The Reaction of Mustard Gas with Nucleic Acids *in vitro* and *in vivo*

By P. BROOKES and P. D. LAWLEY

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

Mustard gas [di-(2-chloroethyl) sulphide] and analogous alkylating agents produce a wide range of biological effects (cf. review by Boyland, 1948). Some of these effects, such as induction of mutations (Auerbach & Robson, 1946) and interference with mitosis and chromosome breakage (Darlington & Koller, 1947), stimulated interest in the reactions of alkylating agents with deoxyribonucleic acid.

Previous studies of such reactions, mainly by physicochemical methods, have led to various suggestions regarding the chemical group or groups in nucleic acids involved. Chanutin & Gjessing (1946) showed by ultraviolet spectroscopy that the nitrogen mustards di-(2-chloroethyl)methylamine and tri-(2-chloroethyl)amine reacted with the bases of deoxyribonucleic acid, but the groups reacting were not specified. Elmore, Gulland, Jordan & Taylor (1948), on the basis of electrometric titration experiments, suggested that both basic and phosphate groups of deoxyribonucleic acid reacted with mustard gas. Press & Butler (1952) found that alkylation of deoxyribonucleic acid by di-(2-chloroethyl)methylamine in sodium bicarbonate solution resulted in a decrease of primary amino nitrogen and of purine nitrogen precipitable as purine silver salt, but no specific sites of reaction were established. Wheeler, Morrow & Skipper (1955) concluded from experiments with methylated xanthines and mustard gas that alkylation of ring nitrogen atoms of purines could occur. Alexander, Cousens & Stacey (1957) postulated that phosphotriesters were produced by alkylation of deoxyribonucleic acid in neutral aqueous solution by a variety of agents. This was based on observations that alkylation was not accompanied by liberation of acid. Reiner & Zamenhof (1957), from experiments on the binding of protamine by deoxyribonucleic acid and paper chromatography of hydrolysed alkylated deoxyribonucleic acid, claimed that whereas dimethyl sulphate reacted exclusively with the purine moieties at N-7, diethyl sulphate reacted exclusively with primary phosphate groups.

The only study of products formed by alkylation *in vivo* of nucleic acid is that by Wheeler & Skipper (1957). After administration of di-(2-chloroethyl)-[¹⁴C]methylamine to rats and mice isotopic labelling of both ribonucleic acid and deoxyribonucleic acid was found, and it was shown that this was associated with the purine fraction.

In most of the earlier chemical studies of alkylation of nucleic acids the conditions were very different from those which would occur *in vivo*, e.g. large concentrations of alkylating agents, and sometimes alkaline pH, were used. Further, the methods used were not such as to enable the sites of reaction to be specified. In view of this uncertainty and the fact that preliminary experiments (Lawley & Wallick, 1957; Lawley, 1957*a*, *b*) had indicated N-7 of guanine moieties to be the most reactive centre towards alkylation in nucleic acid, it was decided to reinvestigate this reaction with a typical alkylating agent. Mustard gas, di-(2-chloroethyl) [³⁵S]sulphide, was chosen since it is known to be one of the most reactive of the alkylating agents and also is obtainable at a high specific radioactivity. The reaction of mustard gas with nucleic acid under mild conditions has therefore been studied chemically and compared with that resulting when tobacco mosaic virus, protoplasts of *Bacillus megaterium*, and the Ehrlich ascites tumour in the mouse are treated with this reagent.

EXPERIMENTAL

Materials. ³⁵S-Labelled mustard gas (about 30 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Ribonucleic acid (RNA) from rat liver and from yeast was supplied by Dr K. S. Kirby, deoxyribonucleic acid (DNA) from calf thymus by Professor J. A. V. Butler, protoplasts of *B. megaterium* by Dr G. D. Hunter and tobacco mosaic virus by Dr H. Fraenkel-Conrat. The Ehrlich ascites tumour was grown in stock mice in this Institute.

Paper chromatography and radioautography. Whatman no. 1 paper was used with the following solvents: methanol–ethanol–conc. HCl–water (50:25:6:19, by vol.) (solvent 1); butan-1-ol–conc. NH₃ soln.–water (86:1:13, by vol.) (solvent 2); ethanol–conc. NH₃ soln.–water (80:2:18, by vol.) (solvent 3); solvents (1) and (3) were run upward-flow and solvent (2) downward-flow. In two-dimensional paper chromatography solvent (1) followed by either solvent (2) or solvent (3) was used. Radioautographs were prepared by placing these papers, after drying, in contact with X-ray film in a light-tight container for up to 4 weeks. The lower limit of detection in this time was about 1 mμc per radioactive spot.

