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Cytotoxicity of Alkylating Agents towards Sensitive and Resistant Strains of *Escherichia coli* in Relation to Extent and Mode of Alkylation of Cellular Macromolecules and Repair of Alkylation Lesions in Deoxyribonucleic Acids

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1. A quantitative study was made of the relationship between survival of colony-forming ability in *Escherichia coli* strains B/r and B_{s-1} and the extents of alkylation of cellular DNA, RNA and protein after treatment with mono- or di-functional sulphur mustards, methyl methanesulphonate or iodoacetamide. 2. The mustards and methyl methanesulphonate react with nucleic acids in the cells, in the same way as found previously from chemical studies *in vitro*, and with proteins. Iodoacetamide reacts only with protein, principally with the thiol groups of cysteine residues. 3. The extents of alkylation of cellular constituents required to prevent cell division vary widely according to the strain of bacteria and the nature of the alkylating agent. 4. The extents of alkylation of the sensitive and resistant strains at a given dose of alkylating agent do not differ significantly. 5. Removal of alkyl groups from DNA of cells of the resistant strains B/r and $15T^-$ after alkylation with difunctional sulphur mustard was demonstrated; the product di(guanin-7-ylethyl) sulphide, characteristic of di- as opposed to mono-functional alkylation, was selectively removed; the time-scale of this effect suggests an enzymic rather than a chemical mechanism. 6. The sensitive strain B_{s-1} removed alkyl groups from DNA in this way only at very low extents of alkylation. When sensitized to mustard action by treatment with iodoacetamide, acriflavine or caffeine, the extent of alkylation of cellular DNA corresponding to a mean lethal dose was decreased to approximately 3 molecules of di(guanin-7-ylethyl) sulphide in the genome of this strain. 7. Relatively large numbers of monofunctional alkylations per genome can be withstood by this sensitive strain. Iodoacetamide had the weakest cytotoxic action of the agents investigated; methyl methanesulphonate was significantly weaker in effect than the monofunctional sulphur mustard, which was in turn weaker than the difunctional sulphur mustard. 8. Effects of the sulphur mustards on nucleic acid synthesis in sensitive and resistant strains were studied. DNA synthesis was inhibited in both strains at low doses in a dose-dependent manner, but RNA and protein synthesis were not affected in this way. 9. DNA synthesis in *E. coli* B_{s-1} was permanently inhibited by low doses of mustards. In the resistant strains $15T^-$ and B/r a characteristic recovery in DNA synthesis was observed after a dose-dependent time-lag. This effect could be shown at low doses in the region of the mean lethal dose. 10. Cellular DNA was isotopically prelabelled and the effect of mustards on stability of DNA was investigated. With resistant strains a dose-dependent release of DNA nucleotide material into acid-soluble form was found; this was much more extensive with the difunctional mustard (about 400 nucleotides released per DNA alkylation) than with the monofunctional mustard (about 10 nucleotides per alkylation). With the sensitive strain no dose-dependent release was found, though the DNA was less stable independent of cellular alkylation. 11. The results are discussed in terms of the concepts that alkylation of cellular DNA induces lesions which interfere with DNA replication, but which can be enzymically 'repaired'. The possible nature of these lesions is discussed in terms of the known reactions of the alkylating agents with DNA.

There is now a considerable body of evidence indicating that cellular DNA is the significant target involved in the cytotoxic action of alkylating agents. In the present work attention is devoted to the extent of alkylation of macromolecular constituents of bacterial cells at which inhibition of cell division results. Two main aspects are considered.

First, different types of alkylating agent are compared; it was known from previous work with bacteriophage (Brookes & Lawley, 1963) that the much greater effectiveness of difunctional alkylating agents, typified by the sulphur mustard [mustard gas, di-(2-chloroethyl) sulphide], in comparison with its monofunctional analogue, half sulphur mustard (2-chloroethyl 2-hydroxyethyl sulphide), could be correlated with an established difference between the modes of reaction of these mustards with bacteriophage DNA. This essential difference lay in the ability of the difunctional agent to yield in DNA a product identified as di(guanin-7-ylethyl) sulphide. It seemed likely that the guanine residues linked by a covalent bond originated in opposite strands of the twin helix of the double-stranded DNA and that alkylation of this type would constitute a powerful hindrance to DNA replication. Evidence for this interstrand cross-linking has been obtained (Lawley & Brookes, 1967). However, the question whether guanine residues in the same strand of the macromolecule could be linked by difunctional alkylating agents cannot yet be conclusively resolved.

Within a group of monofunctional alkylating agents investigated, the bacteriophage studies indicated significant differences in effectiveness, in that half sulphur mustard was a more powerful inhibitor of phage replication than methyl methanesulphonate at a given extent of alkylation of phage DNA. It was therefore decided to investigate whether a comparable difference in effectiveness towards bacteria could be demonstrated.

The second problem concerned the widely different sensitivities of bacteria towards alkylating agents. It seemed unlikely that major differences in reactivity of cellular constituents towards alkylating agents would be found. Therefore, as with the parallel problem of differential sensitivity towards radiations, it seemed that variable abilities to repair cellular alkylation lesions should be sought as the cause.

Some evidence for this has been reported [cf. Harold & Ziporin (1958) on the action of mustards on *Escherichia coli*, and Strauss & Wahl (1964) on the detection of a nuclease in *Micrococcus lysodeikticus* specifically degrading methylated DNA]. Subsequently further studies of recovery of *E. coli* from the effects of alkylation were reported (Loveless, Cook & Wheatley, 1965; Loveless, 1966; Papirmeister & Davidson, 1965).

More detailed evidence was presented that alkylation lesions in DNA of this organism could be removed and that repair replication of DNA ensued (Papirmeister & Davidson, 1964; Kohn, Steigbigel & Spears, 1965; Haynes & Hanawalt, 1965). Preliminary reports of some aspects of our work have been presented (Lawley & Brookes, 1965; Lawley, 1966).

MATERIALS AND METHODS

Materials. 2-Chloroethyl 2-hydroxyethyl sulphide (half sulphur mustard) (Grant & Kinsey, 1946) and di-(2-chloroethyl) sulphide (mustard gas) were prepared by the action of HCl on thiodiglycol; these compounds were obtained isotopically labelled with ^{35}S (specific radioactivities up to 900 mc/m-mole) from The Radiochemical Centre, Amersham, Bucks. The method for preparation of the half mustard involves selective extraction of mustard gas. The purity of the former was therefore investigated, by Dr J. Ogle (The Radiochemical Centre) by using gas–liquid chromatography; mustard gas could be detected in samples of half mustard, but was present to an extent of less than 1·5%.

Methyl methanesulphonate was obtained from Dr A. Loveless; [^{14}C]methyl methanesulphonate (28·1 mc/m-mole) and iodo[1-^{14}C]acetamide (7·04 mc/m-mole) were from The Radiochemical Centre. Isotopically labelled alkylating agents and unlabelled half mustard were kept in ether solution at −20°. Concentration of unlabelled half mustard was determined by reaction of portions (0·25 m-mole) with excess of sodium thiosulphate (0·1 M; 5 ml.) at 50°, the remaining thiosulphate being determined by titration against standard iodine solution; mustard gas was shown in parallel experiments to react with 2 equiv. of thiosulphate, and the half mustard was therefore assumed to react with 1 equiv.

Growth of bacteria. *E. coli* strains B/r and 15T$^-$ were obtained from Dr A. Loveless, and strain B_{s-1} (Hill, 1958) was from Dr W. Harm. Slopes were maintained on nutrient agar, containing 2% (w/v) Difco Bacto-agar and 2·5% (w/v) nutrient broth (Oxoid no. 2), at 4°; subcultures were grown overnight at 37° in 2·5% broth. Experimental cultures were grown in M9 medium. One litre of M9 buffer contained Na_2HPO_4 (6g.), KH_2PO_4 (3g.) NaCl (0·5g.) and NH_4Cl (1g.); to this was added immediately before use as growth medium, glucose (10 ml.; 40%, w/v) and $MgSO_4$ (10 ml.; 0·1 M); these three solutions were sterilized separately. Portions of about 800 ml. volume were inoculated with 10 ml. of the subculture and maintained at 37° with aeration through a sintered-glass bubbler; the exponential phase of growth was rapidly attained with a doubling time of about 60 min. as measured by increase in extinction at 420 mμ; the maximum concentration of cells that could be attained was about 3×10^9/ml. For biochemical studies, the cells were harvested by centrifugation, generally when a concentration of about 5×10^8/ml. had been reached.

For assay of colony-forming ability of cells, the cultures were serially diluted into M9 buffer, and 0·1 ml. portions of the resulting suspensions were spread over nutrient agar plates [1·5% (w/v) Difco Bacto-agar, 2·5% (w/v) nutrient broth]; from preliminary experiments, dilutions for plating

were chosen to give 100–200 colonies per plate; incubation times were generally 16hr. In experiments, when the plating medium contained acriflavine (5μg./ml.) or caffeine (2mg./ml.), about 24hr. was necessary for colonies to attain the requisite size for counting. With acriflavine, plating was performed in subdued light and plates were incubated in the dark.

