6. Preliminary figures for androstenol excretion in urine are given. In the age group 18–45 years they ranged from 19 to 2630 $\mu\text{g}/24$ hr. in men and from 66 to 1010 $\mu\text{g}/24$ hr. in women.

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REFERENCES

- Adkins, H. & Connor, R. (1931). *J. Amer. chem. Soc.* **53**, 1091.
- Allen, W. M. (1950). *J. clin. Endocrin.* **10**, 71.
- Bollenback, G. N., Long, J. W., Benjamin, D. G. & Lindquist, J. A. (1955). *J. Amer. chem. Soc.* **77**, 3310.
- Brooksbank, B. W. L. & Haslewood, G. A. D. (1949). *Biochem. J.* **44**, iii.
- Brooksbank, B. W. L. & Haslewood, G. A. D. (1950). *Biochem. J.* **47**, 36.
- Brooksbank, B. W. L. & Haslewood, G. A. D. (1952). *Biochem. J.* **51**, 286.
- Brooksbank, B. W. L. & Haslewood, G. A. D. (1960). *1st int. Congr. Endocrin., Copenhagen; Short Commun.* no. 516.
- Brooksbank, B. W. L., Haslewood, G. A. D., Pollock, J. A. & Hewett, C. L. (1958). *Biochem. J.* **70**, 14P.
- Burstein, S. & Dorfman, R. I. (1960). *1st int. Congr. Endocrin., Copenhagen; Short Commun.* no. 346.
- Burstein, S. & Lieberman, S. (1958). *J. biol. Chem.* **233**, 331.
- Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
- Engel, L. L., Dorfman, R. I. & Abarbanel, A. R. (1953). *J. clin. Endocrin.* **13**, 903.
- Fishman, W. H. & Green, S. (1955). *J. biol. Chem.* **215**, 527.
- Fishman, W. H., Springer, B. & Brunetti, R. (1948). *J. biol. Chem.* **173**, 449.
- Gallagher, T. F., Bradlow, H. L., Fukushima, D. K., Beer, C. T., Kritchevsky, T. H., Stokem, M., Eidinoff, M. L., Hellman, L. & Dobriner, K. (1954). *Recent Progr. Hormone Res.* **9**, 411.
- Gibson, J. G. & Evans, W. A. (1937). *J. clin. Invest.* **16**, 301.
- Mason, H. L. & Schneider, J. J. (1950). *J. biol. Chem.* **184**, 593.
- Meystre, C. & Miescher, K. (1944). *Helv. chim. acta*, **27**, 231.
- Miescher, K. (1946). *Helv. chim. acta*, **29**, 743.
- Miller, A. M., Rosenkrantz, H. & Dorfman, R. I. (1953). *Endocrinology*, **53**, 238.
- Neher, R. & Wettstein, A. (1952). *Helv. chim. acta*, **35**, 276.
- Pelzer, H. (1959). *Hoppe-Seyl. Z.* **314**, 234.
- Prelog, V. & Ruzicka, L. (1944). *Helv. chim. acta*, **27**, 61.
- Prelog, V., Ruzicka, L. & Wieland, P. (1944). *Helv. chim. acta*, **27**, 66.
- Ruzicka, L., Plattner, P. A., Heusser, H. & Pataki, J. (1946). *Helv. chim. acta*, **29**, 936.
- Sondheimer, F., Mancera, O., Urquiza, M. & Rosenkrantz, G. (1958). *J. Amer. chem. Soc.* **77**, 4145.
- Stitch, S. R. & Halkerston, I. D. K. (1956). *Biochem. J.* **63**, 710.
- Weissberger, A. & Proskauer, E. (1935). *Organic Solvents*. Oxford: The Clarendon Press.
- Wotiz, H. H., Smakula, E., Lichtin, N. N. & Leftin, J. H. (1959). *J. Amer. chem. Soc.* **81**, 1704.

Biochem. J. (1961) **80**, 496

The Reaction of Mono- and Di-functional Alkylating Agents with Nucleic Acids

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In attempts to relate the chemical reactions of the alkylating agents with their biological effects two general principles have emerged. First, a general positive correlation has been established between chemical reactivity, as measured by rate of hydrolysis, and cytotoxicity (Haddow, Kon & Ross, 1948; cf. review by Ross, 1953). Secondly, the difunctional agents, i.e. those possessing two alkylating groups in the molecule, generally exert a markedly more powerful cytotoxic action than the corresponding monofunctional agents (Loveless