Counting techniques. Solid samples of nucleic acid and protein were plated at 'infinite thickness' on 1 cm.² polythene planchets and counted with an end-window Geiger-Müller counter. The specific radioactivity of the samples was obtained by comparison with a ³⁵S-labelled standard of

known activity. Supernatants containing mustard gas and its hydrolysis products in aqueous ethanol were counted by adsorbing a known volume on to a weighed sample of charcoal which was then dried at room temperature under reduced pressure and plated in the usual way. When the amount of solid was insufficient for assay at 'infinite thickness', a sample of a solution of known concentration was evaporated on an aluminium planchet and counted at 'zero self-absorption' with a windowless gas-flow counter. Paper chromatograms of radioactive materials were assayed for radioactivity with a paper-strip counter incorporating a scintillation detector; in this apparatus $1 \mu mc$ gave 4.0 counts/sec. above background from an area of the paper of 6 cm. × 1 cm.

Reaction of mustard gas with guanosine. Guanosine (0·8 g., 2·8 m-moles) was dried at 100° and suspended in acetic acid (10 ml.). Mustard gas (0·55 ml., 4·6 m-moles) was added and the mixture heated at 100° for 2 hr. in a flask fitted with a drying-tube. After this time a clear solution resulted but on cooling a precipitate formed which was removed and shown to consist largely of unchanged guanosine. The filtrate was evaporated to dryness and the residue dissolved in N-HCl (15 ml.). After extraction of unchanged mustard gas with ether, this solution was heated at 100° for 1 hr., cooled and applied to a column of Dowex-50 (H⁺ form, 22 cm. × 3 cm.). The column was developed with N-HCl and 50 ml. fractions were collected automatically; the ultraviolet absorption of these fractions was measured at 260 mμ. Fractions 40–55 contained guanine and fractions 75–120 contained a product of which the sum of the ultraviolet absorption at 260 mμ amounted to 30% of the total. These fractions were evaporated and excess of HCl was removed by further evaporation with water. The residue was dissolved in water and the solution neutralized with conc. NH₃ soln. The resultant precipitate was recrystallized from hot water to yield 7-(β-hydroxy-ethylthioethyl)guanine monohydrate (I) as clusters of colourless needles, m.p. decomp. above 280°. (Found: C, 39·9; H, 5·5; N, 25·5; loss at 150°, 5·8. $C_9H_{13}O_2N_5S,H_2O$ requires C, 39·6; H, 5·5; N, 25·7; H₂O, 6·6%. After drying *in vacuo* at 150°; Found: C, 42·4; H, 4·8. $C_9H_{13}O_2N_5S$ requires C, 42·5; H, 4·7%). Ultraviolet spectra and R_F values are given in Table 1.

Subsequent elution of the column with 4 N-HCl gave a product (III) in about 5% yield which could not be crystallized but had similar ultraviolet spectra (Table 1).

7-(β-*Hydroxyethyl*[³⁵S]*thioethyl*)*guanine* was prepared in a similar experiment with ³⁵S-labelled mustard gas (200 mg., 6 mc). The specific radioactivity of the product was $16.5 \mu c/$ mg.

Reaction of 7-(β-hydroxyethylthioethyl)guanine with hydrogen peroxide. 7-(β-Hydroxyethylthioethyl)guanine (53 mg.) was dissolved in 0·25 N-HCl (1·3 ml.), 100 vol. H₂O₂ (0·2 ml.) added and the mixture was stood at room temp. for 2 hr. The solution was neutralized with conc. NH₃ soln. and the resultant precipitate recrystallized from water as clusters of colourless needles. Yield, 26 mg., m.p. decomp. above 265° (Found: C, 37·6; H, 4·8; N, 24·4; S, 10·6. $C_9H_{13}O_4N_5S$ requires C, 37·6; H, 4·5; N, 24·4; S, 11·1%).

Reaction of mustard gas with ribonucleic acid and deoxy-ribonucleic acid in vitro. RNA or DNA (150 mg.) was dissolved in mM-potassium acetate pH 7·2 (30 ml.) at 37° and ³⁵S-labelled mustard gas (46·7 μg., 1 m-mole/mole of nucleic acid P, in 0·01 ml. of ethanol) was added. After 1, 2,

3, 5 min. and 20 hr., samples (6 ml.) were taken and nucleic acid was precipitated with 2 vol. of ethanol containing 2% potassium acetate. The washed and dried nucleic acid and supernatant fluids were assayed for radioactivity as described above and radioautographs prepared from two-dimensional paper chromatograms.