Treatment of bacteria with alkylating agents. Bacterial suspensions were either treated under growing conditions (about 8×10^5cells/ml. in M9 medium at 37°), or the cultures (400ml.) in exponential growth were centrifuged and the cells resuspended in a smaller volume (20ml.) of M9 buffer, and treated at 37°. The incubation time for sulphur mustards was 15min.; for methyl methanesulphonate or iodoacetamide various times up to 2hr., were used, with a final time for complete reaction of 21hr.

In some experiments, cells treated with mustards were resuspended in M9 growth medium, of volume equal to that of the original culture, and were reincubated with aeration for periods up to 2hr. Treatment of cultures was stopped either by pouring the bacterial suspension on to ice, before isolation of cellular constituents, or by 100-fold dilution into M9 buffer for further dilution and plating.

Isolation of bacterial nucleic acids and protein. Cells (about 2×10^{11}; 400ml. of culture) were harvested by centrifugation and resuspended in 6% (w/v) sodium *p*-aminosalicylate (25ml.). Then, to lyse the cells, 10% (w/v) sodium lauryl sulphate (2·5ml.) was added; lysis was generally achieved after about 15min. at room temperature. Procedures for isolation of DNA, involving removal of protein by extraction into phenol–*m*-cresol, by the method of Kirby (1957) and in later experiments that of Kirby, Fox-Carter & Guest (1967), were then followed. The latter method, in which DNA is selectively precipitated by *m*-cresol, obviates the use of the methoxyethanol–sodium phosphate procedure necessary to remove carbohydrate contaminants from DNA. RNA was obtained by ethanol precipitation from the residual aqueous phase after selective precipitation of DNA. As a final purification stage, the nucleic acids were redissolved in 2% (w/v) sodium acetate–1·5% (w/v) NaCl, centrifuged at 54000*g* for 1hr., and reprecipitated with 1·5vol. of 2-ethoxyethanol (DNA) or 2vol. of ethanol (RNA), washed with ethanol, then ether, and dried in a desiccator. Proteins were precipitated from the first phenol extracts with methanol, washed with methanol and then ether, and dried.

Assay of radioactivity in nucleic acids and protein. DNA was dissolved in 5% (w/v) trichloroacetic acid at 100° (5min.). RNA was dissolved in water. Both nucleic acids were at a concentration of 1mg./ml.; 0·1ml. portions were added to a solution of phosphor and counted in a Packard Tri-Carb scintillation counter. DNA concentrations were determined by measurement of extinction; for a solution diluted to 50μg./ml. E was about 1·0 at 260mμ, about 0·01 or less at 350mμ; from determinations of DNA P (Fiske & Subbarow, 1925) and deoxyribose (Burton, 1956) the value of $E^{1\%}_{1cm.}$ at 260mμ, corrected by subtracting E_{350}, was determined as 260mμ, both for *E. coli* DNA prepared by the method used and for a standard sample of salmon sperm DNA. This value refers to the theoretical dry sodium salt of DNA containing 9·2% of P; all estimations of specific radioactivity of DNA are expressed relative to this value. For RNA in water the same value of $E^{1\%}$ was obtained.

Protein was dissolved in 10% (w/v) tetraethylammonium hydroxide at 37° overnight; 0·1ml. portions were then counted in the scintillation counter. When the amounts of bacterial cultures were too small (about 5ml.) to enable isolation of DNA and protein, material insoluble in 5% (w/v) trichloroacetic acid was isolated by adding portions of the culture to equal volumes of cold 10% (w/v) trichloroacetic acid, washing the precipitate with cold 5% trichloroacetic acid, twice with ethanol and then with ether, and drying. The trichloroacetic acid-insoluble material was then dissolved and assayed as for protein. Even smaller portions of culture (50μl.) were assayed for radioactivity in trichloroacetic acid-insoluble material by absorption on to filter-paper circles (Whatman no. 1, 2·3cm. diam.), which were then washed in cold 5% trichloroacetic acid three times, then in ethanol, acetone and toluene, and placed in counting bottles.

Detection of products of alkylation in nucleic acid and protein. Nucleic acids and proteins isolated from cells treated with isotopically labelled alkylating agents were hydrolysed with HCl: nucleic acids (about 0·5mg.) with 50μl. of N-HCl at 100°, for 10min. (DNA) or for 60min. (RNA). The hydrolysates were applied to Whatman no. 1 paper strips and chromatographed with propan-2-ol–conc. HCl–water (68:16·4:15·6, by vol.) as solvent. Protein (1mg.) was hydrolysed with 6N-HCl in a sealed tube for 24hr. and the hydrolysate was chromatographed by the method of Moore, Spackman & Stein (1958), with an amino acid analyser (Evans Electroselenium Ltd.) fitted with Packard scintillation spectrometer model 3022 and flow detector model 3041.

Identification of the principal minor product from reaction of half sulphur mustard with DNA. Salmon sperm DNA (20mg.) was treated with 2-chloroethyl 2-hydroxyethyl [^{35}S]sulphide (0·24mg.; 0·4μc) in 0·02M-sodium phosphate buffer, pH7·0 (5ml.), at 37° for 15min. The DNA was precipitated and washed as described above, hydrolysed with N-HCl at 100° and chromatographed on a Dowex 50 (H^+ form) column (5cm.×0·5cm.), eluted with a gradient of 0·5–2·5N-HCl (200ml.); 4ml. fractions were collected at 15min. intervals, and 0·25ml. portions of each were assayed for radioactivity. Ultraviolet absorption, monitored at 260mμ, was observed in fractions 0–3 (pyrimidine nucleotides), fractions 17–21 (guanine), fractions 29–34 (containing the ^{35}S-labelled products) and fractions 35–41 (adenine). The peak containing the products was evaporated and a portion was chromatographed on a paper strip as described above. Two radioactive peaks were observed. The principal product, $R_{adenine}$ 1·05, was identified as 7-(2′-hydroxyethylthioethyl)guanine by co-chromatography with authentic unlabelled material (Brookes & Lawley, 1961). The second product, $R_{adenine}$ 1·5, had u.v. absorption characteristic of a 3-alkyladenine ($\lambda_{max.}$ 273mμ) (Brookes & Lawley, 1960). This second product was further identified by comparison with the major product from reaction of half sulphur mustard with adenine, obtained as follows (cf. the methylation of adenine; Pal, 1962). Adenine (3·4mg.) was treated with 2-chloroethyl 2-hydroxyethyl sulphide (11mg.) in 0·1M-sodium phosphate buffer (1ml.) at 37° for 15min. The mixture was then chromatographed on Dowex 50 (H^+ form), as described for alkylated DNA. Ultraviolet-absorbing peaks were observed in fractions 10–13 ($\lambda_{max.}$ 259mμ, characteristic of a 1-alkyladenine), fractions 22–28 ($\lambda_{max.}$ 273mμ, 3-alkyladenine) and fractions 29–33 (adenine). The identity of the labelled and unlabelled

3-alkyladenine products was then established by co-chromatography on paper. The principal minor product from alkylation of DNA extracted from cells treated with half mustard was also identified in this way. This product had been obtained in previous work (cf. Lawley & Brookes, 1963), but not positively identified.

Study of DNA synthesis and degradation. DNA synthesis in bacterial cultures was determined either by estimation of acid-insoluble deoxyribose in portions of the culture, or by uptake of [^{14}C]thymine into acid-insoluble material.

To illustrate the use of the first method, in a typical experiment a culture (1·6l.) of *E. coli* B_{s-1} was grown with aeration. The concentration of cells was determined by measurement of extinction at 420 mμ, according to the relationship, established in a separate experiment, that number of cells/ml. $= 6{\cdot}8 \times 10^8 \times E_{1cm}$. In 30 min. cellular concentration increased from $1{\cdot}2 \times 10^8$ to $1{\cdot}8 \times 10^8$ cells/ml. The culture was then divided into four equal parts, to three of which mustard gas was added to give concentrations of 2·5, 10 and 25 μg./ml. Growth was continued for a further 180 min., during which time the cellular concentration in the untreated control rose to $5{\cdot}7 \times 10^8$ cells/ml. At 10 min. intervals after the additions of mustard gas, 25 ml. portions of the cultures were added to 25 ml. of cold 10% trichloroacetic acid and the resulting precipitates were analysed for deoxyribose by the method of Burton (1956).

As an example of the second method, an exponentially dividing culture of *E. coli* 15T$^-$ (50 ml.; 5×10^8 cells/ml.) in M9 medium containing [^{14}C]thymine (0·02 μc/ml.; 3 μg./ml.), was divided into 10 ml. portions for treatment with mustards; 50 μl. portions of the cultures were taken at 5 min. intervals and assayed for radioactivity by the filter-paper technique.

To investigate effects of mustards on stability of DNA, isotopic prelabelling of cells with thymidine was completed before treatment with mustards.