& Ross, 1950). This has been ascribed to their ability to cross-link fibrous macromolecules, in particular those involved in duplication of the chromosomes (Goldacre, Loveless & Ross, 1949), reaction of this type between di-(2-chloroethyl) sulphide (mustard gas) and deoxyribonucleic acid having been proposed by Elmore, Gulland, Jordan & Taylor (1948). The importance of deoxyribonucleic acid as a site of biological alkylation was also suggested by observation of the mutagenic activity of mustard gas (Auerbach & Robson,

1946) and subsequently of a wide variety of alkylating agents (Fahmy & Fahmy, 1956). The difunctional agents are not markedly more effective as mutagens in comparison with the monofunctional, although differences in the types of mutation produced by these classes of agent have been found (Fahmy & Fahmy, 1961). If the principal biological effects of the alkylating agents are to be ascribed to their reaction with any one cellular constituent, the nature of this reaction must be such as to account for the relationship between chemical structure and biological activity that have been outlined above. The mode of combination of mustard gas with nucleic acids has been established and the reaction shown to occur *in vivo* (Brookes & Lawley, 1960). In order to ascertain whether the criteria for a reaction of biological significance are satisfied, a comparison of alkylating agents of different reactivities, and of mono- and di-functional agents of closely similar reactivities, seemed desirable. Investigations were therefore undertaken of the reactions with nucleic acids of ethyl iodide, diethyl sulphate, methyl methanesulphonate, ethyl methanesulphonate, 1:4-dimethanesulphonoxylbutane (myleran), HN 2[di-(2-chloroethyl)methylamine], nor-HN 2[di-(2-chloroethyl)amine], *NN*-di-(2-chloroethyl)aniline and 2-hydroxyethyl 2-chloroethyl sulphide. The binding of 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide and of ^3H -labelled myleran to cellular constituents in the mouse was also determined. A detailed comparison has been made of the reaction of nucleic acids with 2-hydroxyethyl 2-chloroethyl sulphide and with mustard gas, a preliminary account of which has been given (Brookes & Lawley, 1961*a*).

EXPERIMENTAL

Materials. ^{35}S -Labelled mustard gas (200 $\mu\text{C}/\text{mg.}$) and 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (200 $\mu\text{C}/\text{mg.}$) and labelled starting materials for other radioactive preparations were obtained from The Radiochemical Centre, Amersham, Bucks.

^3H -Labelled myleran was prepared from but-2-ene-1:4-diol by catalytic reduction with tritium and had a specific activity of 3.1 $\text{C}/\text{g.}$ ^{14}C -Labelled myleran (4.0 $\mu\text{C}/\text{mg.}$) was prepared by conversion of [2:3- ^{14}C]succinic acid into 1:4-dihydroxy[2:3- $^{14}\text{C}_2$]butane (Mann & Nystrom, 1951) and subsequent reaction with methanesulphonyl chloride. Di-(2-chloro[$^{14}\text{C}_2$]ethyl)amine hydrochloride (3.6 $\mu\text{C}/\text{mg.}$) was prepared by reaction of [$^{14}\text{C}_2$]ethylene oxide (Cox & Warne, 1951) with ethanolamine and subsequent chlorination of the labelled diol with thionyl chloride. [1- ^{14}C]Ethyl methanesulphonate (9.9 $\mu\text{C}/\text{mg.}$) was prepared by reaction of [1- ^{14}C]ethyl iodide with silver methanesulphonate. *NN*-Di-(2-chloroethyl)[^{14}C]aniline (0.63 $\mu\text{C}/\text{mg.}$) was supplied by Dr J. J. Roberts and Dr G. P. Warwick.

Paper chromatography, radioautography and counting techniques. The methods were as described by Brookes & Lawley (1960).

Reaction of alkylating agents with nucleic acids *in vitro*. RNA or DNA was dissolved in aqueous buffer solution, containing sufficient potassium acetate or sodium phosphate to maintain neutrality during the reaction with the alkylating agents, to give solutions of concentration 0.1–0.01 *M*-nucleic acid P. The alkylating agent was added to the solution, which was maintained at 37°, and after appropriate times samples were taken and nucleic acid was precipitated with 2 vol. of ethanol containing 2% of sodium acetate. When radioactive alkylating agents were used the concentration was in the range 0.001–0.2 mole/mole of nucleic acid P and in other cases up to 1 mole/mole of nucleic acid P. Details of the concentrations used for individual alkylating agents are given in Table 1. The extent of alkylation was determined for labelled alkylating agents by assaying the radioactivity of the precipitated nucleic acids, and for unlabelled reagents by isolation and estimation of the products after acid hydrolysis of the alkylated nucleic acids. When labelled reagents were used the rate of alkylation was determined from the specific radioactivity of the nucleic acid precipitated at suitable time intervals during the course of the reaction (Table 1). With RNA the extent of alkylation increased to a maximum and then remained constant, whereas with DNA the specific radioactivity increased initially at a rate equal to that for RNA, but subsequently decreased, as for ^{35}S -labelled mustard gas (Brookes & Lawley, 1960).