In other experiments where larger amounts of mustard gas were used the concentration of acetate was 4 moles/mole of mustard gas.

Hydrolysis of nucleic acid samples. RNA was hydrolysed with N-HCl (Smith & Markham, 1950). DNA, and the mixed nucleic acids from *B. megaterium* were hydrolysed with formic acid (Wyatt, 1951) to obtain base ratios by ultraviolet spectroscopy; to obtain chromatograms for radioautography incomplete hydrolysis with N-HCl at 100° for 1 hr. was used.

Reaction of mustard gas with tobacco mosaic virus. Tobacco mosaic virus (47·5 mg.) was suspended in 0·1 mM-Na₄P₂O₇ buffer, pH 7·0 (4·75 ml.) at 37° and ³⁵S-labelled mustard gas (6 μg., 0·375 μc) in ethanol (0·01 ml.) was added. In a similar experiment 1·2 μg. of ³⁵S-labelled mustard gas was used. After 45 min. the suspension was centrifuged at 100 000g for 15 min., the tobacco mosaic virus washed by resuspension and recentrifuged. For isolation of RNA and protein the suspension of tobacco mosaic virus in water (1 ml.) was shaken with 90% (w/w) phenol (1 ml.) for 1 hr. and the layers separated by centrifuging at 20 000g. RNA was precipitated from the aqueous layer with ethanol (2 vol.) containing 2% (w/v) sodium acetate, and protein from the phenol layer with a mixture of methanol (5 vol.) and ether (10 vol.). RNA was assayed for radioactivity at 'zero self-absorption' and protein at 'infinite thickness'. Radioautographs were prepared from two-dimensional paper chromatograms of hydrolysed RNA.

Treatment of protoplasts of B. megaterium *with mustard gas.* B. megaterium (500 mg.) was converted into protoplasts, suspended in 'C-phosphate' (60 ml.) (Hunter, Brookes, Crathorn & Butler, 1959) and incubated at 30° for 1 hr. before addition of [³⁵S]mustard gas (6·7 mg., 0·9 mc) in ethanol (0·5 ml.). After a further incubation for 90 min. protoplasts were sedimented and homogenized in 2·5% (w/v) sodium salicylate (20 ml.). Nucleic acid and protein were isolated by the phenol method as for tobacco mosaic virus, above, and assayed for radioactivity. A sample of nucleic acid was hydrolysed with formic acid and a radioautograph prepared of a two-dimensional chromatogram.

Treatment of Ehrlich ascites tumour with mustard gas in vitro. Ehrlich ascites tumour cells were obtained from 7-day-old ascites tumours grown by transplantation in stock mice. The cells were sedimented and washed twice with 0·9% NaCl; 3×10^8 cells were suspended in Hank's medium (Hank & Wallace, 1949) (10 ml.) at 37° and ³⁵S-labelled mustard gas (0·2 mg., 36·2 μc) was added. Samples (2 ml.) were removed after 1, 5, 15, 30 and 60 min., diluted with ice-cold 0·9% NaCl (10 ml.), sedimented, washed twice with ethanol (2 ml.), resedimented, dried and assayed for radioactivity at 'infinite thickness'.

In a similar experiment $1·7 \times 10^9$ cells were suspended in Hank's medium (37 ml.) and treated with ³⁵S-labelled mustard gas (2·67 mg., 440 μc). RNA (23 mg.) was isolated by the method of Colter & Brown (1956) and assayed for radioactivity; a sample was hydrolysed and a radioautograph prepared in the usual way.

Treatment of Ehrlich ascites tumour with mustard gas in vivo. Five mice bearing 7-day-old Ehrlich ascites tumours were each injected with ^{35}S-labelled mustard gas (in one series $10\,\mu g.$, $1\cdot1\,\mu c$; in others $30\,\mu g.$, $2\cdot8\,\mu c$; $100\,\mu g.$, $16\,\mu c$) in arachis oil ($0\cdot1$ ml.). After 30 min. the mice were killed and RNA and protein were isolated from the ascites cells as described above. DNA was isolated from the nuclear material insoluble in $0\cdot14$M-NaCl by the method of Kirby (1959), omitting the ribonuclease treatment and the separation from polysaccharide. [The RNA had $7\cdot5\%$ of P; ϵ_P (extinction/mole of nucleic acid P) in $0\cdot01$N-sodium phosphate pH 7, 7000; molar base ratios, adenine, 1; guanine, $1\cdot89$; cytosine, $1\cdot52$; uracil, $1\cdot07$. DNA had $7\cdot5\%$ of P; ϵ_P in $0\cdot01$N-sodium phosphate pH 7, at $20°$, 6800; after 15 min. at $100°$, 8800; molar base ratios, adenine, 1; guanine, $0\cdot78$; cytosine, $0\cdot75$; thymine, $0\cdot92$; uracil, $0\cdot05$]. The presence of uracil in the DNA shows that RNA was present as an impurity to the extent of about 5%. This results from omission of ribonuclease treatment in order to avoid prolonged dialysis of the DNA with consequent loss of alkylated guanine. Samples of nucleic acid and protein were assayed for radioactivity and radioautographs were prepared in the usual way.

