA polymerase I (deoxynucleoside triphosphate-DNA deoxynucleotidyltransferase, EC 2.7.7.7), was a gift from Dr. D. Sherratt, University of Sussex. The strain carried the ColE1 plasmid and was cured of this by growing for several generations at 42°C and selecting a non-colicin-producing clone as described by Durkacz & Sherratt (1973). *E. coli* DF85 F'-Lac (pro^- , met^- , Bu^R , lac^{DEL} , str^S) was obtained from Dr. D. Freifelder (Freifelder & Freifelder, 1968) and was used to introduce the F'-Lac episome into *E. coli* DS624, cured of ColE1, by conjugation and selection simultaneously for clones that were Lac^+ , thy^- , and Str^R . One of the clones isolated in this way, designated *E. coli* DS624 F'-Lac, was purified and used for experiments reported here. This strain was grown at 33°C, except where stated otherwise, in nutrient broth (Oxoid, London E.C.4, U.K.) (13 g/litre at pH 7.4) supplemented with 2mg of glucose/ml and 10 μ g of thymine/ml, in orbital shakers (150 rev./min) adjusted to appropriate temperatures. The pH of the medium did not vary more than 0.05 unit over the range of temperature used. Treatment of cultures with Acridine Orange was

carried out in the dark to avoid the photodynamic effect of the compound on DNA.

Growth of bacteria was monitored by measurement of A_{650} by using the appropriate medium in the reference cell. Measurement of viable colony-forming units was carried out by plating on nutrient agar (25 g/litre) containing 5 mg of lactose/ml, 20 μ g of thymine/ml, 250 μ g of streptomycin sulphate/ml and 0.5 mg of triphenyltetrazolium chloride/ml (referred to as NA-str-TTZ agar). Elimination of F'-Lac was measured as follows. Samples (1 ml) of an overnight culture of *E. coli* DS624 F'-Lac were added to 19 ml of fresh medium and growth was allowed to continue until A_{650} was 0.1. Acridine Orange (final concn. 25 μ g/ml) was added to experimental cultures. Experimental and control cultures were incubated at either 33 or 42°C and samples (0.5 ml) were withdrawn at intervals, serially diluted and plated on NA-str-TTZ agar. Plates were incubated at 33°C for 36 h and counts made of red (Lac^-), white (Lac^+) and sectorred red/white (Lac^+) colonies. The percentage elimination of F'-Lac at a given time of incubation was calculated as (no. of Lac^- clones + no. of Lac^+ clones) $\times$ 100/total no. of clones.

Measurement of synthesis of DNA

Samples (0.5 ml) of overnight cultures of *E. coli*, grown in SM medium or supplemented nutrient broth as described above, were added to 9.5 ml of appropriate fresh medium and incubation was continued until A_{650} was 0.1. [*methyl*-³H]Thymine was added to all cultures and Acridine Orange (final concn. 25 μ g/ml) was added to experimental cultures. Experimental and control cultures of *E. coli* DF69 were incubated at either pH 7.4 or 7.9; cultures of *E. coli* DS624 F'-Lac were incubated at either 33 or 42°C. Samples were removed at intervals from all cultures and acid-insoluble radioactivity was measured as follows. Samples of cultures (0.2 ml) were pipetted on to filter-paper squares (3 cm $\times$ 3 cm; Whatman 3MM). The filter papers were immersed in 500 ml of trichloroacetic acid (10%, w/v) at 4°C, removed after 1 h and washed twice in 95% (w/v) ethanol, once in ethanol/diethyl ether (1:1, v/v) and once in diethyl ether. They were then separated, air-dried and immersed in 10 ml of scintillation fluid containing 0.4% (w/v) of 2,5-diphenyloxazole and 0.04% (w/v) of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in xylene. Radioactivity was measured in the Packard series 3001 liquid-scintillation spectrometer, with channel limits of 50–1000 (55% gain), which gave 5% counting efficiency.

Demonstration of degradation of DNA

(a) Cultures, grown in the presence of [*methyl*-³H]-thymine as described above, with and without the addition of Acridine Orange, were harvested by

centrifugation of 10000g for 10min. The cells were resuspended in 2ml of buffer containing 0.05M-Tris/HCl, pH 8.0, and 0.05M-EDTA. Lysozyme (200 µg/ml) was added to the suspensions, which were kept at 20°C for 10min, when 0.2ml of sodium dodecyl sulphate solution (10 µg/ml) was added. The resulting lysates were extracted once with phenol saturated with 0.1M-Tris/HCl, pH 7.2, and samples (0.1 ml) of each lysate were layered on to a 5–20% (w/v) sucrose gradient (5ml) containing 0.5M-NaCl and 0.3M-NaOH. The solutions were left for 10min at room temperature (20°C) to allow denaturation of DNA and were then centrifuged at 20°C for 120min at 40000rev./min in the SW65Ti rotor of the Beckman L2-65B centrifuge. Fractions were collected from the bottom of the gradients on to filter papers and acid-insoluble radioactivity was measured as described above.

