Prothidium caused gross loss of motility and clumping of all trypanosomes.

Comparison of the findings for trypanosomes with those for other cells previously studied shows that *T. lewisi* is very similar electrophoretically to freely circulating cells such as lymphocytes and tumour cells. On the other hand, the surface properties of the blood-stream form of *T. rhodesiense* are different electrophoretically from any other freely circulating cell so far studied. If the surface of these cells does contain carboxyl groups, the previous hypothesis that all cells carrying surface carboxyl groups will be adhesive to phagocytes will need modification (Bangham & Pethica, 1960). The absence of net charge on the freely-circulating blood-stream form of *T. rhodesiense* is evidence that van der Waals forces play a very minor role in cell adhesion (Pethica, 1961), although the flagellate motility may adversely influence the adhesion process.

## SUMMARY

1. Electrophoretic results are presented for *Trypanosoma rhodesiense* (blood and culture forms), and for the blood-stream forms of *T. congolense*, *T. equinum*, *T. lewisi* and *T. vivax*.

2. From a study of the electrophoretic behaviour of trypanosomes at various pH values in the presence and absence of $UO_2^{2+}$ ions, it is suggested that the surface of the culture form of *T. rhodesiense* and of the blood-stream form of *T. lewisi* is phosphatide in character, whereas that of the blood-stream form of *T. rhodesiense* may be of a polysaccharide or polypeptide nature.

3. Several trypanocidal drugs decreased the negative mobility of the blood form of *T. lewisi* and culture form of *T. rhodesiense* in a manner similar to that found with Ehrlich ascites-tumour cells; they had little effect on the electrophoretic behaviour of the blood form of *T. rhodesiense*.

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# Further Studies on the Alkylation of Nucleic Acids and their Constituent Nucleotides

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The principal biological effects of alkylating agents, namely cytotoxicity and mutagenesis, have stimulated interest in their action on nucleic acids, and particularly on DNA. This followed from early work such as that of Herriott (1948), who showed that viruses, particularly those containing DNA, were highly susceptible to inactivation by mustard gas [di-(2-chloroethyl) sulphide], and the demonstration by Auerbach & Robson (1946) that mustard gas was mutagenic.

Specificity of effect among alkylating agents falls mainly under two headings. First, the difunctional agents generally exert a more powerful cytotoxic action than the monofunctional, which may be related to the ability of difunctional agents to cross-link the twin strands of the macromolecular structure of DNA (Brookes & Lawley, 1961*a*). Secondly, significant differences in the biological action of the simpler monofunctional alkylating agents have been shown, particular attention

having been paid to agents introducing methyl or ethyl groups. For example, Kølmark (1956) found ethylation to be more effective than methylation in producing mutation at a locus in *Neurospora* resistant to most chemical mutagens, and diethyl sulphate was found to be markedly less toxic than dimethyl sulphate towards this organism. Loveless (1959), in comparing the effects of a number of alkylating agents on T2 bacteriophage *in vitro*, found that ethyl methanesulphonate was the sole efficient mutagen, and that its toxicity was lower than that of the corresponding methylating agent. With *Drosophila*, Fahmy & Fahmy (1961) found that, though methyl methanesulphonate gave a higher yield of mutants than ethyl methanesulphonate at the same dosage, the lower toxicity of the ethylating agent permitted higher doses of the latter to be used with consequent higher maximum mutational yield.

Various differences between the actions of methylating and ethylating agents on DNA have been postulated. Reiner & Zamenhof (1957) and Alexander, Lett & Parkins (1961) claimed that, though methylation of DNA occurred at N-7 of guanine moieties, ethylation of DNA was confined to esterification of phosphate groups. However, experiments with labelled methylating and ethylating agents (Brookes & Lawley, 1961*a*) showed that both reacted similarly to yield 7-alkylguanines. Bautz & Freese (1960) claimed that, though both methylation and ethylation of DNA occurred at guanine moieties, the subsequent fission of the deoxyriboside linkage of the alkylated deoxyguanosine moieties at neutral pH (Lawley, 1957*a*) was more rapid after ethylation than after methylation. This seemed unlikely in view of an approximate estimate of the rate of hydrolysis of 7-ethyldeoxyguanylic acid at pH 7 and 37° which was found to be about the same as that of 7-methyldeoxyguanylic acid (Brookes & Lawley, 1961*b*).