Identification of the products of alkylation of nucleic acids. When ^{14}C - or ^{35}S -labelled alkylating agents were used a sample of alkylated DNA, sufficient to contain at least 5 μC (up to 5 mg.), was hydrolysed with acid, and a radioautograph of a two-dimensional paper chromatogram prepared. The R_F values of the radioactive spots were compared with those of the expected 7-alkylguanines (Brookes & Lawley, 1961*b*).

In other cases the acid hydrolysate from alkylated nucleic acid was chromatographed on Dowex 50 (H^+ form), and the pyrimidine nucleotides and purine bases were eluted with HCl. The amounts of the unchanged purine bases and of the 7-alkylguanine products were estimated by summation of the extinctions at 260 $\text{m}\mu$, and the products were identified by evaporation of the appropriate fractions followed by neutralization of the resulting hydrochlorides and crystallization of the free bases from water.

In a typical example, yeast RNA (240 mg. ; 0.5 *m*-mole) was treated with ethyl methanesulphonate (62 mg. ; 1 mole/mole of RNA P) in 0.4 *N*-potassium acetate buffer (5 ml. ; initial pH 7.2) at 37° for 48 hr. RNA was precipitated with 2 vol. of ethanol containing 2% of potassium acetate and hydrolysed with *N*-HCl (5 ml.) for 1 hr. at 100°, and the resulting solution cooled and chromatographed on a column (17 $\text{cm.} \times 2 \text{ cm.}$) of Dowex 50 (H^+ form; equilibrated with *N*-HCl), with *N*-HCl as solvent. Fractions (50 ml.) were collected automatically and their ultraviolet absorption was measured at 260 and 280 $\text{m}\mu$. Fractions 1–8 contained uridylic and cytidylic acids, fractions 16–24 contained guanine (0.134 *m*-mole), fractions 41–52 contained a product (0.01 *m*-mole) with ultraviolet-absorption spectra similar to those of 7-methylguanine, and fractions 64–100 contained adenine (0.1 *m*-mole). The fractions containing the 7-alkylguanine were evaporated, and the resulting hydrochloride was dissolved in the minimum of water and neutralized, when a crystalline base was deposited. This was shown by comparison of R_F values in

three solvents and ultraviolet-absorption spectra at pH 1, 7 and 12 to be identical with an authentic specimen of 7-ethylguanine (Brookes & Lawley, 1961b).

The results of experiments with labelled and unlabelled alkylating agents together with RNA (from yeast or rat liver) or DNA (from calf thymus) are shown in Table 1.

Hydrolysis of alkylated deoxyribonucleic acid in neutral aqueous solution. After the alkylation of DNA by di-(2-chloro-[^{14}C]ethyl)amine, [^{14}C]ethyl methanesulphonate, [^{14}C]labelled myleran, ^{35}S -labelled mustard gas or 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide, as described above, portions of the reaction mixture were taken after 120 hr. and the DNA was precipitated. The supernatant was evaporated and a two-dimensional paper chromatogram of the residue obtained. Radioautography of this chromatogram showed the presence of the 7-alkylguanines, which had been established as the products of the initial stages of the reaction.

A more detailed study of the liberation of the 7-alkylguanine products was made with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide. DNA (10 mg.) was dissolved in 0.01N-phosphate buffer, pH 7.1 (2 ml.), at 37° and 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (32 μg .) in ether (10 μl .) added. Samples (0.05 ml.) were withdrawn after 30 min. and subsequently at various intervals up to 12 days. The samples were applied to strips of Whatman no. 1 paper and chromatographed with ethanol-conc. NH_3 soln.-water (80:2:18, by vol.) as solvent. After drying, the strips were assayed for radioactivity with a paper-strip counter. Four radioactive areas were found: one at the origin, which was accompanied by ultraviolet absorption, corresponding to alkylated DNA; two, with R_f values 0.2 and 0.45, corresponding to 7- β -hydroxyethylthioethylguanine and its product of oxidation on the paper (Brookes & Lawley, 1960); and one near the solvent front corresponding to [^{35}S]thiodiglycol resulting from hydrolysis of unreacted 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide. Throughout the experiment the radioactivity at the solvent front was a constant proportion (48%) of the total radioactivity on the paper. However, the radioactivity associated with the 7-alkylguanine products increased, being zero after 30 min. and 42% after 14 days, whereas the radioactivity at the origin decreased correspondingly. The results of the experiment are shown in Fig. 1.