RESULTS

The reaction of mustard gas with both RNA and DNA with a low molar proportion of ^{35}S-labelled mustard gas to nucleic acid P at $37°$ and neutral pH was found to occur rapidly and extensively (Fig. 1). In the initial stages the rate of reaction was the same for both RNA and DNA but differences appeared at later times in that a large part of the radioactivity, initially bound to DNA, was released into the supernatant.

Radioautographs of two-dimensional paper chromatograms of hydrolysed [^{35}S]mustard gas-treated nucleic acids were very similar and showed three major radioactive spots (Fig. 2).

In experiments with a higher molar proportion of mustard gas ($0\cdot3$ mole/mole of nucleic acid P) these products were obtained in amounts sufficient for measurement of their ultraviolet-absorption spectra after elution from the paper. The spectra of (I), (II) and (III) (Fig. 2) were virtually identical and
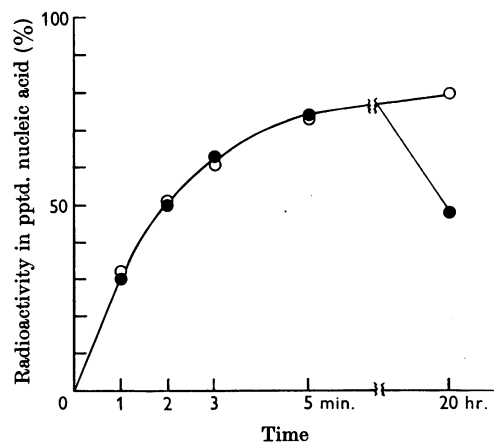

Fig. 1. Rate of reaction of ^{35}S-labelled mustard gas with RNA (yeast) and DNA (calf thymus). The reaction mixture contained 1 m-mole of mustard gas/mole of nucleic acid P in neutral aqueous solution at $37°$. $\bigcirc$, RNA; $\bullet$, DNA.
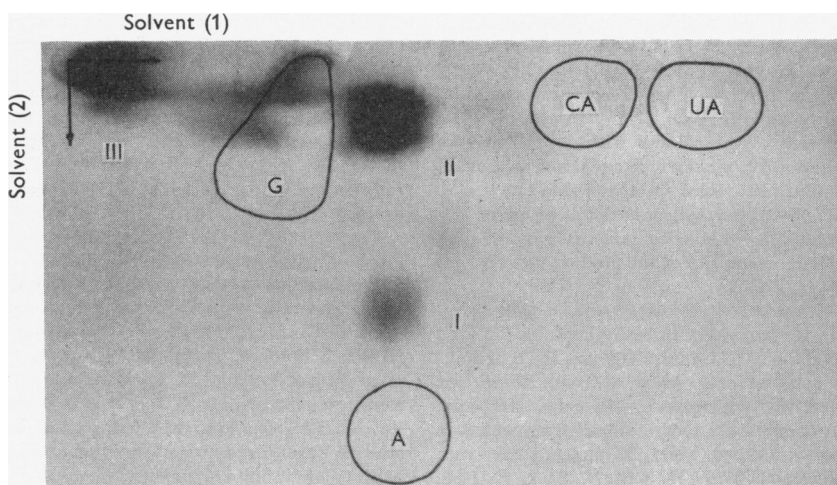

Fig. 2. Typical radioautograph of a two-dimensional paper chromatogram of hydrolysed RNA after reaction with ^{35}S-labelled mustard gas (1 m-mole/mole of RNA P). The positions of unchanged bases of RNA on the paper chromatogram, as shown, were determined by examination of the paper under ultraviolet light (λ, 2537 Å). A, Adenine; G, guanine; CA, cytidylic acid; UA, uridylic acid. Solvent (1), methanol:ethanol:HCl:water, (50:25:6:19, by vol.); solvent (2), butan-1-ol:conc. NH$_3$:water, (86:1:13, by vol.).