Marked differences between methylation and ethylation of nucleic acids with respect to either site of reaction or subsequent effects of alkylation seem unlikely *a priori*. Both reagents would be expected to attack the most reactive nucleophilic centres in the nucleic acid molecule, which have been shown experimentally to be N-7 of guanine moieties (Lawley & Wallick, 1957; Lawley, 1957*b*; Brookes & Lawley, 1960*a*, 1961*b*), in agreement with theoretical studies (Nakajima & Pullman, 1958; Pullman & Pullman, 1959). The subsequent destabilization of alkylated deoxyguanosine moieties would result principally from the quaternization of N-7 which would deplete the electrons in the guanine-ring system. Dependence on the nature of the alkyl substituent would then be expected to reflect differences in the inductive effects of the alkyl groups and to be relatively small.

Since previous studies of the alkylation of nucleic acids by a variety of agents had failed to suggest any reason for the known differences in biological effect, it was thought desirable to extend the work by a more detailed study of these reactions with particular regard to minor reaction products, and to differences in the stability of these products.

## EXPERIMENTAL

*Materials.* [$^{14}$C]Methyl methanesulphonate and [$^{14}$C]-ethyl methanesulphonate were obtained as described by Brookes & Lawley (1961*a*) at a specific radioactivity of 5 mc/m-mole, and kept in ether solution at a concentration of 10 mg./ml. at −20°. 9-Ethyl-7-methylguanine was kindly supplied by Dr C. L. Leese of this Institute. 7,9-Di-(2′-hydroxyethyl)guanine was obtained as described by Brookes & Lawley (1961*b*). RNA samples from yeast or rat liver were kindly supplied by Dr K. S. Kirby. Salmon-sperm DNA (highly polymerized) was obtained from Mann Research Laboratories Inc., New York. Calf-thymus DNA was kindly supplied by Professor J. A. V. Butler. DNA samples from *Escherichia coli* and T2 or T4 bacteriophages were prepared by the phenol method (Kirby, 1957). With *E. coli*, to disrupt the cells 1% (w/v) sodium dodecyl sulphate was included in the aqueous phase which was maintained at 60° for 10 min. before extraction with phenol. With the phages, ribonuclease treatment was omitted.

*Solvents for paper chromatography.* These were: (1) methanol–conc. HCl–water (7:2:1, by vol.); (2) butan-1-ol–aq. $NH_3$ (sp.gr. 0·88)–water (85:2:12, by vol.); (3) saturated aq. ammonium sulphate–propan-2-ol–0·1 M-phosphate, pH 7·2 (79:2:19, by vol.); (4) aq. 5% (w/v) $Na_2HPO_4$ adjusted to pH 8 saturated with 3-methylbutan-1-ol, both aqueous and non-aqueous phases being present in the tank; (5) butan-1-ol–water (43:7, by vol.); (6) isobutyric acid–water (5:3, by vol.) adjusted to pH 7 with aq. $NH_3$ (sp.gr. 0·88). Whatman no. 1 paper was used with upward flow for solvents (1), (3) and (4), and downward flow for solvents (2), (5) and (6).

*Radioactivity.* DNA was dissolved in water at a concentration of 0·5 mg./ml. and treated with 0·01 ml. of a solution of deoxyribonuclease (1 mg./ml.) at 37° for 10 min. A sample (0·2 ml.) of the resulting non-viscous solution was assayed in a Packard Tri-Carb liquid-scintillation counter giving an efficiency for $^{14}$C of approx. 80% and for $^{3}$H of approx. 10%. Paper-strip counting and radioautography were as described by Brookes & Lawley (1960*a*).