In a similar experiment with the sodium salt of deoxyguanylic acid (39 mg.) and 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0.1 mg.) the hydrolysis of the 7-alkyldeoxyguanylic acid to yield 7- β -hydroxyethylthioethylguanine was found to be a first-order reaction with half-life of 8 hr.

Determination of the proportion of combined ^{35}S -labelled mustard gas present as di-(β -guanin-7-ylethyl) sulphide in the reaction of mustard gas with nucleic acids. Nucleic acid (RNA, DNA or DNA denatured by heating at 100° for 10 min. in 0.01N-phosphate buffer, pH 7.1) (40 mg.) was dissolved in 0.02N-phosphate buffer, pH 7.1 (10 ml.), at 37°, and ^{35}S -labelled mustard gas (0.35 mg.; 55 μC) in ether (0.01 ml.) was added. After 0.5, 1, 2, 5 and 15 min. samples (2 ml.) were taken, nucleic acid was precipitated with 2 vol. of ethanol containing 2% of sodium acetate cooled to 0°, and dried, and the radioactivity assayed. The samples were hydrolysed with N-HCl (0.5 ml.), and 0.05 ml. was applied to a strip of Whatman no. 1 paper and chromatographed with methanol-conc. HCl-water (7:2:1, by vol.) as solvent. The paper was dried and the radioactivity assayed on a paper-strip counter. Two areas of radioactivity

were found, one at the origin corresponding to di-(β -guanin-7-ylethyl) sulphide, and another with R_f 0.1-0.4 corresponding to 7- β -hydroxyethylthioethylguanine and related products of monofunctional alkylation. The ratio of the radioactivity of the former to the total was a measure of the extent of difunctional alkylation, as shown in Fig. 2.

Binding in vivo of 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide and of ^3H -labelled myleran. Four mice bearing 7-day-old Ehrlich-ascites tumours were each injected intraperitoneally with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0.2 mg.; 31 μC) in arachis oil (0.1 ml.). After 1 hr. the mice were killed and RNA, DNA and protein were isolated from the ascites cells as described by Brookes & Lawley (1960). In a similar experiment tumour-bearing mice were injected with ^3H -labelled myleran (1 mg.; 3 mC) suspended in arachis oil (0.1 ml.). Mice were killed after 24, 72 and 120 hr.

In another series of experiments C3H mice with advanced virus-induced lymphoblastic leukaemia were used and RNA, DNA and protein were isolated from leukaemic spleen and lymphocytes 22 and 66 hr. after injection of ^3H -labelled myleran. The RNA contained 6.8% of P; molar base ratios, adenine 1, guanine 1.75, cytosine 1.58, uracil 0.68. DNA contained 7.1% of P; molar base ratios, adenine 1, guanine 0.79, cytosine 0.72, thymine 0.88, uracil 0.09. The presence of uracil in the DNA shows that RNA was present as an impurity to the extent of about 9%. This results from omission of ribonuclease treatment in order to avoid prolonged dialysis of DNA with consequent loss of alkylated guanine.

Samples of RNA, DNA and protein were assayed for radioactivity (Table 2) and when 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide had been used, radioautographs from chromatograms of hydrolysed nucleic acids were prepared in the usual way.

Tumour growth inhibition by mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide. A C3H mouse bearing the 12-day-old Gardner-ascites tumour was injected with ^{35}S -labelled mustard gas (0.1 mg.; 1 μC) in arachis oil (0.1 ml.), and after 1 hr. the mouse was killed, the ascites cells (3×10^6) were removed and washed with 0.9% NaCl soln., and 10^6 cells injected into each of six C3H mice. A portion of the remainder of the cells was dried and assayed for radioactivity. Similarly, a tumour-bearing mouse was injected with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0.1 mg.; 15.2 μC) and a further six mice were injected with 10^6 mustard-treated ascites cells. A control group of six mice were injected at the same time with the same number of untreated ascites cells. The assay of radioactivity showed that the mustard gas-treated cells contained 5 μg . of bound mustard gas/g. of cells and that the 2-hydroxyethyl 2-chloroethyl sulphide-treated contained 4 μg . of bound 2-hydroxyethyl 2-chloroethyl sulphide/g. The mice were examined at intervals. After 8 days the control and 2-hydroxyethyl 2-chloroethyl sulphide-treated groups had developed tumours, whereas the mustard gas-treated group had no visible tumour growth. The numbers of survivors after 28 days were: controls, 0; 2-hydroxyethyl 2-chloroethyl sulphide-treated, 1; mustard gas-treated, 6.

In similar experiments doses of 30 μg . of mustard gas and 1 mg. of 2-hydroxyethyl 2-chloroethyl sulphide were used. Again the mustard gas-treated cells did not yield tumours on reinjection, whereas the 2-hydroxyethyl 2-chloroethyl sulphide-treated gave visible tumours within 14 days.