Table 1. R_F *values and ultraviolet-absorption spectra of products of alkylation*
of nucleic acid by mustard gas

Product	R_F			$\lambda_{max.}$ (mμ)		
	Solvent (1)	Solvent (2)	Solvent (3)	pH 1	pH 7	pH 12
(I)	0·37	0·20	0·45	250 (ϵ 11 400)	284 (ϵ 7400)	281 (ϵ 7300)
(II)	0·37	0·04	0·20	250	284	281
(III)	0·03	0·0	0·0	250	284	281
(IV)	0·14	0·04	0·20	250	284	281

closely resembled those of 7-methylguanine (Lawley, 1957a), (Table 1).

The reaction of guanosine and mustard gas was therefore investigated by column chromatography and yielded two products. The major product was purified and analysed for 7-(β-hydroxyethylthioethyl)guanine; the minor product, which could be eluted from the column only with concentrated acid, could not be purified but its behaviour on paper chromatography was identical with that of (III). The major product on paper chromatography ran mainly with R_F values identical with those of (I), but always gave a small proportion of a second component having the same R_F in solvent (1) but lower R_F in solvents (2) and (3). The R_F values of this second component were identical with those of (II) (Table 1), and further when (I) was eluted from the paper and re-run partial conversion into (II) was found. It appears, therefore, that (I) is 7-(β-hydroxyethylthioethyl)guanine and that (II) is a closely related substance formed from it during paper chromatography. When (II) was eluted from the paper and re-run it was partially converted into
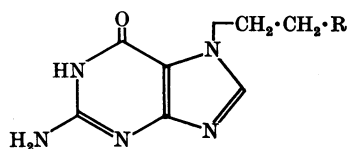
R = –S·CH$_2$·CH$_2$·OH (I);   –SO$_2$·CH$_2$·CH$_2$·OH (IV)

a product (IV) with lower R_F in solvent (1). A substance with the chromatographic properties of (IV), probably 7-(β-hydroxyethylsulphonylethyl)-guanine, was obtained when (I) was treated with hydrogen peroxide under mild conditions; the ultraviolet-absorption spectra of this substance were again identical with those of (I). It appears, therefore, that modification of the side chain of (I) occurs readily under mild conditions, such as those of paper chromatography, and the experiments with hydrogen peroxide suggest that oxidation may be involved. When very small amounts of these products were present on the paper chromatograms, as with experiments *in vivo*, the proportion of (II) relative to (I) was higher, with (II) sometimes predominating.

Table 2. *Specific radioactivity of ribonucleic acid and*
deoxyribonucleic acid

RNA (rat liver) and DNA (calf thymus) were treated with ^{35}S-labelled mustard gas (1 m-mole/mole of nucleic acid P) at pH 7, and 37° for the time stated, before isolation of nucleic acid by precipitation. Specific radioactivity of solid nucleic acid was obtained at 'infinite thickness', and was also calculated from strip-counting of radioactive spots on paper chromatograms of hydrolysed samples of nucleic acid.

	RNA	RNA	DNA	DNA
Time (min.)	1	15	1	15
Specific radioactivity of solid (μc/g.)	36	66	18	37
Specific radioactivity from paper chromatogram (μc/g.)	42	89	17	37

Since the products show a degree of instability, the effect of conditions used for hydrolysis of nucleic acids on 7-(β-hydroxyethyl[35S]thioethyl)-guanine was investigated. N-Hydrochloric acid at 100° for 1 hr. had no effect, but formic acid at 175° for 1 hr. caused partial decomposition of this compound with formation of guanine, indicated by paper chromatography, and of isotopically-labelled products which ran at the solvent front in solvent (1). These products are probably derived from the aliphatic side chain since mustard gas and its products of hydrolysis in aqueous solution behave similarly.

As shown in Fig. 1 the radioactivity of DNA treated with ^{35}S-labelled mustard gas rapidly reaches a maximum and then decreases slowly, and this is accompanied by a decrease in the viscosity of the solution. The radioactivity which is lost appears in the supernatant fluid when the DNA is precipitated, and paper chromatograms of the supernatant showed it to contain products (I), (II) and (III). When DNA was treated with 0·3 mole of mustard gas/mole of P a precipitate began to form after about 20 hr. This was isolated and paper chromatography showed it to be mainly (III) together with traces of (I) and (II).