*Preparation of 7-methylguanosine.* Triacetylguanosine (890 mg.), prepared by the method of Bredereck (1947), was dissolved in methanol (30 ml.) and acetone (5 ml.), and treated at 0° with an etheral solution of diazomethane in ether (17 mg./ml.) until a permanent yellow colour signified the presence of excess of reagent. On standing overnight at 0° a precipitate (220 mg.) formed which recrystallized from methanol as prisms, m.p. 165°, of 7-methylguanosine hemihydrate (Found: C, 43·1; H, 5·9; N, 22·6. $C_{11}H_{15}N_5O_5,\frac{1}{2}H_2O$ requires C, 43·1; H, 5·2; N, 22·9%). This analysis was obtained after drying *in vacuo* at 100°.

*Preparation of 7-ethylguanosine.* By using ethereal diazoethane and the method as described above a precipitate was obtained which recrystallized from ethanol as prisms,

m.p. 320°, of 7-*ethylguanosine hemihydrate* (Found: C, 44·7; H, 5·5; N, 21·9. $C_{12}H_{17}N_5O_5,\frac{1}{2}H_2O$ requires C, 45·0; H, 5·5; N, 21·9%). A *picrate* recrystallized from ethanol as needles, m.p. 300° (Found: C, 40·5; H, 3·7. $C_{18}H_{20}N_8O_{12}$ requires C, 40·0; H, 3·7%).

*Preparation of diacetyldeoxyguanosine.* Deoxyguanosine (2 g.) was treated with acetic anhydride (15 ml.) and pyridine (18 ml.) at 100° for 16 hr. when all the solid had dissolved. The addition of light petroleum (b.p. 40–60°) gave a brown precipitate which after recrystallization twice from ethanol gave slightly brown plates (1·09 g.), m.p. 175°, but after a further three recrystallizations from ethanol colourless plates (118 mg.), m.p. 202°, were obtained (Found: C, 48·3; H, 4·9; N, 20·0. $C_{14}H_{17}N_5O_6$ requires C, 48·3; H, 5·0; N, 20·3%).

*Attempted methylation of diacetyldeoxyguanosine.* Diacetyldeoxyguanosine (500 mg.) was treated with excess of diazomethane as described above for triacetylguanosine. No precipitate formed on standing, and the addition of ether gave a hygroscopic solid. The ultraviolet-absorption spectrum of this material showed that it was not the required 7-methyldeoxyguanosine.

*Preparation of 1-methyldeoxycytidine.* Deoxycytidine hydrochloride (210 mg.) was converted into the free base by using Dowex 1 ($OH^-$ form), and the deoxycytidine was methylated as described for cytidine by Brookes & Lawley (1962), giving *1-methyldeoxycytidine methosulphate* in good yield, m.p. 143–144° (Found: C, 37·3; H, 5·2; N, 12·0. $C_{10}H_{16}N_3O_4,CH_3SO_4$ requires C, 37·4; H, 5·4; N, 11·9%).

*Alkylation of nucleic acids and identification of the products.* Nucleic acids at a concentration of 10 mM-nucleic acid phosphate were dissolved in 0·01 M-sodium phosphate buffer, pH 7·2, together with sufficient sodium acetate to maintain neutrality during the alkylation, and were treated at 37° with amounts of isotopically-labelled alkylating agents at concentrations generally in the range 1–10 mM. The times of treatment were: for 2-chloroethyl 2-hydroxyethyl [$^{35}$S]sulphide, 20 min.; for [$^{14}$C]methyl methanesulphonate, 4, 6 or 18 hr.; for [$^{14}$C]ethyl methanesulphonate, 18 hr. The alkylated nucleic acids were then precipitated, assayed for radioactivity, hydrolysed with acid, and paper chromatograms and radioautographs of the hydrolysates were obtained as described by Brookes & Lawley (1961*a*). The following methods of hydrolysis were used: to obtain purine bases from DNA, hydrolysis in N-HCl for 5 min. at 100°; to obtain purine bases and pyrimidine nucleotides from RNA, hydrolysis in N-HCl for 1 hr. at 100° (Smith & Markham, 1950); to obtain purine and pyrimidine bases from DNA, hydrolysis in formic acid (Wyatt, 1951) or perchloric acid (Marshak & Vogel, 1951); to obtain purine and pyrimidine bases from RNA, hydrolysis in perchloric acid. The radioactive products were identified by co-chromatography with the following authentic bases: 7-methylguanine and 7-ethylguanine (Brookes & Lawley, 1961*b*); 1-methyladenine, 3-methyladenine and 6-methylaminopurine (Brookes & Lawley, 1960*b*); 1-methylcytosine (Brookes & Lawley, 1962); 1-methyluracil (Brown, Hoerger & Mason, 1955). The relevant $R_F$ values are given in Table 1. The relative amounts of radioactive products on the paper chromatogram were determined with a paper-strip counter.