(b) *E. coli* DF69 was grown overnight in SM medium at pH 7.4 containing [methyl-³H]thymine (10 µCi/ml; specific radioactivity 100 Ci/g). A sample (4ml) was diluted to 80ml with fresh medium at 37°C containing labelled thymine as above and was incubated until A_{650} was 0.1. Cells were harvested at 37°C and resuspended in 8ml of SM medium at pH 7.4 at 37°C, but containing no thymine. Samples (1ml) of the suspension were added to 19ml of SM medium at 37°C previously adjusted to pH 7.4 or 7.9. Acridine Orange (25 µg/ml) was added to experimental cultures, but no addition was made to control cultures. Cultures were incubated at 37°C and samples (1ml) were withdrawn at intervals and added with thorough mixing, together with 0.1ml of bovine serum albumin (2.5mg/ml), to 1ml of 20% (w/v) trichloroacetic acid at 0°C. After being left for 1h, the precipitates were removed by centrifugation at 10000g for 15min at 4°C. Samples (0.2ml) of the supernatants, taken for measurement of acid-soluble radioactivity, were applied to filter paper, dried in air and radioactivity was measured as described above.

Results

Effects of Acridine Orange on growth of E. coli and maintenance of F'-Lac plasmid

The effects of Acridine Orange (25 µg/ml) on the growth of *E. coli* DF69 at pH 7.4 and 7.9 are shown in Fig. 1(a). There was no significant effect at pH 7.4, but at pH 7.9 growth was severely inhibited after 60min. A similar, but smaller, inhibition was observed at pH 7.7 (results not shown). In the absence of the drug, growth was the same at all pH values used.

The effects of Acridine Orange on growth of *E. coli* DS624 F'-Lac at 33 and 42°C are shown in Figs. 1(b) and 1(c) respectively. In the absence of Acridine

Fig. 1. Growth of *E. coli* in the presence of Acridine Orange

Samples (1 ml) from cultures of *E. coli* grown overnight as described in the text were added to fresh medium (19ml). Acridine Orange (25 µg/ml) was added to experimental cultures, but not to controls. Cultures were incubated with shaking under the conditions indicated and A_{650} was measured at intervals. Samples (0.5ml) were withdrawn at intervals and colony-forming units were counted after plating on NA-str-TTZ agar. (a) *E. coli* DF69 grown in medium at pH 7.4 (△) and pH 7.9 (□) in the presence of Acridine Orange; ○, control cultures grown at either pH. (b) *E. coli* DS624 F'-Lac grown in the presence (—) and absence (---) of Acridine Orange at 33°C. ●, A_{650} ; ▲, colony-forming units. (c) Growth was measured as described under (b), except that incubations were carried out at 42°C.

Orange, *E. coli* DS624 F'-Lac grew equally well at 33 and 42°C. At 33°C, a permissive temperature for DNA polymerase I in this strain (Durkacz & Sherratt, 1973), growth was not affected by Acridine Orange up to 150 min and only retarded thereafter (Fig. 1*b*). However, at 42°C, a non-permissive temperature for DNA polymerase I, growth, as indicated by measurements of A_{650} , stopped at 2 h incubation and there was a progressive decrease in the number of colony-forming units (Fig. 1*c*).

Fig. 2 shows that incubation of *E. coli* DS624 F'-Lac with Acridine Orange at 33°C has little or no effect on the retention of the F'-Lac plasmid by the cells, but that at 42°C, under otherwise similar conditions, there is a rapid elimination of F'-Lac after 1 h incubation.

Effects of Acridine Orange on synthesis and degradation of DNA in E. coli

The results shown in Figs. 3(*a*) and 3(*b*) indicate that Acridine Orange is without significant effect on

Fig. 2. Elimination of the F'-Lac plasmid from *E. coli* DS624 F'-Lac by Acridine Orange

E. coli DS624 F'-Lac was grown as described in Figs. 1(*b*) and 1(*c*) at 33°C (▲) and 42°C (●), in the presence (—) and absence (----) of Acridine Orange. Samples (0.5 ml) were withdrawn at intervals and incubated on NA-str-TTZ agar plates at 33°C for 36 h. Red (Lac⁻), white (Lac⁺) and sectorial (Lac⁺) colonies were counted and the percentage elimination of the F'-Lac plasmid was calculated as (no. of Lac⁻ clones + no. of Lac⁺ clones) × 100/total no. of clones.