The extent of the reaction between ^{35}S-labelled mustard gas and nucleic acid was estimated from the specific radioactivity of the isolated treated nucleic acid. It was also estimated by assaying the

31

Table 3. *Amount of reaction of* [35S]-*labelled mustard gas with tobacco mosaic virus B. megaterium protoplasts and Ehrlich ascites tumour in the mouse*

Tobacco mosaic virus and *B. megaterium* protoplasts were treated in buffer for the time stated. Ehrlich ascites tumour was treated by injection of tumour-bearing mice with [35S]-labelled mustard gas in arachis oil, the mice being killed after the time stated. The figure for dose given to mice is based on an average weight of 50 g./mouse. The figure for the amount of mustard gas that reacted with nucleic acid is based on an average P content for nucleic acid of 7·5%. Nucleic acid from *B. megaterium* was about 70% RNA and 30% DNA.

	Time (min.)	Dose of [35S]mustard gas (μc/g.)	Mustard gas found in whole virus or whole cells (% of dose)	Specific radioactivity (μc/g.)			Amount of mustard gas which reacted with nucleic acid (μmoles/mole of nucleic acid P)	
				Protein	RNA	DNA	RNA	DNA
Tobacco mosaic virus	45	7·9	37	2·8	4·9	—	248	—
Tobacco mosaic virus	45	1·5	47	0·7	0·8	—	40	—
B. megaterium	90	1750	1	22	31	—	700	—
Tumour	15	0·16	—	—	0·6	0·6	20	20
Tumour	30	0·32	—	1·9	2·4	2·2	40	37
Tumour	70	0·06	1·8	0·30	0·25	0·25	7	7
Tumour	30	0·02	1·2	0·09	0·08	0·09	2·4	2·7

radioactivity on a two-dimensional paper chromatogram of hydrolysed nucleic acid with a paper-strip counter. The amount of nucleic acid on the paper was determined by eluting the adenine spot and measuring its ultraviolet absorption. The estimates by the two methods were in good agreement (Table 2), thus confirming that the products on the paper chromatograms accounted for the whole of the reaction.

In order to ascertain the effect of excess of protein and other cellular constituents as competitors of nucleic acid in reaction with [35S]-labelled mustard gas the reaction with tobacco mosaic virus and *B. megaterium* was studied. In both cases the specific radioactivity of nucleic acid was greater than that of protein (Table 3). Radioautographs of paper chromatograms, obtained in the usual way, showed that the reaction with nucleic acid was of the characteristic type described above.

Since mustard gas is known to be an inhibitor of tumour growth (Bass & Freeman, 1946) it was of interest to study its reaction with tumour cells, and the Ehrlich ascites tumour was chosen since the cells show a high degree of uniformity and are readily available. A study *in vitro* showed that mustard gas was taken up rapidly by the whole cells, attaining a maximum of 12% of the given amount after 30 min., and that the isolated RNA had reacted in the characteristic way. In further experiments, mice bearing the tumour were injected with [35S]-labelled mustard gas and the RNA, DNA and protein were subsequently isolated from the tumour cells. The results were similar to those obtained *in vitro* except that a lower proportion of

the mustard gas administered was taken up by the tumour cells (Table 3). The specific radioactivities of the RNA and DNA were approximately equal. To avoid loss of alkylated guanine from DNA during its isolation, traces of RNA and polysaccharide were not removed since this would have involved prolonged dialysis. The analytical data for the nucleic acids showed that the amount of impurity was small.

DISCUSSION

The present work, which was designed to establish the nature of the reaction of a low concentration of mustard gas with nucleic acid, when only the most reactive centres would be attacked, has clearly established that alkylation of guanine is the only reaction occurring in detectable amount. The technique used enabled the reaction products to be identified when the extent of the reaction was about 30 μmoles of mustard gas reacting/mole of nucleic acid phosphorus. This level of reaction was that which occurred with the nucleic acid of the Ehrlich ascites tumour in the mouse after injection of 0·1 mg. of mustard gas.

Earlier work (Butler, Gilbert, James & Ross, 1951) had suggested that esterification of phosphate groups might be an important reaction of alkylating agents with nucleic acids. If this had occurred to any extent in the case of mustard gas, hydrolysis of partially esterified nucleic acid would be expected to result in the formation of simple hydrolysis products of mustard gas, but these were not found in the present investigation.