*Hydrolysis of alkylated nucleosides, nucleotides and nucleic acids in aqueous solutions: spectrophotometric methods.* Solutions of 7-methylguanosine, 7-ethylguanosine, 9-ethyl-7-methylguanine, 7,9-di-(2-hydroxyethyl)guanine, 1-methyldeoxycytidine or 1-methylcytidine in 0·1 M-sodium phosphate or bicarbonate buffers, or in HCl or NaOH solutions of the required pH, were prepared at room temperature, and the changes in absorption spectra with time of the solutions maintained at constant temperature were determined, either by a constant-temperature cell-housing in the Unicam SP. 500 spectrophotometer, or by withdrawing samples of the solutions and measuring their spectra at room temperature. Other alkylated nucleosides or nucleotides which could not be isolated as solids, namely the 7-methyl and 7-ethyl derivatives of deoxyguanosine and of deoxyguanylic acid, and the 1-alkyl derivatives of adenylic acid and of deoxyadenylic acid, were isolated by paper chromatography as previously described (Lawley, 1957*a*; Brookes & Lawley, 1960*a*, *b*), with solvent (3) for nucleotides and solvent (4) for nucleosides; the alkylation products were eluted from the paper at neutral pH and room temperature, and after adjustment of pH to the required value the changes in absorption spectra at constant temperature were determined. The rates of reaction were expressed as plots of $\log(E_t - E_\infty)$ against time, where $E_t$ denotes the extinction of a solution at a certain wavelength at time $t$, and 'infinite' time is greater than five times the half-life of the reaction, and these plots were found to be linear up to periods greater than the half-life. For very slow reactions, such as the conversion of 1-alkyladenylic acids at neutral pH and 37°, the 'infinity' reading was obtained by heating the solution for 3 hr. at 100°.

*Methods used with radioactive products.* The solution containing the radioactive alkylated product was maintained at constant temperature and portions were chromatographed on paper at various times, the radioactivity in the products being determined with a paper-strip counter. The following solvents were used: for alkylated DNA, solvent (6); for alkylated deoxyguanylic acid, solvent (4); for alkylated deoxyadenosine, solvents (4) and (5).

In general, to follow the rate of hydrolysis of alkylated DNA one-dimensional chromatography in solvent (6) was used, when the bases that had split off from the DNA ran

Table 1. *$R_F$ values of alkylated bases, relative to those of adenine*

The $R_F$ values of adenine were 0·37 in solvent (1), and 0·33 in solvent (2). Solvents (1) and (2) are defined in the text.

| | $R_{adenine}$ | |
|---|---|---|
| | Solvent (1) | Solvent (2) |
| 1-Methyladenine | 1·3 | 0·6 |
| 3-Methyladenine | 1·5 | 0·95 |
| 7-Methylguanine | 0·8 | 0·45 |
| 7-Ethylguanine | 1·0 | 1·0 |
| Suspected 1-ethyladenine | 1·5 | 1·2 |
| Suspected 3-ethyladenine | 1·8 | 1·5 |
| 6-Methylaminopurine | 1·4 | 1·9 |
| Suspected 3-(2-hydroxyethyl-thioethyl)adenine | 1·9 | 0·3 |
| 1-Methylcytosine | 1·6 | 1·0 |
| 1-Methylcytidylic acid | 2·2 | 0·0 |
| 5-Hydroxymethyl-1-methyl-cytosine | 1·8 | 0·6 |
| 1-Methyluracil | 2·4 | 1·8 |
| 1-Methyluridylic acid | 2·4 | 0·0 |

near the solvent front leaving the unhydrolysed DNA at the origin. To determine the nature and relative proportions of the alkylated bases hydrolysed from the DNA, two-dimensional chromatography was employed with solvent (6) followed by solvent (1). The first solvent separated the free alkylated bases [$R_F$ values in solvent (6): 3-methyladenine, 0·68; 7-methylguanine, 0·63; suspected 3-(2-hydroxyethylthioethyl)adenine, 0·95; 7-(2-hydroxyethylthioethyl)guanine, 0·80]; the acidity of the second solvent liberated the alkylated purines that had remained attached to the DNA at neutral pH, and also achieved their separation.

