INTERSTRAND CROSS-LINKS IN THE DNA OF ESCHERICHIA COLI B/r AND B_{s-1} AND THEIR REMOVAL BY THE RESISTANT STRAIN

S. Venitt

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3.

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It has been suggested (Brookes and Lawley, 1961) that the characteristic cytotoxic action of the difunctional alkylating agent mustard gas, di-(2-chloroethyl)sulphide, is due to its ability to cross-link the twin strands of the DNA helix, thus preventing its replication. Some evidence for this view has been obtained: Lawley and Brookes (1965) found that the resistant strain of Escherichia coli, B/r, but not the sensitive strain B_{s-1}, could selectively remove the characteristic product of difunctional alkylation, di-(guanin-7-yl)ethyl sulphide, from its DNA when the cells were incubated under normal growth conditions after treatment with mustard gas. Kohn, Steigbigel and Spears (1965) showed on the basis of renaturation studies that B/r, but not B_{s-1}, could remove cross-links from its DNA after treatment with nitrogen mustard.

Previous techniques did not permit the detection of cross-linking of cellular DNA at low doses, in the region of the mean lethal dose, by direct physical measurement, since the number of such cross-links would be small and would require the isolation of DNA of high molecular weight. The technique of McGrath and Williams (1966) was therefore employed in the present study

and this has permitted unequivocal demonstration of the induction of interstrand cross-links in the DNA of E.coli strains B/r and B_{s-1} and the removal of these cross-links in the resistant strain, B/r. Chromatographic studies of purified DNA from mustard gas-treated cultures indicated that the time course of the loss of the diguaninyl product in B/r parallels the disappearance of inter-strand cross-linked macromolecules, whilst in B_{s-1} the absence of such excision mirrors the non-disappearance of cross-linked DNA molecules.

METHODS AND MATERIALS

E.coli B/r was obtained from Dr. A. Loveless and E.coli B_{s-1} (Hill, 1958) from Dr. W. Harm.

All experiments were performed on cells grown at 37°C in a salts-glycerol-casamino acids medium (Venitt, Brookes and Lawley, 1968). It was confirmed that the mean lethal doses of mustard gas under these conditions were 6.0 μg/ml for B/r and 0.8 μg/ml for B_{s-1} (Lawley and Brookes, 1965). A dose of 6 μg/ml of mustard gas was used in all subsequent experiments.

For the sucrose density gradient studies, cells were pre-labelled with [^{3}H] thymidine by the method of Boyce and Setlow (1962), treated with mustard gas for 10 minutes with aeration and resuspended in [^{3}H] thymidine-free medium. Samples were then taken at zero time and after 80 minutes incubation with aeration. Non-alkylated prelabelled cells were subjected the same treatment in each experiment. All samples were kept at 0°C until the end of the experiment. The cells were then converted to spheroplasts, subjected to sucrose density gradient centrifugation at pH 12, and the gradients assayed for TCA-insoluble radioactivity by the methods of McGrath and Williams (1966).

The time course of excision of diguaninyl products from the purified DNA of [^{35}S]labelled mustard gas-treated cells and the extent of binding of mustard gas to DNA were determined by the methods of Lawley and Brookes (1965), using [^{35}S]mustard gas at a specific radioactivity of 2 mc/mg.

RESULTS AND DISCUSSION

In confirmation of the work of Lawley and Brookes (1965) figure 1 shows that during incubation in conditions permitting growth, cells of B/r selectively excise di-(guanin-7-yl)ethyl