Alkylation of guanine moieties of nucleic acids

also accounts for the observed difference in behaviour between RNA and DNA after alkylation, namely that whereas alkylated RNA appears to be stable in neutral aqueous solution at 37°, alkylated DNA decomposes with loss of 7-alkylguanine, since it has been shown (Lawley, 1957a) that methylated deoxyguanylic acid hydrolyses at the deoxyriboside linkage under these conditions. On the other hand, esterification of phosphate groups of nucleic acids to give phosphotriester groups would be expected to result in destabilization of RNA but not of DNA, since it has been shown (Brown, Magrath & Todd, 1955) that dimethyl and dibenzyl uridine 3'-phosphate are unstable at pH 7 and 37°. This instability was attributed to the formation of an unstable intermediate by a cyclization involving the phosphorus atom and the oxygen of the vicinal hydroxyl of ribose. As the deoxyribose moiety of DNA has no free hydroxyl group such an intermediate could not be formed, and therefore phosphotriester groups in DNA would be expected to have the stability normally associated with a trialkyl phosphate (Brown et al. 1955).

The suggestion (Butler et al. 1951) that esterification of phosphate groups might occur on alkylation of nucleic acids by compounds of the mustard type appears to be supported by observations that alkylation at pH 7 occurs without liberation of acid (Stacey, Cobb, Cousens & Alexander, 1958). These latter authors stated that this evidence showed unambiguously that alkylation of DNA in vivo would be confined entirely to esterification. They appear not to have considered the fact that alkylation of ring nitrogen atoms to yield quaternary ammonium derivatives would result in the same experimental finding.

The experiments of Press & Butler (1952) showing that alkylation of nucleic acid by di-(2-chloroethyl)methylamine caused a decrease in the amount of purine nitrogen precipitable by silver and in purine amino nitrogen might appear to be in disagreement with the present findings that N-7 of guanine moieties is the site of alkylation. However, their experiments used alkaline conditions under which it is known that the product of alkylation at this site would be unstable (Lawley & Wallick, 1957), yielding a product non-precipitable by silver. Further, the excess of alkylating agent used would be expected to result in alkylation at less reactive sites, such as N-1 or N-3 of adenine (Brookes & Lawley, 1960), with consequent loss of primary amino nitrogen.

It has been suggested, on the basis of physicochemical evidence, that alkylation of DNA results initially in its partial aggregation (Elmore et al. 1948; Alexander & Stacey, 1958; Bendich, di-Mayorca, Rosoff & Rosenkranz, 1959), but with a subsequent decrease in its molecular weight (Con-

way, Gilbert & Butler, 1950). The present work does not establish chemical mechanisms which account for these observations but the product (III) has properties which are not inconsistent with its having resulted from linkage, by mustard gas, of two guanine moieties, which may have been derived from different molecular chains of DNA. Subsequent decrease in the molecular weight of alkylated DNA might follow the loss of 7-alkylguanine since a mechanism for the fission of the DNA molecular chain after such a loss of a purine moiety has been suggested (Brown & Todd 1955).

The studies reported here of the reaction of mustard gas with tobacco mosaic virus, B. megaterium and the Ehrlich ascites tumour have established that the presence of large excess of protein and other cellular constituents does not prevent the alkylation of nucleic acid, in agreement with similar findings in other systems (Butler, 1956). Further, the experiments with the Ehrlich ascites tumour in the mouse show that the same reaction occurs in vivo. The amount of ^{35}S-labelled mustard gas taken up by the tumour cells in vivo was small (Table 3) but as usual the specific radioactivity of nucleic acid was about the same as that of the protein or of the whole cells. The extent of reaction with tumour nucleic acid after the injection of tumour-bearing mice with 0·1 mg. of mustard gas/mouse can be expressed as about 1 molecule of mustard gas/molecule of DNA of molecular weight 6×10^6. This dose of mustard gas is of the order of that shown to inhibit growth of certain mouse tumours (Bass & Freeman, 1946). Tests of mustard gas against the Ehrlich ascites tumour do not appear to have been reported, but the nitrogen mustard di-(2-chloroethyl)methylamine is an effective inhibitor of growth of this tumour at a dose of 0·2 mg./kg. (Creech, 1958).

SUMMARY

1. A study has been made of the reaction of ^{35}S-labelled mustard gas with nucleic acid in neutral aqueous solution at 37° with a low concentration of mustard gas.

2. The only detectable reaction with both ribonucleic acid and deoxyribonucleic acid was at N-7 of guanine moieties, the hydrolysis products having chromatographic properties identical with those obtained from guanosine and mustard gas.

3. The principal product from nucleic acid and mustard gas, 7-(β-hydroxyethylthioethyl)guanine, has been synthesized.

4. Ribonucleic acid alkylated by mustard gas is stable in neutral aqueous solution but alkylated deoxyribonucleic acid decomposes with loss of 7-alkylguanine.