With *E. coli* 15T$^-$, a subculture (33 ml. of M9 medium containing [^{14}C]thymine, 0·5 μc/ml.; 2·5 μg./ml.) was grown overnight. The cells, collected by centrifugation, were washed with, and then resuspended in, M9 medium (40 ml.), incubated with aeration for 10 min. to exhaust the pool of non-incorporated labelled thymine, and diluted into M9 medium (350 ml.) containing unlabelled thymine (5 μg./ml.). At this stage the concentration of the culture was about 10^8 cells/ml.; incubation with aeration for 30 min. established exponential growth of the culture, which was then subdivided and treated with mustard at various concentrations as described above. At 15 min. intervals after treatment, portions (1 ml.) of culture were added to cold 10% trichloroacetic acid (1 ml.), and after standing for 30 min. at 4° the precipitates were centrifuged; portions (0·25 ml.) of the supernatants were assayed for radioactivity.

Some samples of precipitate were heated for 10 min. at 100° with 2 ml. of 5% trichloroacetic acid, and 0·25 ml. portions of the resulting supernatant after recentrifuging were then assayed for radioactivity. In a typical example, the radioactivities obtained were: for 0·125 ml. of the culture before treatment, 4090 counts/min.; for 0·25 ml. of the digest of trichloroacetic acid-insoluble material, 3750 counts/min.; for a control culture not treated with mustard, 20–40 counts/min., during an incubation period of 150 min. with no significant dependence on time.

For strains not dependent on added thymine, such as *E. coli* B_{s-1}, DNA was prelabelled by the technique of Boyce & Setlow (1962); subculture (15 ml. of M9 medium) for prelabelling contained deoxyadenosine (250 μg./ml.) and [^{3}H]thymidine (10 μc/ml.). In a typical example, after dilution of the subculture into 100 ml. of M9 medium and growth for 30 min., 0·25 ml. of hot-trichloroacetic acid digest, obtained as described above, gave 15 600 counts/min.

The rate of RNA synthesis was investigated by diluting a subculture of *E. coli* into M9 medium (50 ml.) containing cytosine (50 μg./ml.) and [^{14}C]uracil (10 μg./ml.; 0·4 μc/ml.). When exponential growth was attained, the culture was subdivided and treated with mustards at various concentrations, as described above, and the filter-paper technique for assay of radioactivity in trichloroacetic acid-insoluble material was followed.

Renaturation of DNA. The hyperchromicity of DNA isolated from *E. coli* was investigated by heating, then rapidly cooling, solutions of DNA in 0·5 mM-sodium citrate buffer, pH 7·0. DNA was dissolved at 0·6 mg./ml., and a portion of this solution was added to 10 vol. of buffer maintained at 68°. After 10 min. and 120 min., portions of the solution were withdrawn and cooled in an ice bath. Hyperchromicity was determined relative to the absorption of an unheated solution at the same dilution; for various samples this value, $E^{1\%}_{1\,cm.}$, ranged from 145 to 155.

RESULTS

Alkylation of cellular macromolecules in vivo. With the various isotopically labelled alkylating agents, the extents of alkylation of macromolecular cellular constituents of the bacterial cells were determined.

First, the time-courses of these alkylations were followed. As expected, these were closely similar to the rates of hydrolysis of the various agents in neutral solution in the absence of added cells. Both the sulphur mustards reacted rapidly with a half-life of about 1·5 min. (cf. Fig. 1, showing the time-course of reaction of ^{35}S-labelled mustard gas with acid-insoluble material of *E. coli* B/r). Iodoacetamide (Fig. 1) reacted somewhat more slowly (half-life about 30 min.), and methyl methanesulphonate (Fig. 2) even more so (half-life about 5 hr.).

The extents of reaction in relation to dosage, as measured by concentration of reagent in the medium, were also determined. These can be conveniently summarized in terms of a factor K, defined as μg. of reagent combined with 1 g. of cellular constituent after reaction is complete, divided by original concentration of reagent (μg./ml.). At the relatively low doses of reagents employed, this factor showed no appreciable dependence on dose. Fig. 3 shows that for mustard gas the values of K were 2·8 for DNA and 5·9 for protein; for half sulphur mustard they were 2·3 for DNA and 2·7 for protein. Values for RNA (not shown) were closely similar to those for DNA. For iodo[^{14}C]acetamide after 21 hr., K for protein was 33, but effectively zero for either RNA or DNA

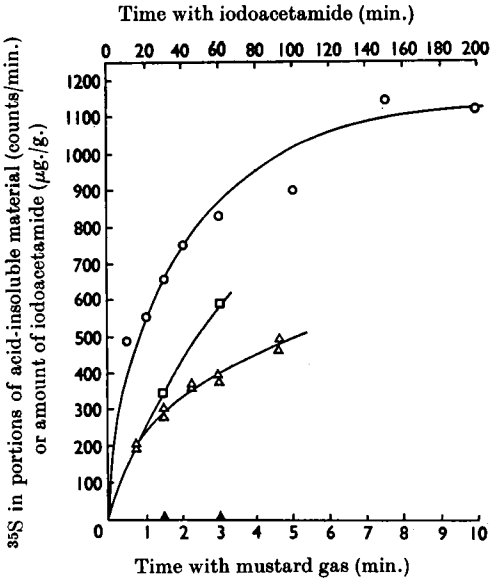

Fig. 1. Rate of reaction of ^{35}S-labelled mustard gas, or of iodo[^{14}C]acetamide, with protein of *E. coli*. Solutions of the reagents were added to suspensions of the cells (1×10^9–5×10^9/ml.) in M9 buffer at 37°. Mustard gas was in ether solution (0·2 ml.) and iodoacetamide in acetone (0·2 ml.). After various times the extent of reaction with material insoluble in 5% trichloroacetic acid was determined by adding portions (2·5 ml.) of the suspension to equal volumes of cold 10% trichloroacetic acid and assaying the precipitate for radioactivity as described in the text. The extent of reaction of iodoacetamide with DNA or protein of *E. coli* B_{s-1} was determined after isolating these constituents as described in the text. The ordinates show extent of reaction of mustard gas with acid-insoluble material of *E. coli* B/r on an arbitrary scale of counts/min.; reaction with iodoacetamide is expressed as μg. of iodoacetamide/g. of cellular constituent. The concentration of mustard gas was 1 μg. (1·07 μc)/ml.; the concentration of iodoacetamide was 25 μg. (0·96 μc)/ml. The extents of reaction of iodoacetamide after 21 hr. were: with acid-insoluble material, 530 μg./g.; with protein, 825 μg./g.; with DNA, < 1 μg./g. ○, Acid-insoluble material with mustard gas; △, acid-insoluble material with iodoacetamide; □, protein with iodoacetamide; ▲, DNA with iodoacetamide.

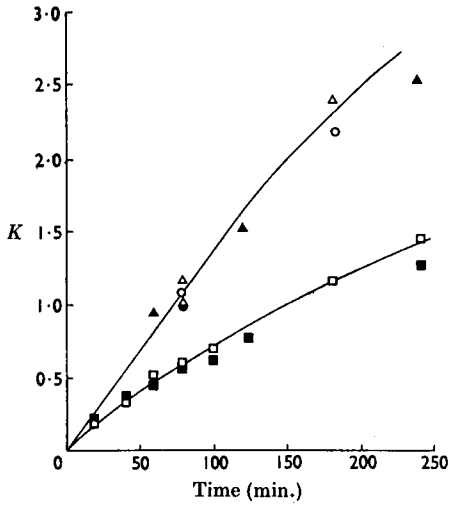

Fig. 2. Rate and extent of reaction of cellular constituents of *E. coli* with [^{14}C]methyl methanesulphonate. Suspensions of cells (about 5×10^8/ml.) in M9 buffer at 37° were added to the reagent at zero time and after various times samples (about 20 ml.) were poured on to ice, the cells were collected by centrifugation and DNA, RNA and protein were isolated as described in the text. The ordinate represents the factor *K* (μg. of methyl methanesulphonate reacted/g. of cellular constituent divided by the original concentration of the reagent in the medium, μg./ml.). After 21 hr. treatment, the values of *K* were: for RNA or DNA, 5·7; for acid-insoluble material, 4·3. DNA: △, strain B_{s-1}; ▲, strain B/r. RNA: ○, strain, B_{s-1}; ●, strain B/r. Material insoluble in 5% trichloroacetic acid: □, strain B_{s-1}; ■, strain B/r. Concentrations of methyl methanesulphonate: when DNA was isolated, for strain B/r (after 60, 120, 240 min. and 21 hr.), 15·4 μg./ml. and (after 80 min.) 1·81 mg./ml., and for strain B_{s-1} (80 min.), 1·81 mg./ml. and (180 min., 21 hr.) 21 μg./ml.; when acid-insoluble material was isolated, 2·6 mg./ml.

(*K* less than 0·1). A small amount of ^{14}C label was found with some samples of RNA, but this probably reflected the presence of traces of incompletely removed protein. Methyl methanesulphonate, like the mustards, reacted with all the three cellular constituents examined. Values of *K* after 21 hr. were 5·7 for RNA or DNA, and 4·3 for acid-insoluble material, mainly protein.

In all cases no significant differences between the sensitive and resistant strains of *E. coli* with respect to extents of reaction with the various cellular constituents were found.