Table 1. *Reaction of alkylating agents with nucleic acids at pH 7 and 37°*

Concn. of nucleic acid (M)	Concn. of reagent (M)	Concn. of buffer (M)	Half-life of reagent (hr.)		Extent of reaction as % of reagent combined with nucleic acid	Product(s)
			Reaction with nucleic acid	Hydrolysis in water		
RNA (yeast), 0.05	Methyl methanesulphonate, 0.05	Phosphate, 0.4	—	9.2	1.9	7-Methylguanine
RNA (yeast), 0.05	Ethyl iodide, 0.05	Phosphate, 0.4	—	—	1.1	7-Ethylguanine
RNA (yeast), 0.05	Di-(2-chloroethyl)methylamine, 0.025	Phosphate, 0.4	—	2.2	18	β -(Guanin-7-yl)ethyl- β -hydroxy- ethylmethylaniline and di-(β - guanin-7-ylethyl)methylamine
RNA (yeast), 0.1	Methyl methanesulphonate, 0.1	Acetate, 0.4	—	—	5.7	7-Methylguanine
RNA (yeast), 0.1	Ethyl methanesulphonate, 0.1	Acetate, 0.4	—	10.4	1.8	7-Ethylguanine
RNA (rat liver), 0.1	[¹⁴ C]Ethyl iodide, 0.002	Phosphate, 0.01	~40	—	0.95	7-Ethylguanine
RNA (rat liver), 0.05	[¹⁴ C]Ethyl methanesulphonate, 0.01	Phosphate, 0.05	10	—	0.75	7-Ethylguanine
RNA (rat liver), 0.05	¹⁴ C-Labelled myleran, 0.008	Phosphate, 0.03	9	2	1.8	7- δ -Hydroxybutylguanine and $\alpha\delta$ -di-(guanin-7-yl)butane
RNA (rat liver), 0.067	<i>NN</i> -Di-(β -chloroethyl)[¹⁴ C]- aniline, 0.0024	Acetate, 0.02, 50% ethanol	8	—	65	See text
RNA (rat liver), 0.01	Di-(2-chloro[¹⁴ C]ethyl)amine, 0.0002	Phosphate, 0.002	10	—	67	See text
RNA (rat liver), 0.01, or DNA, 0.01	³⁵ S-Labelled mustard gas, 0.0001	Phosphate, 0.001	0.02	—	80	7- β -Hydroxyethylthioethyl- guanine and di-(β -guanin-7- ylethyl) sulphide
RNA (rat liver), 0.01, or DNA, 0.01	2-Hydroxyethyl 2-chloroethyl [³⁵ S]sulphide, 0.0001	Phosphate, 0.001	0.02	0.02	75	7- β -Hydroxyethylthioethyl- guanine
DNA, 0.1	Ethyl sulphate, 0.1	Acetate, 0.4	—	—	0.7	7-Ethylguanine
DNA, 0.1	¹⁴ C-Labelled myleran, 0.005	Acetate, 0.05	—	—	0.7	7- δ -Hydroxybutylguanine and $\alpha\delta$ -di(guanin-7-yl)butane
DNA, 0.01	[¹⁴ C]Ethyl methanesulphonate, 0.0025	Phosphate, 0.02	~12	—	0.4	7-Ethylguanine
RNA, 0.01	[¹⁴ C]Ethyl methanesulphonate, 0.0025	Phosphate, 0.02	~12	—	0.8	7-Ethylguanine
DNA, 0.01	[¹⁴ C]Methyl methanesulphonate, 0.0025	Phosphate, 0.02	8	—	4	7-Methylguanine
RNA, 0.01	[¹⁴ C]Methyl methanesulphonate, 0.0025	Phosphate, 0.02	8	—	8	7-Methylguanine

RESULTS

The alkylation of nucleic acids in neutral aqueous solution by a variety of agents has been shown, as expected, to proceed at a rate approximately equal to the rate of hydrolysis of the alkylating agents in water (Table 1). The extent of reaction with nucleic acids was not necessarily proportional to the rate of reaction, e.g. with di-(2-chloroethyl)amine and *NN*-di-(2-chloroethyl)-aniline the major part of the reagent reacted with nucleic acid, whereas with ethyl methanesulphonate and myleran the extent of reaction with nucleic acid was small, although in all four cases the rate of reaction was approximately the same.

The site of alkylation of the nucleic acids was shown in all cases to be N-7 of guanine moieties by comparison of the products isolated from the acid-hydrolysed alkylated nucleic acids with those from the alkylation of guanosine or guanylic acid (Brookes & Lawley, 1961*b*). The difunctional agents gave in all cases two types of product. The first type had R_F values greater than those for guanine, i.e. were 7-alkylguanines, e.g. (I), resulting from reaction of one alkylating group with a guanine moiety and the second with water. The second type had zero R_F values, characteristic of di-(guanin-7-yl) derivatives e.g. (II) resulting from reaction of both alkylating groups with guanine moieties.