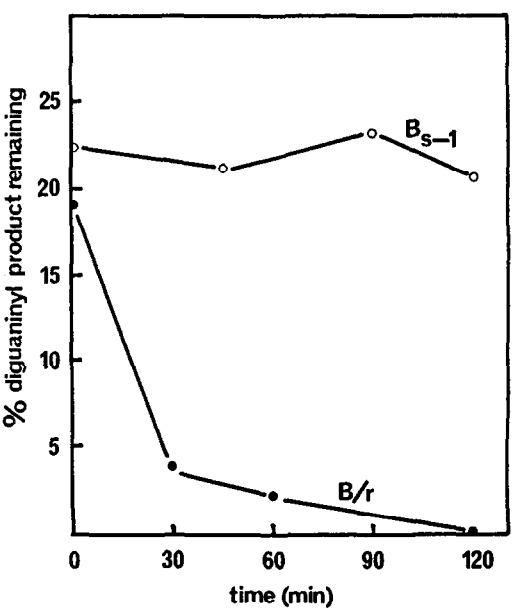

FIGURE 1: PERCENTAGE OF [^{35}S] DI-(GUANIN-7-YL)ETHYL SULPHIDE REMAINING BOUND TO DNA AT VARIOUS TIMES AFTER ALKYLATION OF E.COLI B/r OR B_{s-1} WITH [^{35}S] -LABELLED MUSTARD GAS. Fifty ml cultures at 10^{10} cells/ml were alkylated with 6 µg/ml [^{35}S] mustard gas (sp. act. 2mc/mmole) for 10 min and then diluted into 3 l of medium and incubated with aeration at 37°C. 750 ml samples were taken at intervals, rapidly chilled and DNA extracted by m-cresol precipitation of the phenol-extracted cell lysate. After centrifugation at 30,000g for 2 hr, DNA was precipitated from solution with 2-ethoxy-ethanol, washed in ethanol, and dried in ether. Samples of dry DNA were hydrolysed in 1 N HCl for 15 min at 100°C, and chromatographed on paper. Radioactivity was assayed in a gas-flow chromatogram scanner, and the results are expressed as percentage difunctionally alkylated guanine moieties relative to total alkylated guanine residues.

sulphide from their DNA whereas those of B_{s-1} cannot, when both are treated with 6 μg/ml of mustard gas. DNA isolated from cells of B/r contains less than 5% diguaninyl residues 1 hour after treatment and no detectable cross-linked product after 2 hours, in contrast to B_{s-1}, where the percentage remains constant, at about 22%, throughout the 2 hour period.

The sedimentation data presented in figure 2 indicate that at zero time after mustard gas treatment in both strains there is a peak of radioactivity corresponding to an average molecular weight ($\overline{MW}$) of 4.5×10^8 and that there is proportionally less radioactivity in the position of the control. The control value of $\overline{MW}$ 2.1×10^8 agrees closely with that reported by McGrath and Williams (1966). Since DNA is denatured at pH 12 and above (Studier, 1965) the control band of $\overline{MW}$ 2.1×10^8 must consist of single strands, and therefore the material of $\overline{MW}$ 4.5×10^8 (approximately double the control value) seen in the mustard gas-treated preparations must be due to alkaki-stable inter-strand cross-linked DNA.

The complete disappearance of this material in preparations from B/r after 80 minutes incubation (figure 2 (a)) and the fact that the sedimentation profile exactly corresponds to that of the control is overwhelming evidence that there is, in this strain, selective removal of interstrand cross-links from high molecular weight DNA, followed by a process of repair. The time course of the process as demonstrated by this physical technique agrees very well with the chemical data presented in figure 1. The sedimentation profiles shown in figure 2 (b) indicate that cells of B_{s-1} treated with mustard gas at 6 μg/ml have no capacity whatever for the selective removal of interstrand cross-links followed by repair since the DNA released from cells after 80