Fig. 3. Incorporation of radioactivity into acid-insoluble material during growth of *E. coli* in the presence of [methyl-³H]thymine and Acridine Orange

Samples (1 ml) from cultures of *E. coli* grown overnight as described in the text were added to fresh medium containing [methyl-³H]thymine (19 ml) and incubation was continued until A_{650} was 0.1. Acridine Orange (25 µg/ml) was added to experimental cultures, but not to controls, and incubation was continued. Samples (0.2 ml) were removed at intervals after addition of Acridine Orange and acid-insoluble radioactivity was measured as described in the text. (a) *E. coli* DF69 grown at pH 7.4 (●) and 7.9 (▲). (b) *E. coli* DS624 F'-Lac grown at 33°C (Δ) and 42°C (□). ○, Control cultures grown in the absence of Acridine Orange, but otherwise under the same conditions as experimental cultures.

the incorporation of [*methyl*-³H]thymine into acid-insoluble material during growth of *E. coli* DF69 at pH7.4 and *E. coli* DS624 F'-Lac at 33°C. However, there is a marked inhibition of incorporation by Acridine Orange in *E. coli* DF69 at pH7.9 and in *E. coli* DS624 F'-Lac at 42°C. There is also evidence of degradation of DNA in *E. coli* DF69 at pH7.9, since there is a decrease in the acid-insoluble radioactivity on prolonged incubation.

Examination of DNA isolated from treated and control cells, by using rate sedimentation in alkaline sucrose gradients, indicated that, under certain conditions of treatment, Acridine Orange gives rise to fractions of single-stranded DNA having lower molecular weight than that from control cells. The results shown in Fig. 4(a) indicate that DNA isolated from *E. coli* DF69 treated with Acridine Orange at pH7.4 is confined to the same region of the gradient as that occupied by DNA from untreated cells. However, DNA from cells treated with Acridine Orange at pH7.9 contains a major fraction sedimenting more slowly. The results shown in Fig. 4(b) reveal no significant effect of Acridine Orange on the sedimentation behaviour of DNA isolated from *E. coli* DS624 F'-Lac treated at 33°C. The results shown in Fig. 4(c) indicate that approx. 48% of the DNA isolated from *E. coli* DS624 F'-Lac after treatment with Acridine Orange at 42°C sediments

more slowly than the major component, in contrast with only 30% of that from untreated cells grown at 42°C.

To investigate the possibility that Acridine Orange fails to penetrate *E. coli* cells at pH7.4, degradation of DNA in cells under the two conditions was examined by measurement of the appearance of acid-soluble material. The results shown in Fig. 5 indicate that labelled DNA is converted into acid-soluble material during incubation with Acridine Orange at both pH values, whereas no such effect is observed in untreated cells.

Discussion

Previous work referred to above had shown that high pH increases the growth-inhibitory effects of Acridine Orange on *E. coli* and also its ability to cause loss of a merogenote. It has been suggested (Bastarrachea & Willetts, 1968) that the effect of high pH on curing may be due to the high permeability of *E. coli* to acridine dyes under such conditions. In the present experiments, a close correlation was found between the effects of high pH and of inactivity of DNA polymerase I on the growth-inhibitory properties of Acridine Orange (Figs. 1a, 1b and 1c). A similar correlation was found between the previously known effect of high pH on the elimination of the

Fig. 4. Sedimentation of DNA in alkaline sucrose gradients

E. coli was grown in the presence of [*methyl*-³H]thymine, as described in the text, until A_{650} was 0.1. Acridine Orange (25 µg/ml) was added to experimental cultures, but not to control cultures, and incubation was continued for 90 min. Cells were harvested by filtration, lysed as described in the text and samples (0.1 ml) of the lysates were centrifuged on an alkaline sucrose gradient and fractions were analysed for ³H. (a) *E. coli* DF69 grown at pH7.4 (●) and 7.9 (▲); ○, controls grown in the absence of Acridine Orange at either pH7.4 or 7.9. (b) *E. coli* DS624 F'-Lac grown in the presence (■) and absence (○) of Acridine Orange at 33°C. (c) *E. coli* (DS624) F'-Lac grown in the presence (■) and absence (○) of Acridine Orange at 42°C.

Fig. 5. Degradation of DNA in *E. coli* DF69 during incubation in the presence of Acridine Orange

Cells were grown in 80ml of medium containing [methyl- ^3H]thymine until the A_{650} was 0.1 as described in the text. Cells were harvested and resuspended in fresh medium containing no [methyl- ^3H]thymine at the pH indicated. Acridine Orange (25 $\mu\text{g}/\text{ml}$) was added to experimental cultures, but not to controls, and incubation was continued. Samples (1ml) were withdrawn at intervals and acid-soluble radioactivity was measured as described in the text. Experimental cultures incubated at pH 7.4 ($\blacktriangle$) and pH 7.9 ($\blacksquare$); $\circ$, control cultures incubated at either pH.