To determine the nature of the products formed by alkylation of nucleic acids and proteins in the cells, these constituents were isolated, hydrolysed with hydrochloric acid and chromatographed by conventional procedures.

The sulphur mustards were thus shown (cf. Fig. 4) to give the products in nucleic acids previously identified (Lawley & Brookes, 1963); the principal product was 7-(2′-hydroxyethylthioethyl)guanine. The principal minor product, of higher R_F, was identified as 3-(2′-hydroxyethylthioethyl)adenine. Mustard gas gave in addition a product of low R_F, previously identified (Brookes & Lawley, 1960) as di(guanin-7-ylethyl) sulphide. Iodoacetamide did not react with nucleic acids in the cells. Methyl methanesulphonate gave the previously identified products (Lawley & Brookes, 1963) 7-methylguanine and 3-methyladenine.

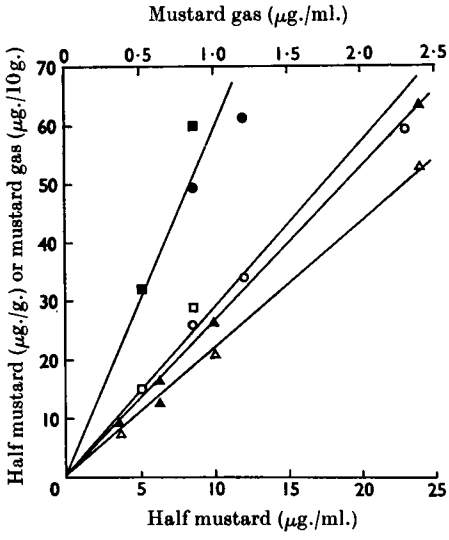

Fig. 3. Extent of reaction of ^{35}S-labelled mustard gas or half sulphur mustard with cellular constituents of *E. coli*. Suspensions of cells (about 5×10^9/ml.) in M9 buffer at 37° were treated for 10 min., then DNA and protein were isolated as described in the text and assayed for radioactivity. Mustard gas with DNA: ○, strain B_{s-1}; □, strain B/r. Mustard gas with protein: ●, strain B_{s-1}; ■, strain B/r. Half mustard with DNA: △, strain B_{s-1}. Half mustard with protein: ▲, strain B_{s-1}.

The principal products from the reaction of iodo[^{14}C]acetamide and of [^{14}C]methyl methanesulphonate with cellular protein were isolated chromatographically and identified by comparison with authentic unlabelled amino acids in the same chromatographic system. Iodoacetamide gave mainly a single product, eluted between cysteic acid and aspartic acid, and accounting for 95% of the radioactivity, which was identified as carboxymethylcysteine. A small amount of a product eluted between phenylalanine and lysine accounted for the remainder of the radioactivity.

Methyl methanesulphonate gave as principal product *S*-methylcysteine (elution time relative to cysteic acid, $t_{rel.}^{Cys}$, 3·3; 64% of recovered radioactivity), and one other acidic or neutral amino acid product, $t_{rel.}^{Cys}$ 6·4, 4% of recovered radioactivity, not identified. Three basic products were obtained, elution times relative to histidine, $t_{rel.}^{His}$, 0·9, 1·1 and 1·2, 2, 21 and 9% respectively of recovered radioactivity. These were not positively identified, but the major product had an elution time identical with that of 1-methylhistidine, and it is probable that the more rapidly eluted product was 3-methylhistidine, and the remaining product 1,3-dimethylhistidine (cf. the analogous pattern of products obtained from proteins of rat liver after incubation

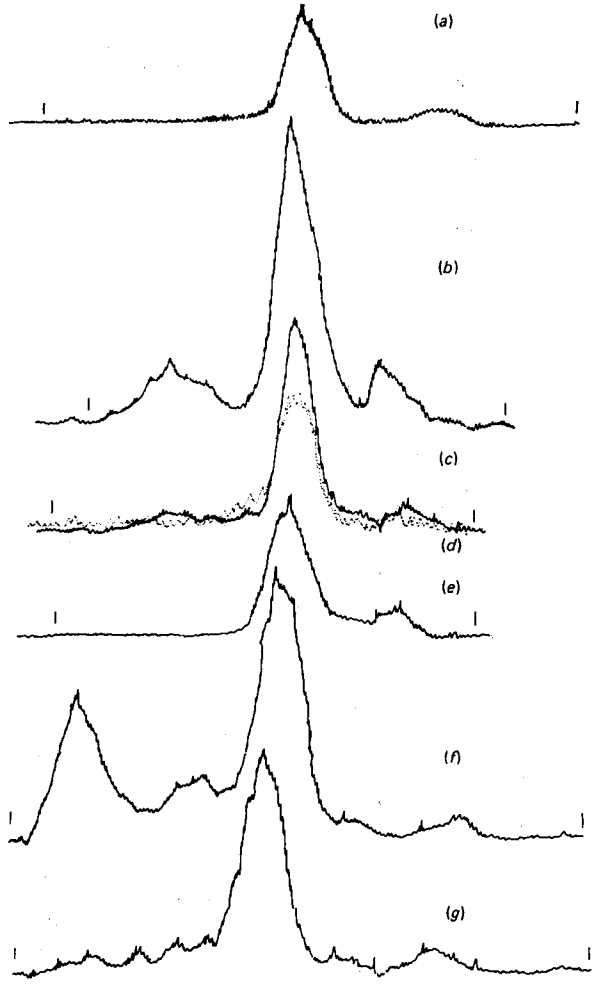

Fig. 4. Products from alkylation *in vivo* of nucleic acids in *E. coli*. Suspensions of cells were treated with isotopically labelled alkylating agents, and nucleic acids were isolated as described in the text, hydrolysed with N-HCl, and chromatographed on Whatman no. 1 paper strips with propan-2-ol–conc. HCl–water (17:4·1:3·9, by vol.) as solvent. The distribution of radioactivity in the dried and neutralized papers was then determined with a Packard radiochromatogram scanner. The ordinates represent counts/min. as traced by the recorder; about 10^4 counts/min. were applied; background was 20 counts/min. The abscissae represent distance along the paper. Origin at left and solvent front at right are indicated by vertical lines. (*a*) DNA from strain B_{s-1} treated with [^{14}C]methylmethanesulphonate (21 μg./ml.) for 3 hr. (*b*) DNA from strain B_{s-1} treated with ^{35}S-labelled mustard gas (2·3 μg./ml.) for 10 min. (*c*) RNA from strain B_{s-1} treated with ^{35}S-labelled mustard gas (1·5 μg./ml.) for 10 min. (*d*) RNA from strain B/r, treated as for (*c*). (*e*) DNA from strain B_{s-1} treated with ^{35}S-labelled half mustard (20 μg./ml.) for 10 min. (*f*) DNA from strain 15T⁻ treated with ^{35}S-labelled mustard gas (0·5 μg./ml.) for 10 min. (*g*) DNA from strain 15T⁻ treated as for (*f*), then incubated for 60 min. at 37° before isolation of DNA.

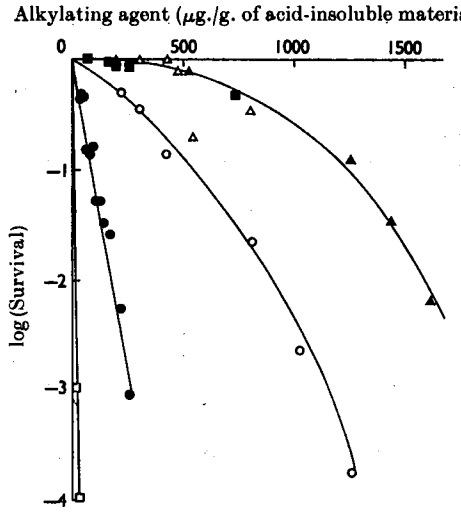

Fig. 5. Survival of *E. coli* B_{s-1} or B/r in relation to extent of reaction of isotopically labelled alkylating agents with the acid-insoluble fraction of cells. The various extents of reaction were achieved either by varying the concentration of reagent for a fixed reaction time (15 min., with mustard gas and half sulphur mustard), or by varying reaction time (up to 120 min.) at a given concentration of reagent (with methyl methanesulphonate and iodoacetamide). The method for assay of radioactivity in material insoluble in 5% trichloroacetic acid is described in the text. Strain B_{s-1}: □, mustard gas; ●, half mustard; ○, methyl methanesulphonate; △, iodoacetamide. Strain B/r: ■, half mustard; ▲, methyl methanesulphonate.