With *NN*-di-(2-chloroethyl)aniline the products from guanosine were rapidly oxidized in air and could not be analysed. With di-(2-chloroethyl)-amine the R_F values of the products were closely similar to the analogous products from HN 2 [di-(2-chloroethyl)methylamine] and their structures have been assigned on that basis as β -(guanin-7-yl)-ethyl- β -hydroxyethylamine and di-(β -guanin-7-ylethyl)amine.

It had been shown (Lawley, 1957; Brookes & Lawley, 1961*b*) that after alkylation of deoxyguanylic acid the product was hydrolysed at neutral pH yielding 7-alkylguanine. In the present work alkylated DNA hydrolyses in a similar way. In the alkylation of DNA with labelled alkylating agents the specific radioactivity of DNA increased initially at the same rate as for RNA, but subsequently decreased, the lost radioactivity being found in the supernatant after precipitation of DNA. This radioactivity was shown to be associated with 7-alkylguanines and di-(guanin-7-yl) derivatives resulting from hydrolysis of the alkylated DNA.

In a more detailed study of the time course of the hydrolysis of DNA at pH 7 and 37° after its alkylation by 2-hydroxyethyl 2-chloroethyl [^{35}S]-sulphide (Fig. 1) the initial rate of hydrolysis was about the same as for the corresponding 7-alkyl-deoxyguanylic acid, but subsequently the rate decreased, the overall half-life being about 52 hr. After 300 hr. less than 10% of the alkyl groups remained attached to DNA. This residual alkylated DNA was hydrolysed and the products were identical with those initially found.

Comparison of the reaction with nucleic acids of mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide showed that the only difference was the absence of formation of di-(β -guanin-7-ylethyl) sulphide in the latter case. This was also the only difference observed for the reaction of the reagents with nucleic acids *in vivo*, although the difunctional agent was shown to be at least 30 times as effective as the monofunctional in inhibiting the growth of an ascites tumour.

In a comparison of these mustards with myleran, with regard to their binding to cellular constituents

Fig. 1. Hydrolysis in 0.01 *N*-phosphate buffer, pH 7.1, at 37°, of DNA alkylated with 2-hydroxyethyl 2-chloroethyl [^{35}S]sulphide (0.01 mole/mole of DNA P).

Table 2. Amount of reaction of ³⁵S-labelled mustard gas, 2-hydroxyethyl 2-chloroethyl [³⁵S]sulphide and ³H-labelled myleran with cellular constituents in the mouse

Type of cell	Reagent	Dose		Time (hr.)	Specific radioactivity ($\mu\text{C/g.}$)				Amount of reaction with nucleic acid ($\mu\text{moles/mole of nucleic acid P}$)	
					Protein					
		(mg./kg.)	($\mu\text{C/g.}$)		Cytoplasm	Nucleus	RNA	DNA		
Ascites	^{35}S -Labelled mustard gas	2	0.32	0.5	1.9	1.9	2.4	2.2	40	37
Ascites	2-Hydroxyethyl 2-chloroethyl [^{35}S]sulphide	4	0.63	1	2.9	2.6	4.0	3.6	75	70
Ascites	^3H -Labelled myleran	20	60	24	10.6	7.1	2.1	4.3	1.2	2.4
Ascites	^3H -Labelled myleran	20	60	72	5.6	4.0	2.2	4.2	1.2	2.3
Leukaemic	^3H -Labelled myleran	40	120	22	3.3	1.6	5.3	6.4	3.3	3.7
Leukaemic	^3H -Labelled myleran	40	120	66	1.9	1.7	4.0	4.0	2.2	2.2

Mice bearing the Ehrlich-ascites tumour or with virus-induced lymphoblastic leukaemia were injected intraperitoneally with the reagent in arachis oil and killed after the time stated. Cellular constituents were isolated from ascites or leukaemic cells.

Fig. 2. Proportion of combined mustard gas present as di-(β-guanin-7-ylethyl) sulphide in the reaction of nucleic acids with ³⁵S-labelled mustard gas: m-mole of mustard gas combined/mole of nucleic acid P after 15 min.: x, DNA, 1.6; +, DNA, 6.3; Δ, heat-denatured DNA, 1.4; ○, RNA, 2.9; ●, RNA, 16.

in vivo (Table 2), it was found that the mustards were bound to an equal extent, which was about 20 times that found for myleran given at a tenfold higher dose. A similar low extent of binding of myleran to leukaemic cells was found.