F'-Lac plasmid (Hirota, 1960; Bastarrachea & Willetts, 1968) and its stimulation under conditions preventing operation of DNA polymerase I (Fig. 2). These results suggested that high pH may have some more specific influence in addition to the possible effect of increasing the permeability of cells to Acridine Orange.

The results shown in Fig. 3(b) indicate that, in the absence of Acridine Orange, incorporation of [^3H]thymidine into DNA in *E. coli* DS624 F'-Lac is not significantly different at 33 and 42°C, which is in agreement with the view that DNA polymerase I is not essential for DNA synthesis and replication (DeLucia & Cairns, 1969). At the permissive temperature, Acridine Orange is shown by Fig. 3(b) to

be without significant effect on the rate of DNA synthesis in *E. coli* DS624 F'-Lac, but at 42°C an initial decrease in rate followed by complete cessation of synthesis occurs. In parallel with these observations, the results shown in Fig. 3(a) indicate that the rate of incorporation of [^3H]thymidine into DNA of *E. coli* DF69 is the same at pH 7.4 and at pH 7.9 in the absence of Acridine Orange. Moreover, the rate of DNA synthesis is affected to only a small extent by Acridine Orange at pH 7.4. However, at pH 7.9, Acridine Orange severely inhibits DNA synthesis and, on continued incubation with the dye at pH 7.9, there is evidence of degradation of labelled DNA.

The influence of Acridine Orange on both growth and elimination of plasmids have been ascribed to its effect on DNA. The results shown in Fig. 4(b) indicate that Acridine Orange is without significant effect on the DNA isolated from *E. coli* DS624 F'-Lac after growth at 33°C. However, as shown in Fig. 4(c), treatment with Acridine Orange of cells grown at 42°C results in an increase in the proportion of DNA having lower molecular weight. These results are in agreement with the findings of Southwick *et al.* (1972). A parallelism is again seen with the effect of pH as shown in Fig. 4(a). In this case, a major proportion of the DNA isolated from *E. coli* DF69 treated with Acridine Orange at pH 7.9 has a molecular weight lower than that obtained from untreated cells and from cells treated at pH 7.4.

Although the results obtained at different pH values with *E. coli* DF69 appeared to be similar to those obtained at different temperatures with *E. coli* DS624 F'-Lac, it was necessary to consider the possibility that the absence of degradation of DNA isolated from cells treated with Acridine Orange at pH 7.4 is due to failure of the dye effectively to penetrate the cell. However, Fig. 5 shows that treatment of *E. coli* DF69 with Acridine Orange at both pH 7.4 and 7.9 results in the degradation of DNA, giving rise to the appearance in the acid-soluble fraction of ^3H derived from DNA. It is therefore concluded that, even at pH 7.4, Acridine Orange penetrates the cell and causes degradation of DNA. However, since no change in the sedimentation behaviour is seen in DNA isolated from cells after treatment under these conditions (Fig. 4a), it is concluded that the degradation is repaired. In contrast, the presence of material of lower molecular weight in DNA isolated from cells treated at pH 7.9 (Fig. 4a) suggests that repair processes are not fully operative under these conditions. It is possible that the influence of high pH on cell permeability may produce an additional effect, but cannot account fully for our observations. Owing to the difficulty of measuring uptake of Acridine Orange (Silver *et al.*, 1968), it is not possible to assess quantitatively the part played by permeability in producing the overall effects of pH.

On the basis of the above conclusions, it is suggested

that the stimulation of the elimination of the F'-Lac plasmid in the presence of Acridine Orange requires impairment of DNA replication processes and that this condition is fulfilled either at high pH or in the *polA*⁻ mutant at a non-permissive temperature. At the non-permissive temperature for DNA polymerase I, Acridine Orange also exerts a marked inhibitory effect on growth (Fig. 1c), and this is believed to be responsible for the observation that the elimination of the F'-Lac under these conditions results in the production of a high proportion of sectorial colonies on NA-str-TTZ agar plates. Microscopic examination of cultures treated under these conditions shows a high proportion of filamentous cells. Sectorial colonies showing some areas exhibiting Lac⁺ and some Lac⁻ properties are believed to have developed from cells in the process of losing the F'-Lac plasmid and subject to delayed division.

The experimental results described suggest that elimination of the F'-Lac plasmid by Acridine Orange is due not to a specific effect of the drug on extra-chromosomal DNA, but that its effects both on cell growth and on plasmid elimination depend on a general degradative effect on DNA and are fully observed only if replicative processes are impaired. Similar observations have been made on degradation of bacterial DNA during irradiation and photodynamic treatment (Ziebell *et al.*, 1977). Further experiments with more repair-deficient mutants are necessary before it can be decided whether inactivation of enzymes other than DNA polymerase I produces such effects and what specific metabolic effects are produced by medium of high pH.

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