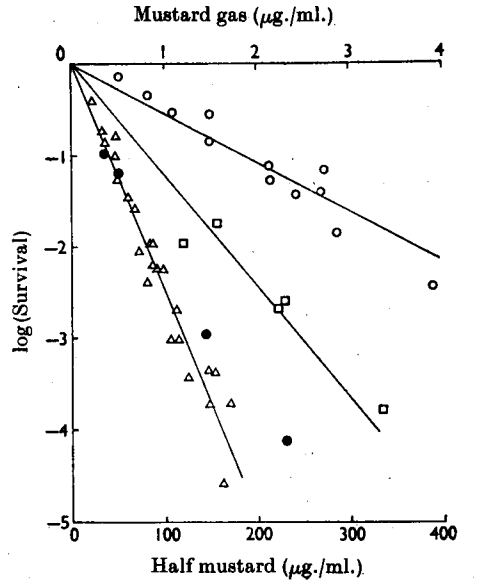

Fig. 6. Survival of colony-forming ability of *E. coli* B_{s-1} after treatment with ^{35}S-labelled mustard gas or half mustard. Suspensions of cells (about 5×10^9/ml.) in M9 buffer were treated at 37° for 10 min., then diluted into M9 buffer and plated on nutrient agar: ○, mustard gas; △, half mustard. In some experiments (●) cells were pretreated for 45 min. with iodoacetamide (0·87 mM) before mustard treatment; in others (□) the plating medium contained caffeine (2 mg./ml.).

with [^{14}C]dimethylnitrosamine reported by Magee & Hultin, 1962).

The elution patterns of products from protein hydrolysates of cells treated with ^{35}S-labelled half mustard and mustard gas were much more complex, though they were reproducible. Since these products have not been positively identified, it may suffice to summarize the results briefly. Both mustards gave two groups of products. The first, in the neutral and acidic amino acids, had similar elution times to products obtained from the reaction of ^{35}S-labelled mustard gas with cysteine. The principal products from protein and half mustard had $t_{rel.}^{Cys}$ 1·4 and 1·5, accounting for 30% of recovered radioactivity, and a product with $t_{rel.}^{Cys}$ 3·8 accounted for a further 15%. Mustard gas gave mainly a product with $t_{rel.}^{Cys}$ 1·4, 67% of recovered radioactivity. In the basic amino acids, half mustard yielded two products, $t_{rel.}^{His}$ 1·0 and 1·5, 14 and 29% respectively of recovered radioactivity; mustard gas gave four products, $t_{rel.}^{His}$ 1·0, 1·1, 1·5 and 2·1, 2, 2, 2 and 17% respectively of recovered radioactivity. Mustard gas and histidine gave three products, $t_{rel.}^{His}$ 0·9, 1·2 and 1·9, 78, 7 and 12% respectively of recovered radioactivity. It is therefore likely that these products derive from reaction of mustards with cysteine and histidine residues in cellular proteins, and that the products with mustard gas may in some cases differ from the products with half mustard. The multiplicity of products may be due to the effects of prolonged acid hydrolysis on the mustard side chains of the initially formed products.

Correlation between cytotoxicity and extents of alkylation of cellular macromolecules. Results relating extent of inactivation of colony-forming ability of treated cells to the extent of alkylation of their macromolecular constituents are presented in Fig. 5. The four alkylating agents used differ markedly in effectiveness, especially with regard to their action on the sensitive strain B_{s-1}. Mustard gas was by far the most potent cytotoxic agent of this series, half mustard and methyl methanesulphonate being progressively weaker, and iodoacetamide was inactive except at very high extents of reaction. The resistant strain B/r was markedly less susceptible to the action of the mustards. For mustard gas a dose of 6 μg./ml. was required to decrease survival to 37%, compared with the corresponding mean lethal dose of 0·8 μg./ml. for

Table 1. *Effect of sulphur mustards and methyl methanesulphonate on colony formation by* E. coli

Details of methods are described in the text. The mean lethal doses, i.e. those decreasing survival to 37% of that of controls (D_{37}), were derived from plots of log (survival) versus dose, and the extents of alkylation of cellular DNA at these doses were derived from data relating this quantity with dose, as described in the text. The treatment time for mustards was 10 min. at 37°; for methyl methanesulphonate the time-course of inactivation was determined. Values in parentheses denote moles of di(guanin-7-yl)ethyl sulphide (estimated number of cross-links) per genome of 9×10^6 moles of DNA P.

Strain	Reagent	Sensitizing agent	D_{37} (μg./ml.)	D_{37} (μmoles of alkyl/mole of DNA P)	D_{37} (moles of alkyl/9×10^6 moles of DNA P)
B/r	Mustard gas	None	6	36	320 (64)
B/r	Mustard gas	Iodoacetamide (3 mM)	2	12	107 (22)
B/r	Mustard gas	Caffeine (2 mg./ml.)	3	18	160 (32)
B/r	Mustard gas	Acriflavine (5 μg./ml.)	0·7	4·2	37 (8)
B/r	Half mustard	None	~350	1920	17300
B/r	Half mustard	Iodoacetamide (3 mM)	90	494	4450
B/r	Half mustard	Acriflavine (5 μg./ml.)	90	494	4450
B/r	Methyl methanesulphonate	None	2560 (43 min.)	4840	43500
B_{s-1}	Mustard gas	None	0·8	4·8	43 (9)
B_{s-1}	Mustard gas	Iodoacetamide (3 mM)	0·12	1·4	13 (3)
B_{s-1}	Mustard gas	Caffeine (2 mg./ml.)	0·36	2·1	19 (4)
B_{s-1}	Mustard gas	Acriflavine (5 μg./ml.)	0·27	1·6	14 (3)
B_{s-1}	Mustard gas	Acriflavine (10 μg./ml.)	0·30	1·8	16 (3)
B_{s-1}	Half mustard	None	16	88	790
B_{s-1}	Half mustard	Acriflavine (5 μg./ml.)	12	66	590
B_{s-1}	Methyl methanesulphonate	None	1270 (30 min.)	1720	15400

the sensitive strain B_{s-1}. Half mustard proved to be as weakly toxic towards strain B/r as were methyl methanesulphonate and iodoacetamide.

Both strains could be sensitized towards mustards by pretreatment with iodoacetamide, or by growing in plating medium containing caffeine or acriflavine (cf. Fig. 6, and Table 1 showing doses required to lower survival to 37% after various treatments). The extents of reaction of the mustards with cellular DNA were derived from the known relationship between extent of alkylation of cellular DNA and dose (cf. Fig. 3). For methyl methanesulphonate extents of alkylation were derived by combining the data relating survival with time of treatment (Fig. 7) and that relating extents of reaction with these times (Fig. 2).

Effects of alkylation on nucleic acid synthesis. Since the extent of alkylation of cellular constituents did not itself determine cellular survival, it was decided to investigate the possible biochemical effects subsequent to initial alkylation of cells. It was expected that, if cellular DNA were the most susceptible target, its effectiveness as a template for DNA synthesis might be decreased at alkylation doses of about the mean lethal dose. The mustards were chosen for this type of investigation, since their rapid reaction permits detailed studies of nucleic acid metabolism subsequent to completion of the cellular alkylation.

A marked difference in response between sensitive and resistant strains was found. Fig. 8 shows that

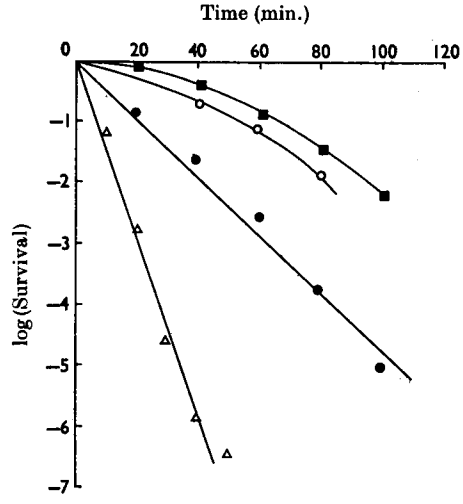

Fig. 7. Survival of colony-forming ability of *E. coli* treated with methyl methanesulphonate. The reagent was added to a suspension of cells (about 5×10^9/ml.) in M9 buffer at 37° and after various times portions were diluted for plating on nutrient agar. Concentrations of methyl methanesulphonate (mg./ml.) were: strain B/r: ■, 2·56; strain B_{s-1}: ○, 1·8; ●, 2·56; △, 6·3.

DNA synthesis in exponentially growing cultures of the strain B_{s-1} is rapidly and irreversibly inhibited by low doses of mustard gas. The resistant strains

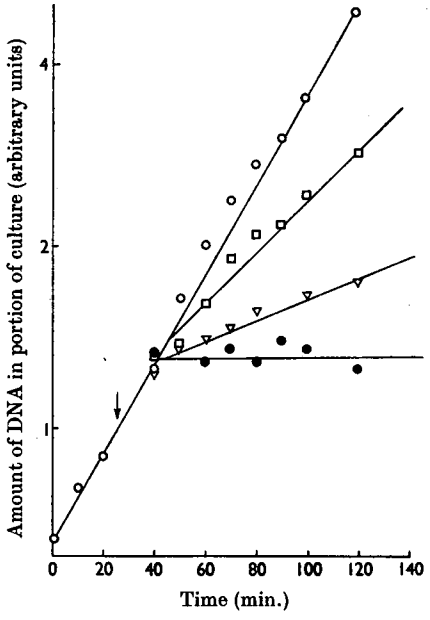

Fig. 8. Effect of mustard gas on DNA synthesis in an exponentially growing culture of *E. coli* B_{s-1}. The culture was subdivided at the time shown by the arrow and portions were treated with mustard gas: ○, none; □, 2·5 μg./ml.; ▽, 10 μg./ml.; ●, 25 μg./ml. After various times portions were taken for determination of DNA content by the diphenylamine method as described in the text. The ordinates represent relative amounts of DNA in cultures on an arbitrary logarithmic scale.