Reaction of difunctional alkylating agents with both RNA and DNA was shown to yield a proportion of di(guanin-7-yl) derivatives. Studies of the rate of formation of di-(β-guanin-7-ylethyl) sulphide (Fig. 2) showed that as expected it was less than that of the primary alkylation reaction. The proportion of combined mustard gas present as di-(β-guanin-7-ylethyl) sulphide was greatest for DNA, less for heat-denatured DNA and least for RNA (Fig. 2).

DISCUSSION

Comparisons of the rates and extents of alkylation of nucleic acids by a variety of alkylating agents have shown that in general with the mustard type of reagent a high extent of alkylation is attained, although the rates of reaction varied from half-lives of a few minutes to several hours (Table 1). On the other hand, the methanesulphonate, sulphate or halide type of reagent reacted to a much lower extent. This difference was also found when the binding to cellular constituents *in vivo* of mustard gas or of 2-hydroxyethyl 2-chloroethyl sulphide was compared with that of myleran (Table 2).

The finding reported by Brookes & Lawley (1960) that the sole site of alkylation of nucleic acids by mustard gas when the extent of reaction is

small, is at N-7 of guanine moieties, has now been confirmed for a number of alkylating agents. It has also been established that with the difunctional alkylating agents used products are formed by linkage of two guanine moieties by alkyl chains, e.g. (II).

Myleran, mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide were chosen for studies *in vivo* since they represent respectively weakly reactive difunctional, highly reactive difunctional and highly reactive monofunctional alkylating agents. In tests against the Ehrlich-ascites tumour in the mouse, myleran was inactive at a dose of 20 mg./kg./day (Sugiura, 1956), and the present work has shown that 2-hydroxyethyl 2-chloroethyl sulphide is inactive at a similar dose level, whereas mustard gas is active at one-thirtieth of this dose. The low anti-tumour activity of myleran is perhaps expected in view of the low level of binding to the cellular constituents found (Table 2). The present findings do not suggest any explanation of the effectiveness of myleran against chronic myelogenous leukaemia (Haddow & Timmis, 1953).

Consideration of the overall extent of binding to cellular constituents clearly cannot explain the marked difference in cytotoxic activity found for the mono- and di-functional sulphur mustards. The explanation must therefore be sought in differences in the mode of binding of these reagents. With nucleic acids both mustards react rapidly and to the same extent to give identical products, except that the difunctional reagent yields a proportion of a cross-linked guanine product. If the biological activity of these agents is to be ascribed to reaction with nucleic acid then the formation of this type of product must be of importance, and it is clearly of interest to attempt to determine the relative dispositions of the linked guanine moieties in the nucleic acid structure.

The model proposed by Crick & Watson (1954) for the structure of DNA shows that the N-7 atoms of guanine moieties are sterically well available for reaction. For a pair of these atoms to be situated at a distance apart such that they could be linked by an extended alkyl chain of 4 or 5 atoms, i.e. about 8 Å, it appears from this model that the sequence of bases along the DNA macromolecular chain must be guanine-cytosine along one strand (and by the necessary complementary pairing of bases, cytosine-guanine on the other strand), and in one direction only, namely deoxyguanylyl-(3' → 5')-deoxycytidylyl. With this sequence guanine moieties on each of the twin strands could be readily cross-linked, as represented diagrammatically in Fig. 3. If the order of the guanine-cytosine sequence is reversed the cross-linking of the guanine moieties is no longer possible. Linkage of adjacent guanine moieties on the same molecular strand of

DNA cannot be eliminated on steric grounds, but would require the alkyl chain to assume a less probable non-extended configuration.

Support for the model of cross-linking represented in Fig. 3 comes from consideration of the proportion of cross-linked guanine resulting from alkylation of DNA, denatured DNA and RNA (Fig. 2). Whereas DNA yields 26% of the total alkylation products in this form, the proportion becomes progressively less in passing to denatured DNA and RNA, in spite of the higher proportion of guanine in RNA. However, this is the order of decreasing extent of twin-stranded structure of the nucleic acids according to the Crick-Watson model, and decreasing yield of a product derived from such a structure would therefore be expected.