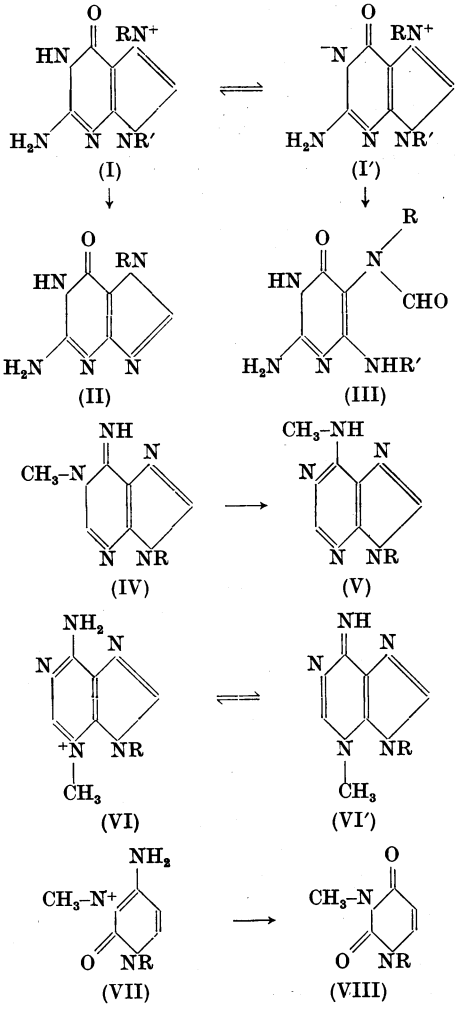
*Conversion of 1-methylcytidine and 1-methyldeoxycytidine in aqueous solution.* The methylated nucleoside methosulphates were dissolved separately in 0·02 M-potassium phosphate buffer, pH 7·4, or in aq. 1·5 N-ammonia, pH 10·9, at concentrations of 10 mg./ml. After 4 hr. and 1, 2, 3, 4, 7, 10, 13, 40 and 70 days at 37°, 0·025 ml. portions of the solution were chromatographed on paper with solvent (5), and ultraviolet-absorption spectra of the solutions after dilution to 0·1 mg./ml. were determined.

*Attempted isolation of principal minor methylation product from T2-phage deoxyribonucleic acid.* T2-phage DNA (100 mg.) was dissolved in 2·5 M-sodium acetate (25 ml.), and dimethyl sulphate (1·25 ml.) was added. The solution was shaken vigorously and kept at 37° for 16 hr., when a further 0·5 ml. of dimethyl sulphate was added. After a further 1 hr. at 37°, 0·1 vol. of conc. HCl was added, and the solution was heated at 100° for 15 min., then diluted to 100 ml. and applied to a column (15 cm. × 3 cm.) of Dowex 50 ($H^+$ form) previously washed neutral. The column was developed with 0·67 N-HCl (3250 ml.), N-HCl (2500 ml.) and finally 2 N-HCl. Fractions (50 ml.) were collected automatically and their extinctions measured at 260 and 280 mμ. No significant ultraviolet-absorbing material was eluted with 0·67 N- or 1·0 N-HCl, but 2 N-HCl eluted guanine in fractions 130–135 followed immediately (fractions 136–140) by a product having $\lambda_{max.}$ in acid at 273 mμ. Evaporation of fractions 136–140 gave a small amount of solid which was redissolved in water and chromatographed on paper, showing that guanine was present in these fractions in addition to the methylation product. The latter was separated from guanine by two-dimensional paper chromatography with solvents (1) and (2), eluted from the paper with 0·1 N-HCl and its absorption spectra determined at pH 1, 7·5 and 12. The $R_F$ values and absorption spectra of this product were identical with those of 3-methyladenine (Brookes & Lawley, 1960*b*).