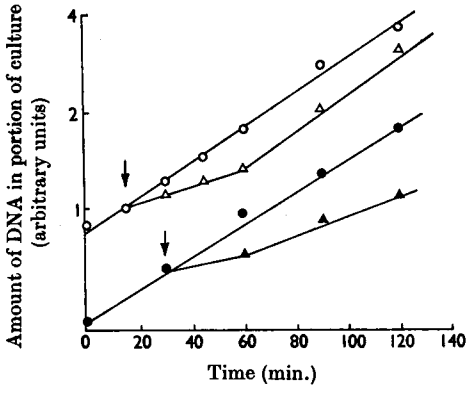

Fig. 9. Effect of mustard gas (50 μg./ml.) on DNA synthesis in resistant strains of *E. coli*. The reagent in ether solution (0·1 ml.) was added to exponentially growing cultures in M9 medium (400 ml. supplemented with thymine, 5 μg./ml., for strain 15T⁻) at the times denoted by the arrows. After various times portions were withdrawn for determination of the amount of DNA in the acid-insoluble fraction by the diphenylamine method. Strain 15T⁻: ○, untreated; △, treated with mustard gas at 15 min. Strain B/r: ●, untreated; ▲, treated with mustard gas at 30 min.

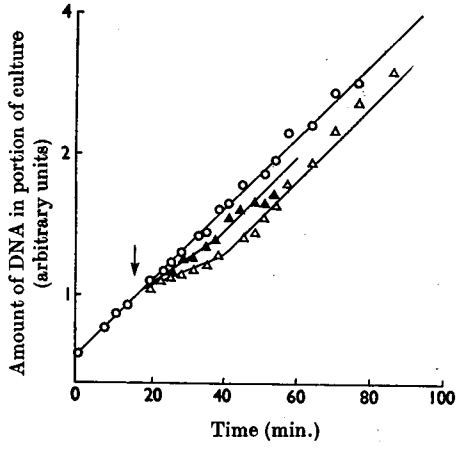

Fig. 10. Effect of mustard gas on DNA synthesis in exponentially growing cultures of *E. coli* 15T⁻. The culture in M9 medium supplemented with [^{14}C]thymine (3 μg./ml.; 0·02 μc/ml.) was subdivided at the time shown by the arrow for treatment with mustard gas. At various times portions were withdrawn for assay of incorporation of radioactivity into material insoluble in 5% trichloroacetic acid. Concentration of mustard gas: ○, none; ▲, 8 μg./ml.; △, 25 μg./ml.

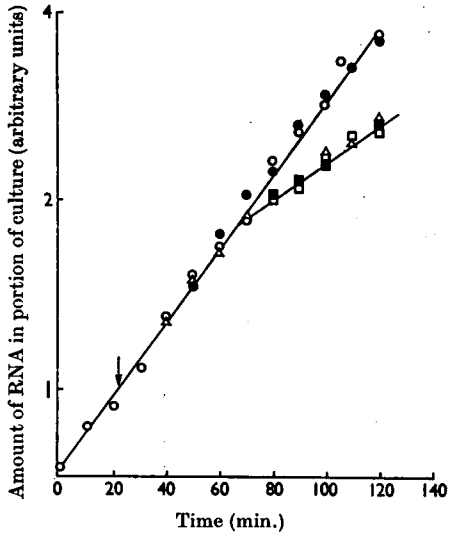

Fig. 11. Effect of sulphur mustards on RNA synthesis in an exponentially growing culture of *E. coli* B_{s-1}. The culture was divided and treated at the time shown by the arrow. Concentration of mustard gas: ○, none; □, 10 μg./ml.; ■, 25 μg./ml. Concentration of half mustard: ●, 50 μg./ml.; △, 200 μg./ml. Amount of RNA in the culture was determined by extent of incorporation of [^{14}C]uracil into material insoluble in 5% trichloroacetic acid as described in the text.

Table 2. *Selective removal of di(guanin-7-ylethyl)* [^{35}S]*sulphide from DNA of* E. coli *treated with* ^{35}S*-labelled mustard gas*

Cells were treated with ^{35}S-labelled mustard gas for 10 min. at 37°, in either M9 buffer or M9 growth medium, and then incubated for various times at 37°. The conditions of incubation are denoted as follows: r, cells alkylated in M9 buffer at high concentration (5×10^9 cells/ml.) were resuspended in M9 growth medium at a density (1×10^8–5×10^8 cells/ml.) permitting growth as measured by increase in extinction of the suspension; g, cells in an exponentially growing culture were treated with mustard gas and allowed to continue growth; ng, cells alkylated at high density in M9 buffer were incubated in this state, i.e. under 'non-growing' conditions. After various incubation times cells were collected by centrifugation and DNA was isolated, assayed for radioactivity or hydrolysed with N-HCl at 100° and chromatographed, as described in the text. The relative amounts of DNA in the incubated cultures at various times were determined by the method of Burton (1956), and the relative amount of ^{35}S remaining in DNA was found from its specific radioactivity; the values are expressed as a percentage of the amount at the commencement of incubation. The proportion of di-(guanin-7-ylethyl) [^{35}S]sulphide in alkylpurines liberated from DNA by hydrolysis was determined by paper chromatography, as described in the text; typical radiochromatogram traces are shown in Fig. 4.

Strain	B/r	B/r	B/r	B/r	B/r	B/r	B/r	B/r	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}	$15T^-$	$15T^-$	B/r	B/r	B/r	B_{s-1}	B/r	B_{s-1}	Br	B_{s-1}
Concn. of mustard gas (μg./ml.)	100	100	100	50	50	50	25	25	50	50	25	25	0·5	0·5	1	1	1	0·5	0·5	1	1	1
Incubation conditions	r	r	r	r	r	r	r	r	r	r	r	r	r	r	g	g	g	g	g	ng	ng	ng
Incubation time (min.)	0	60	120	0	30	60	0	60	120	0	60	0	30	60	0	60	5	15	45	75	75	75
Percentage of ^{35}S remaining in DNA	100	52	43	100	59	55	100	71	47	45	100	135	100	92	99	100	52	100	78	60	63	—	—	—
Percentage of di(guanin-7-ylethyl) sulphide in DNA alkylpurines	—	—	—	21	9	0	—	—	—	—	24	28	—	—	—	22	4	20	17	13	3	17	4	20	19

B/r and 15T$^-$ show a quite different pattern of response to this agent (Fig. 9). A block to DNA synthesis was apparent immediately after mustard treatment, but after a dose-dependent lag period DNA synthesis resumed at approximately the normal rate. This response could be detected at doses as low as the mean lethal dose (Fig. 10). RNA synthesis was unaffected during this period, even by high doses of mustards (Fig. 11); a lysis that was not dose-dependent resulted after prolonged incubation of the heavily alkylated cells.

Breakdown of cellular DNA induced by alkylation. Since this typical response of the resistant strains was possibly mediated by a removal of alkylation 'lesions' from the DNA template, the degradation of cellular DNA after alkylation was investigated. Lawley & Brookes (1965) showed that ^{35}S label was lost from the DNA of resistant, but not sensitive, cells when such cells were incubated after alkylation under conditions permitting growth, as measured by continued RNA and protein synthesis. No alkyl groups were removed from RNA or protein. This phenomenon was investigated in more detail (Table 2), showing that both strains B_{s-1} and B/r were able to remove selectively the product of difunctional alkylation, di(guanin-7-ylethyl) sulphide, from their DNA, after treatment with very low doses of mustard gas. However, only the resistant strain B/r was effective in this respect at higher doses. Neither strain could remove this product when incubated under conditions of high cell density not permitting continued growth.

The difunctional mustard induces interstrand cross-links into DNA (Lawley & Brookes, 1967), associated with the presence of the product of difunctional alkylation. A technique permitting the demonstration of the presence of heat-labile interstrand cross-links in DNA, previously described (Lawley & Brookes, 1967), was therefore applied to DNA isolated from cells after treatment with mustard gas and subsequent incubation (Table 3). This method detects cross-linkage by the low hyperchromicity of DNA heated for a short period (10 min.) and then cooled. The heat-lability of the cross-links is shown by the increase in hyperchromicity after prolonged heating. These results showed that removal of di(guanin-7-yl)ethyl sulphide from DNA, detected chromatographically, was accompanied by loss of such cross-links, as indicated by increase in hyperchromicity of DNA renatured after short periods of heating.