The greater cytotoxic effect of the difunctional alkylating agents could clearly be explained by accepting this model of cross-linking of the twin strands of DNA. According to the Crick-Watson model separation of these twin strands is necessary for cell division and this would be inhibited if the strands were joined through a covalent bond as proposed. Further, the loss of 7-alkylguanine from DNA, which has been shown to follow its alkylation, would lead to fission of the sugar phosphate chain (Brown & Todd, 1955), but breaking of the twin-stranded DNA molecule would be expected

Fig. 3. Diagrammatic representation of cross-linking by a difunctional alkylating agent of the twin strands of DNA according to the model of Crick & Watson. The order of bases along the molecular chain is shown by their initial letters; dotted lines represent the hydrogen bonds between the bases; the alkyl chain is indicated by the zig-zag line; the required sequence of bases is deoxyguanylyl-(3' → 5')-deoxycytidylyl along either chain.

only if alkylation on each strand at nearly opposite points had occurred. At low degrees of alkylation by monofunctional agents this would occur only rarely, being in proportion to the square of the degree of alkylation; with difunctional agents about a quarter of the alkylations would be of this type. It would also be expected that fission of the DNA molecule could seriously interfere with its biological function.

SUMMARY

1. The rates and extents of alkylation of ribonucleic acid and of deoxyribonucleic acid in neutral aqueous solution at 37° by a variety of alkylating agents have been determined.

2. Alkylation has been shown to occur at N-7 of guanine moieties, monofunctional agents yielding 7-alkylguanines, and difunctional agents yielding in addition di(guanin-7-yl) derivatives.

3. Alkylated ribonucleic acid is stable in neutral aqueous solution but alkylated deoxyribonucleic acid decomposes with loss of the alkylated guanine products.

4. The extent of binding has been determined of ³H-labelled myleran, ³⁵S-labelled mustard gas and of 2-hydroxyethyl 2-chloroethyl [³⁵S]sulphide to cellular constituents of the Ehrlich-ascites tumour and of ³H-labelled myleran to leukaemic cells in the mouse.

5. The only difference found *in vivo* or *in vitro* for reaction of mustard gas and 2-hydroxyethyl 2-chloroethyl sulphide with nucleic acids is that mustard gas yields di-(β-guanin-7-yl) sulphide.

6. Mustard gas is at least 30 times as effective as 2-hydroxyethyl 2-chloroethyl sulphide as an inhibitor of the growth of an ascites tumour in the mouse.

7. The mode of combination of monofunctional and of difunctional alkylating agents with nucleic acids is discussed in terms of the Crick-Watson model for the structure of deoxyribonucleic acid.

8. The possible relationship between the alkyl-

ation of deoxyribonucleic acid and the biological properties of the alkylating agents is discussed.

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REFERENCES

- Auerbach, C. & Robson, J. M. (1946). *Nature, Lond.*, **157**, 302.
 Brookes, P. & Lawley, P. D. (1960). *Biochem. J.* **77**, 478.
 Brookes, P. & Lawley, P. D. (1961*a*). *Biochem. J.* **78**, 2*r*.
 Brookes, P. & Lawley, P. D. (1961*b*). *J. chem. Soc.* (in the Press).
 Brown, D. M. & Todd, A. R. (1955). In *The Nucleic Acids*, vol. 1, p. 444. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
 Cox, J. D. & Warne, R. J. (1951). *J. chem. Soc.* p. 1893.
 Crick, F. H. C. & Watson, J. D. (1954). *Proc. Roy. Soc. A*, **223**, 80.
 Elmore, D. T., Gulland, J. M., Jordan, D. O. & Taylor, H. F. W. (1948). *Biochem. J.* **42**, 308.
 Fahmy, O. G. & Fahmy, M. J. (1956). *J. Genet.* **54**, 146.
 Fahmy, O. G. & Fahmy, M. J. (1961). *Genetics*, **46**, 446.
 Goldacre, R. J., Loveless, A. & Ross, W. C. J. (1949). *Nature, Lond.*, **163**, 667.
 Haddow, A., Kon, G. A. R. & Ross, W. C. J. (1948). *Nature, Lond.*, **162**, 824.
 Haddow, A. & Timmis, G. M. (1953). *Lancet*, **i**, 207.
 Lawley, P. D. (1957). *Proc. chem. Soc., Lond.*, p. 290.
 Loveless, A. & Ross, W. C. J. (1950). *Nature, Lond.*, **166**, 1113.
 Mann, K. M. & Nystrom, R. F. (1951). *J. Amer. chem. Soc.* **73**, 5894.
 Ross, W. C. J. (1953). *Advanc. Cancer Res.* **1**, 397.
 Sugiura, K. (1956). *Ann. N.Y. Acad. Sci.* **63**, 962.

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The Relative Stabilities of the Skeletal-Muscle Myosins of some Animals

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It is sometimes found that the stabilities of the members of a series of closely related proteins differ widely, examples being the haemoglobins and the collagens. It has been proposed (Haurowitz, Hardin & Dicks, 1954) that the relative

stabilities towards alkaline denaturation of the haemoglobins of various animals are due to different degrees of complementarity of the globin molecules to the haem group. Differences in the hydrothermal stability of collagens from certain animals