*Methylation of 5-hydroxymethylcytosine.* 5-Hydroxymethylcytosine (1 m-mole) was treated with dimethyl sulphate (5 m-moles) in 2·5 M-sodium acetate (5 ml.) for 18 hr. at 37°. The resulting solution was applied to a column (15 cm. × 3 cm.) of Dowex 50 ($H^+$ form) and eluted with 0·67 N-HCl, 50 ml. fractions being collected. Fractions 36–53 contained a mixture of 5-hydroxymethylcytosine and a product that could not be resolved, although the later fractions were shown by their ultraviolet-absorption spectra to contain a higher proportion of the product. Fractions 47–53 were evaporated, and the product was redissolved in methanol and reprecipitated with ethyl acetate. Although insufficient was obtained for analysis, the product was sufficiently pure to run as a single spot on paper, and gave the following ultraviolet-absorption spectra: at pH 2, $\lambda_{max.}$ = 278 mμ, $\epsilon_{280\,m\mu}/\epsilon_{260\,m\mu}$ = 1·9; at pH 12, $\lambda_{max.}$ = 296 mμ, $\epsilon_{280\,m\mu}/\epsilon_{260\,m\mu}$ = 3·5; the variation of absorption spectra with pH corresponded to a value of $pK_a'$ of 7·1. These properties are closely similar to those found for 1-methylcytosine (Brookes & Lawley, 1962), and since the product was not identical in $R_F$ with 1-methylcytosine it was considered to be 5-hydroxymethyl-1-methylcytosine.

## RESULTS

Methylation of RNA with [$^{14}$C]methyl methanesulphonate followed by acid hydrolysis, liberating the purine bases and pyrimidine nucleotides, yielded four isotopically labelled products. The major product (60–70 % of the total) was 7-methylguanine (II, R = $CH_3$), and in addition 1-methyladenine (IV, R = H), 1-methylcytidylic acid [VII, R = ribose 2′(3′)-phosphate] and 3-methyladenine (VI, R = H) were identified. The proportions of the various products obtained are shown in Table 2. Similar methylation of DNA samples from various



sources with subsequent hydrolysis showed that, though the same products were obtained, the preponderance of 7-methylguanine was greater than that found with methylated RNA. 1-Methylcytosine (VII, R = H) was detected only at the higher levels of methylation, and the proportion of 1-methyladenine was low. However, the proportion of 3-methyladenine was increased, so that this became the major methylation product from adenine. Because of the similar $R_F$ values of 1-methylcytosine and 3-methyladenine particular attention was paid to the identification of the latter. It was shown to be liberated from methylated DNA by mild acid hydrolysis, which liberates purine bases but not pyrimidine bases. When formic acid hydrolysis or perchloric acid hydrolysis was used, at low levels of methylation no products were found additional to those liberated by hydrochloric acid hydrolysis, and co-chromatography of the hydrolysates with 1-methylcytosine (or with 5-hydroxymethyl-1-methyl-cytosine for the T2- or T4-phage DNA samples) showed the presence of detectable amounts of methylated cytosine in only one case, amounting to 1 % of the total methylation products. Methylation of T2-phage DNA with a large excess of dimethyl sulphate, followed by hydrochloric acid hydrolysis of the methylated DNA, enabled the chromatographic isolation of sufficient of the principal minor methylation product to measure its ultraviolet-absorption spectra, which were identical with those of 3-methyladenine (Brookes & Lawley, 1960*b*). Heat-denatured DNA gave on methylation the same methylated purines as did undenatured DNA, but in relative yields more similar to those found with RNA than with undenatured DNA, 1-methyladenine being the major adenine product.