Degradation of DNA in alkylated cells was also demonstrated by prelabelling cellular DNA with isotopically labelled thymidine and measuring release of label into the acid-soluble fraction after alkylation of washed cells and their subsequent incubation. It was further found that this radioactivity was rapidly released into the medium,

Table 3. *Renaturation of DNA isolated from* E. coli *treated with mustard gas*

Treated cells were resuspended in growth medium and incubated for various times, before isolation of DNA, as described in the text. DNA was dissolved in 0·5 mM-sodium citrate buffer, pH 7·0; hyperchromicity of DNA was determined by diluting a portion of the solution (about 0·5 mg./mL) into 10 vol. of 0·5 mM-sodium citrate buffer maintained at 68°, then after various times, *t*, portions of this solution were cooled in ice and their extinctions, E_t, were determined. Hyperchromicity $= (E_t - E_0)/E_0$, where E_t refers to the heated and cooled solution and E_0 to an unheated solution of the same concentration; values of 21–25% were found for non-cross-linked DNA; lower values indicate cross-linkage; higher values probably indicate degradation of DNA.

Strain	B/r	B/r	B/r	B/r	B_{s-1}	B_{s-1}	B/r	B/r	B/r	B/r	B/r	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}	B_{s-1}
Concn. of mustard gas (μg./ml.)	70	70	70	70	5	5	25	25	25	25	25	25	25	25	25	25	25	25	25	25
Time of incubation (min.)	0	0	120	120	10	10	0	0	60	60	210	0	0	30	30	60	60	120	240	240
Time of heating of DNA (min.)	10	120	10	120	10	120	10	150	10	120	150	10	120	10	120	10	120	10	120	120
Hyperchromicity (%)	7	25	22	31	18	28	12	27	21	27	39	12	27	15	28	13	28	14	24	13	28

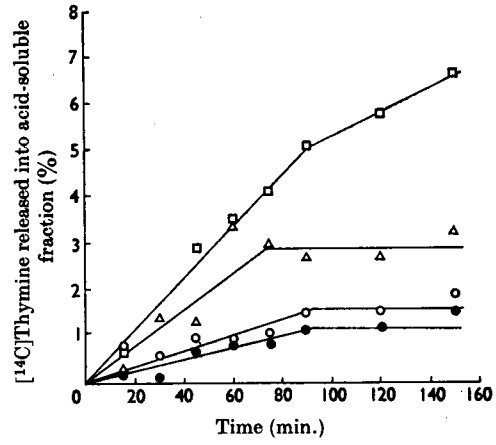

Fig. 12. Release of material containing [^{14}C]thymine into the acid-soluble fraction in suspensions of *E. coli* 15T$^-$ after treatment with mustard gas. Experimental details are described in the text. The ordinates represent the amount of ^{14}C found in the supernatant, after precipitation of cellular material from portions of the suspension at various times with 5% trichloroacetic acid, as a percentage of [^{14}C]thymine incorporated before treatment with mustard gas at zero time, less the amount released from non-treated cells. Concentrations of mustard gas (μg./ml.): ●, 12·5; ○, 25; △, 50; □, 100.

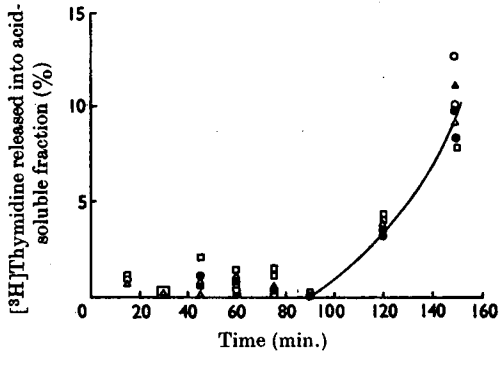

Fig. 13. Release of material containing [^{3}H]thymidine into the acid-soluble fraction in suspensions of *E. coli* B_{s-1} after treatment with mustard gas. Experimental details are described in the text. The ordinates represent the amount of ^{3}H found in the supernatant, after precipitation of cellular material from portions of the suspensions at various times with 5% trichloroacetic acid, as a percentage of [^{3}H]thymidine incorporated before treatment with mustard gas at zero time, less the amount released from non-treated cells. Concentration of mustard gas (μg./ml.): ●, 2·5; ○, 10; △, 25; □, 50.

accompanied by u.v.-absorbing material. Alkylated resistant cells released DNA degradation products in this way in a dose-dependent manner (Fig. 12); after about 90 min. incubation further lysis of

heavily alkylated cells resulted. Some breakdown of alkylated sensitive cells was indicated throughout the incubation period, but it was independent of the alkylation dose (Fig. 13). After 90min., non-dose-dependent lysis of alkylated cells became more marked than that of controls. The extent of release from strains B/r or 15T$^-$ after treatment with mustard gas corresponded to about 400 nucleotides per alkyl group in cellular DNA; that after treatment with half mustard was estimated to be about 10 nucleotides per alkyl group.

DISCUSSION

For the series of alkylating agents examined, the extents of alkylation of cellular constituents in relation to dosage were quite similar, with one marked exception, namely that iodoacetamide reacted most extensively with cellular protein, but not with nucleic acids. Since iodoacetamide was the weakest cytotoxic agent of the series, this finding strongly suggests that extensive alkylation of cellular proteins is not of itself a cause of inhibition of cell division. However, a difference between iodoacetamide and the remaining agents with respect to mode of reaction with protein was evident, in that iodoacetamide reacted almost exclusively with thiol groups. The mustards and methyl methanesulphonate, though reacting mainly with such groups, also alkylated the basic histidine residues.

The concept that cellular DNA is the most susceptible target to alkylating agents was supported by the following observations. First, both the sensitive and resistant strains were inactivated by mustard gas at doses so low that, on the basis of the number of alkylations per molecule, most of the smaller macromolecules such as RNA and protein would escape alkylation, unless some considerable specificity of attack on particular macromolecules occurred, for which there is as yet no evidence.

Moreover, it is known that a significant difference exists between the mode of reaction with DNA of the difunctional and monofunctional mustards, namely that the former can cross-link twin-stranded DNA (Lawley & Brookes, 1967). This reaction, according to the concept of semi-conservative replication of DNA, would be expected to inhibit DNA synthesis and to constitute an effective block to cell division.

A second factor is the observation that at low doses of mustards, of the order of the mean lethal dose, inhibition of DNA synthesis is the sole detectable biochemical lesion. RNA synthesis, as shown here and in more detail elsewhere (Venitt, Brookes & Lawley, 1968), is much less susceptible to the action of either mustard and, further, no difference between the mono- and di-functional mustards was detected. Similar considerations apply to protein synthesis (Lawley & Brookes, 1965), as measured both by incorporation of amino acids into unfractionated cellular protein and by the synthesis of a specific inducible protein, β-galactosidase (Venitt *et al.* 1968).

The simplest interpretation of the results would therefore be that a cross-link induced in the cellular DNA template could prevent synthesis of this macromolecule, which according to current views is required to be replicated intact in order to permit cell division.

However, examination of the quantitative data relating extent of DNA alkylation corresponding to the mean lethal dose, here assumed to be that required to lower survival of colony-forming ability to 37%, revealed that this simple view was incorrect. Obviously for the resistant strain B/r the mean lethal dose expressed in this way is far greater than one cross-link per molecule of DNA (Table 1).

Two factors contribute uncertainty to these correlations. First, though it is now accepted that cellular DNA is present as large macromolecules, and that probably the complete bacterial genome is contained in a single double-stranded molecule, its exact size is not yet known. Cairns (1963) deduced a minimal molecular weight of $2{\cdot}8 \times 10^9$ daltons for *E. coli* DNA; the total amount of cellular DNA in exponentially growing cultures of *E. coli* B/r or B_{s-1} was determined by us to be 9×10^9 daltons. Witkin (1951) has shown that two to four nuclear bodies are present in such cells. It seems reasonable therefore to take an approximate value of 3×10^9 daltons for the molecular weight of the bacterial genome, corresponding to 9×10^6 moles of DNA phosphorus or nucleotide units.

The second uncertainty derives from lack of exact knowledge of the number of cross-links induced by mustard gas in DNA. It is known that the number of difunctional alkylations, as determined by chemical analysis, is at least approximately equal to the number of interstrand cross-links detected by physicochemical methods (Lawley & Brookes, 1967). However, no positive method for estimation of the number of intrastrand cross-links has yet been found. We therefore quote for the present the number of inter-guanine linkages determined by chemical analysis, while recognizing that this is a maximal estimate for the number of interstrand cross-links. The presence of such interstrand links has been unequivocally demonstrated in cells of *E. coli* after mustard gas treatment, not only by the present work with an optical method, but also by gradient centrifugation (Venitt, 1968). This latter method shows the presence of linked strands of DNA from cells alkylated at the mean lethal dose, this DNA being in the denatured form in

an alkaline sucrose gradient, according to the technique of McGrath & Williams (1966). The molecular weight of this cross-linked DNA is 5×10^8 daltons, i.e. double that of the large fragments present in DNA from non-alkylated cells.

With the assumptions outlined above, the mean lethal doses of the various agents can then be expressed as number of alkylations per cellular genome of 9×10^6 moles of DNA phosphorus (cf. Table 1). This number approximates to unity only for the case of cross-links in DNA of the sensitive strain B_{s-1}, which when further sensitized to the action of mustard gas by other treatments requires about three cross-links/molecule for inactivation. Nevertheless, when the extremely low extents of alkylation of other cellular macromolecules at this mean lethal dose for either strain are considered, it seems implausible to attribute lethal action to any other cause than the induction of cross-links in cellular DNA.