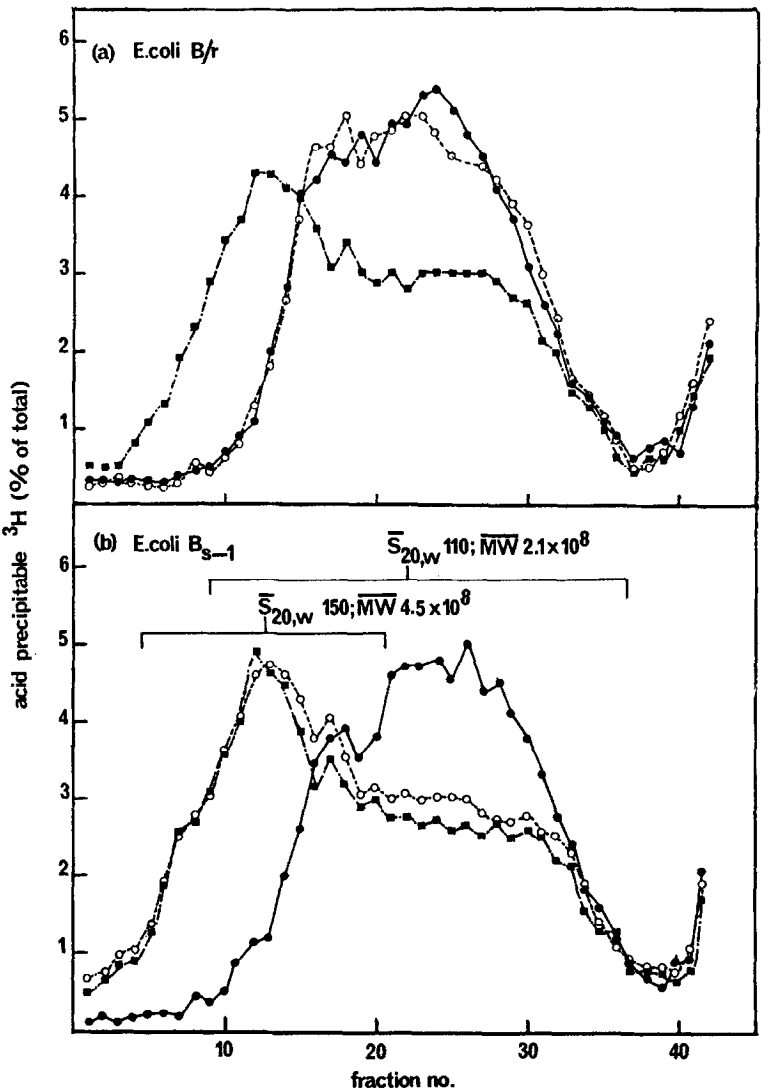

FIGURE 2 : THE EFFECT OF MUSTARD GAS ON THE SEDIMENTATION OF [^{3}H]-LABELLED DNA FROM E.COLI B/r AND B_{s-1} IN ALKALINE SUCROSE GRADIENTS. Cultures, prelabelled with [^{3}H] thymidine (sp. act. 18c/mmole) in the presence of 250 μg/ml deoxyadenosine, were divided into two portions when uptake of TCA-insoluble radioactivity had reached 10,000 cpm per 10 μl culture. One portion was treated with 6 μg/ml mustard gas and the other left as control. Both were incubated for 10 minutes, washed and resuspended in [^{3}H] thymidine-free medium. Samples were taken at zero time and after 80 minutes incubation with aeration. Cells were converted to spheroplasts with lysozyme-EDTA-sucrose, pH 8. A 10 μl aliquot of spheroplasts, containing 10,000 cpm, was placed on 0.1 ml 0.5 M NaOH layered on top of a 5-20% linear sucrose gradient (0.9 M in NaCl, 0.2 M in NaOH, pH 12). Gradients were held at 20°C for 30 min to release the DNA and then run at 30,000 rpm for 90 min, at 20° in the MSE

3 x 5 swinging-bucket rotor. Four-drop fractions were collected on to filter discs (Whatman 3MM) which were then washed in ice-cold 5% TCA, ethanol and dried in ether, and counted by immersion in toluene scintillation fluid.

—●— control —·—■·— zero time mustard gas ···○··· 80 min incubation after mustard gas

The bars above the curves in (b) indicate the portion of the curve used to determine the mean values for the average sedimentation coefficients, $\overline{S}_{20,w}$, and hence the average molecular weights, $\overline{MW}$.

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minutes of incubation sediments in exactly the same position as the DNA released from cells at zero time after alkylation, again in agreement with the chemical data.

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