5. Tobacco mosaic virus, *Bacillus megaterium* and cells of the Ehrlich ascites tumour have been treated with ^{35}S-labelled mustard gas; the extent of reaction with nucleic acid has been determined and shown to be about the same as that with protein. The site of reaction with nucleic acid has been shown, as in the case of pure nucleic acid, to be at N-7 of guanine moieties.

6. Mice bearing the Ehrlich ascites tumour have been injected with ^{35}S-labelled mustard gas and the nucleic acid of the tumour cells shown to have reacted in the same way as *in vitro*.

7. Previous work on the reactions of alkylating agents with nucleic acids is discussed in relation to the present findings.

REFERENCES

Alexander, P., Cousens, S. F. & Stacey, K. A. (1957). In *Ciba Foundation Symp., Drug Resistance in Micro-organisms*, p. 294. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.

Alexander, P. & Stacey, K. A. (1958). *Ann. N.Y. Acad. Sci.* **68**, 1225.

Auerbach, C. & Robson, J. M. (1946). *Nature, Lond.*, **157**, 302.

Bass, A. D. & Freeman, M. L. H. (1946). *J. nat. Cancer Inst.* **7**, 171.

Bendich, A., diMayorca, G., Rosoff, M. & Rosenkranz, H. S. (1959). *Trans. Faraday Soc.* **55**, 491.

Boyland, E. (1948). *Symp. biochem. Soc.* **2**, 61.

Brookes, P. & Lawley, P. D. (1960). *J. chem. Soc.* p. 539.

Brown, D. M., Magrath, D. I. & Todd, A. R. (1955). *J. chem. Soc.* p. 4396.

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.

Butler, J. A. V. (1956). In *Ciba Foundation Symp., Ionizing Radiations and Cell Metabolism*, p. 67. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.

Butler, J. A. V., Gilbert, L., James, D. W. F. & Ross, W. C. J. (1951). *Nature, Lond.*, **168**, 985.

Chanutin, A. & Gjessing, E. C. (1946). *Cancer Res.* **6**, 599.

Colter, J. S. & Brown, R. A. (1956). *Science*, **124**, 1077.

Conway, B. E., Gilbert, L. & Butler, J. A. V. (1950). *J. chem. Soc.* p. 3421.

Creech, H. J. (1958). *Ann. N.Y. Acad. Sci.* **68**, 868.

Darlington, C. D. & Koller, P. C. (1947). *Heredity*, **1**, 187.

Elmore, D. T., Gulland, J. M., Jordan, D. O. & Taylor, H. F. W. (1948). *Biochem. J.* **42**, 308.

Hank, J. H. & Wallace, R. E. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 196.

Hunter, G. D., Brookes, P., Crathorn, A. R. & Butler, J. A. V. (1959). *Biochem. J.* **73**, 369.

Kirby, K. S. (1959). *Biochim. biophys. Acta*, **36**, 117.

Lawley, P. D. (1957a). *Proc. chem. Soc., Lond.*, p. 290.

Lawley, P. D. (1957b). *Biochim. biophys. Acta*, **26**, 450.

Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.

Press, E. M. & Butler, J. A. V. (1952). *J. chem. Soc.* p. 626.

Reiner, B. & Zamenhof, S. (1957). *J. biol. Chem.* **228**, 475.

Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.

Stacey, K. A., Cobb, M., Cousens, S. F. & Alexander, P. (1958). *Ann. N.Y. Acad. Sci.* **68**, 682.

Wheeler, G. P., Morrow, J. S. & Skipper, H. E. (1955). *Arch. Biochem. Biophys.* **57**, 133.

Wheeler, G. P. & Skipper, H. E. (1957). *Arch. Biochem. Biophys.* **72**, 465.

Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

The Simple Monosubstituted Guanidines of Mammalian Brain

By J. P. BLASS

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

Although observations have been made on individual monosubstituted guanidine derivatives of the brain, there appears to have been no systematic investigation of their total amount or number. Such a study has now been performed. Values for the concentration of total monosubstituted (Sakaguchi-positive) guanidines have been obtained, in guinea-pig, ox and rat brain; paper-chromatographic studies have confirmed the presence of arginine, glycocyamine (guanidoacetic acid) and γ-guanidobutyric acid, and have indicated the occurrence of taurocyamine (guanidoethane sulphonic acid). Approximate values for concentrations of the individual bases have also been obtained.