When the monofunctional agents are considered, the wide range of lethal doses presents the most striking feature. Evidently even the sensitive strain B_{s-1} can withstand multiple DNA alkylations of the monofunctional type. This conclusion parallels that deduced from previous work with other systems, e.g. studies with bacteriophage (Loveless & Stock, 1959; Brookes & Lawley, 1963) and transforming DNA (Strauss, 1963).

The question then arises whether alkylation itself can inactivate the DNA template, or whether hydrolytic degradation of alkylated DNA (Lawley & Brookes, 1963) induces the essential lesions.

Quantitative determination of the number of depurinations that correspond to these mean lethal doses presents difficulties. The rates of hydrolysis of alkylated DNA at neutral pH are known (Lawley & Brookes, 1963). Clearly the number of potential depurinations in cellular DNA will be time-dependent, but the significant time involved, namely that ensuing between the alkylation itself and that when DNA is used as a biological template *in vivo*, can only be roughly estimated. Since the normal division time in liquid medium is about 1 hr., it may be reasonable to assume such an average latent period before DNA replication in plating medium. Such an assumption still leads to the conclusion that multiple depurinations would have occurred after administration of the mean lethal doses, even for the sensitive strain (the estimated values are about 20 depurinations after treatment with half mustard and about 130 after treatment with methyl methanesulphonate).

A further possibility concerns the number of single-strand breaks, which would in turn result after hydrolytic depurination. Here the rate is less certain, though some data are available for mustard-treated calf thymus DNA (Laurence, 1963), and for mustard-treated ϕX-174 DNA (K. V. Shooter, P. A. Edwards & P. D. Lawley, unpublished work). This shows that a single depurination in DNA would yield a chain break by hydrolysis at neutral pH with a half-life of about 2000 hr. Evidently only for the resistant strain B/r would monofunctional alkylation at the mean lethal dose be expected to cause any significant DNA strand breakage by hydrolysis.

The significant difference in response of *E. coli* B_{s-1} to the two monofunctional alkylating agents used parallels that found with T-even phage (Brookes & Lawley, 1963). As concluded for the latter, this difference does not appear to be accounted for completely by the difference in rates of depurination of DNA subsequent to its alkylation. A possible explanation might be that the attachment of the larger alkyl group derived from the mustard can itself exert a relatively inefficient block to the DNA polymerase system, which studies of this system *in vitro* might reveal, whereas methylation of DNA would cause an even weaker effect of this type. Evidently neither of these monofunctional agents is effective in the resistant strain, and both are about as weakly cytotoxic as iodoacetamide. This finding suggests that repair of monofunctional alkylation lesions in DNA is very efficient, and the residual cytotoxic action may be principally due to alkylation of other cellular constituents. A possible target is the repair enzyme system itself, which has been shown to be inhibited by iodoacetamide in *E. coli* (Lawley & Brookes, 1965). It has also been suggested (Loveless, 1966) that the sensitization of yeast towards radiation by ethyl methanesulphonate (Patrick & Haynes, 1964), could be, at least in part, due to ethylation of the repair enzymes.

Consideration of the difference in response to the alkylating agents of the sensitive and resistant strains leads immediately to the conclusion that neither alkylation nor any subsequent hydrolytic process could account for the observed phenomenon of resistance. Evidence has been obtained that this resistance, for the case of treatment with mustard gas, is mediated by removal of alkylation lesions from cellular DNA. The time-scale of this removal cannot be accounted for by spontaneous chemical hydrolysis, nor can the observed selective removal of difunctional alkylations. The mediation of an enzymic repair process is therefore indicated.

Stages of such enzymic repair processes operative after u.v. irradiation of *E. coli* have been defined by biochemical and genetic studies, and cross-resistance to effects of alkylating agents and u.v. or X-irradiation has been extensively investigated (Setlow, 1964; Howard-Flanders & Boyce, 1966; Greenberg, 1967; Mattern, Zwenk & Rörsch, 1966). These studies led to the conclusion that four types

of enzymic action were involved. First, alkylation- or u.v.-radiation-induced lesions in cellular DNA are removed by a specific endonuclease (or endonucleases); this stage appears to be unnecessary for repair of damage due to ionizing radiation, consistent with the concept that the primary lesions involve strand-breakage in the cellular DNA molecules; further, a strain of *Bacillus subtilis* unable to remove u.v.-induced lesions from transforming DNA was found to be able to repair methylated DNA (Reiter & Strauss, 1965), indicating that either the excising enzymes differ for these two types of lesion, or that excision is also unnecessary in repair of methylation damage. The initial excision process appears to involve removal of only a small segment of DNA as an oligonucleotide (Setlow, 1964); a second excision stage, presumably mediated by an exonuclease, then enlarges the gap thus formed. Presumably this enlargement is necessary to permit the third process in repair, i.e. that of repair replication; since this involves replacement of a deleted single-stranded portion of DNA it differs from normal replication in being 'non-semi-conservative'. This difference can be recognized by appropriate techniques (Pettijohn & Hanawalt, 1964) and has been shown to occur in *E. coli* after treatment with nitrogen mustard (Haynes & Hanawalt, 1965).

Although in our work we have demonstrated that excision of alkylation lesions occurs, we have not obtained evidence in detail for the operation of the second and third stages of repair, as outlined above. However, we have shown that, in the resistant strain, DNA synthesis resumes at the normal rate after such excision. Further, the extents of degradation of prelabelled cellular DNA after alkylation can be correlated with the extents of alkylation of DNA, showing that, within a 90min. incubation period after alkylation, about 2000 nucleotides are removed for each cross-link induced in DNA by the difunctional mustard, and about 10 for each alkylation by the monofunctional mustard. It remains unknown in what form these alkyl groups are removed.

The fourth stage in the repair process involves the rejoining of repair-replicated DNA to the residual normal DNA. Genetic evidence suggests that the enzyme responsible in bacteria is identical with that performing the analogous function in the process of host-cell reactivation of radiation-damaged bacteriophage (Howard-Flanders & Boyce, 1966; Greenberg, 1967). Again we have no direct evidence for the operation of such an enzyme from the present work, but the survival of resistant cells after alkylation to extents lethal to cells of the sensitive strain, accompanied by a recovery in DNA synthesis, would be difficult to envisage without the occurrence of such a process to complete the restitution of the partially degraded cellular DNA template.

With respect to u.v. radiation, it has been shown that the sensitivity of *E. coli* B_{s-1} is due to two mutant genes, one associated with sensitivity to u.v. light, and the other also conferring a loss of the host-cell-reactivating property (Greenberg, 1967; Mattern *et al.* 1966). It therefore seems likely that these mutations have caused deficiencies in activity of the enzymes carrying out the first (excision) and last (rejoining) stages of the repair process. Again, in our work, we have evidence only for a deficiency of the first kind, but the second is not, of course, ruled out.

One unexpected finding in our work was that DNA synthesis in the sensitive strain did not cease immediately after alkylation, but did so after a period of about one-third of a generation time. In contrast with the resistant strain, these sensitive cells did not show a dose-dependent lag in DNA synthesis immediately after mustard treatment. A possible explanation is that DNA synthesis that has already started proceeds to completion independently of whether the template is blocked, whereas a new round of DNA synthesis cannot begin on the alkylated template. This would imply that the polymerase enzyme can by-pass the blocked site.

The finding (Lawley & Brookes, 1965) that the diguaninyl derivative, formed in DNA after treatment of resistant cells with mustard gas, can be selectively excised implies that a lesion spanning both strands of the template double helix can be enzymically removed. This contrasts with the original proposals for the mechanism of action of the endonuclease removing u.v.-induced lesions, which were envisaged to involve excision from a single strand of DNA only. Thus the strand opposite that containing the lesion remained available as a repair template. It has been suggested that interstrand cross-links in DNA due to reaction with mustard gas require a base sequence ...GpC... in either affected strand (Brookes & Lawley, 1961). Thus it is conceivable that, provided that the endonuclease action cleaved this sequence leaving a pC... end group to each chain, sufficient genetic information would remain to specify the original base sequence after completion of repair.

Two mechanisms can therefore be envisaged for repair of this difunctional alkylation lesion. First, the excision could be confined initially to a single strand, which then undergoes repair replication before the second half of the lesion is excised. Alternatively, if both ends of the cross-link are simultaneously removed, rejoining of a double-strand break would be required.

The latter mechanism might formerly have been considered less likely. However, recent evidence

strongly suggests that double-strand breaks induced by ionizing radiation can be repaired, not only in micro-organisms but in mammalian cells. This conclusion follows from the fact that non-lethal radiation doses are high enough to cause double-strand breaks in the DNA of these cells; further, for *Micrococcus radiodurans*, positive evidence for rejoining of such breaks has been obtained (Dean, Hamilton & Lett, 1968; personal communication from Dr C. J. Dean).

Clearly, for alkylating agents, more detailed studies of the repair process will be required to decide between these alternatives.

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