Secondary changes in the methylated nucleic acids consequent on the initial alkylation were investigated by studies of the time-course of hydrolysis of the methylated nucleic acids in neutral aqueous solution at 37°. With methylated RNA two changes were found (Table 2): first, a slow loss of 1-methyladenine occurred accompanied by the appearance of a corresponding amount of a product identified as 6-methylaminopurine (V, R = H); secondly, an even slower loss of 1-methylcytosine, accompanied by the formation of a product identified as 1-methyluracil (VIII, R = H). With [$^{14}$C]methylated DNA a loss of radioactivity was found (Fig. 1), and two products that split off from the methylated DNA were identified as 3-methyladenine and 7-methylguanine, the rate of liberation of 3-methyladenine being the greater (Fig. 2). The slower transformation of the 1-methyladenine moieties in methylated denatured DNA to 6-methylaminopurine moieties was shown to occur, as with RNA (Table 2).

Table 2. *Relative yields of products from methylation of nucleic acids with* [$^{14}$C]*methyl methanesulphonate*

Nucleic acids were methylated at neutral pH and 37° for the times stated up to 18 hr., precipitated and hydrolysed as described in the text. For incubations longer than 18 hr. the methylated nucleic acids were redissolved in 0·02 M-sodium phosphate buffer, pH 7·4, and maintained at 37°, then reprecipitated and hydrolysed after the stated time. Denatured DNA was obtained by heating a solution of concentration 2 mg./ml. in 0·01 M-sodium acetate for 15 min., then rapidly cooling, before methylation. Deoxyguanosine and deoxyadenosine were treated together, each at a concentration of 20 mM in 0·05 M-sodium acetate with 4 mM-[$^{14}$C]methyl methanesulphonate and hydrolysed as for DNA.

| | RNA | | | | | | DNA | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Deoxyguanosine + deoxyadenosine | Yeast | | | | Rat liver | Salmon sperm | | Calf thymus | *E. coli* | T2-phage | T4-phage | Heat-denatured salmon | Heat-denatured sperm | |
| Extent of reaction (m-moles of [$^{14}$C]methyl/mole of nucleic acid phosphate) | — | 4 | 16 | 13 | 13 | 13 | 37 | 3 | 16 | 14 | 14 | 11 | 14 | 5 | 5 |
| Time of incubation (hr.) | 18 | 18 | 18 | 18 | 240 | 1056 | 18 | 18 | 18 | 18 | 18 | 4 | 18 | 18 | 560 |
| Method of hydrolysis | HCl | HCl | $HClO_4$ | $HClO_4$ | $HClO_4$ | $HClO_4$ | HCl | HCl | $HCO_2H$ | HCl | HCl | HCl | $HCO_2H$ | HCl | HCl |
| Relative yield: | | | | | | | | | | | | | | | |
| 7-Methylguanine | 69 | 61 | 64 | 64 | 61 | 63 | 71 | 86 | 86 | 86 | 91 | 78 | 80 | 63 | 21 |
| 1-Methyladenine | 29·5 | 23 | 21 | 22·5 | 12 | 3 | 16 | 5 | 2 | 4 | 1 | 2 | 3 | 31 | 65 |
| 1-Methylcytosine* | — | 16 | 14 | 12 | 9 | 7 | 12 | — | 1 | — | — | — | — | — | — |
| 3-Methyladenine | 1·5 | — | — | — | — | — | 1 | 9 | 11 | 10 | 8 | 20 | 17 | 6 | — |
| 6-Methylaminopurine | — | — | — | 1 | 15 | 20 | — | — | — | — | — | — | — | — | 14 |
| 1-Methyluracil* | — | — | — | 0·5 | 3 | 7 | — | — | — | — | — | — | — | — | — |

* Pyrimidine nucleotides were obtained in HCl hydrolysates of methylated RNA samples.

Similar studies on the hydrolysis of [$^{14}$C]-ethylated DNA showed that the loss of radioactivity was slower than from methylated DNA, whereas that from the 2-chloroethyl 2-hydroxyethyl [$^{35}$S]sulphide-treated DNA was more rapid than from methylated DNA (Fig. 1). Two products from hydrolysis of 2-chloroethyl 2-hydroxyethyl sulphide-treated DNA were found. The one that was released more slowly was identified as 7-(2-hydroxyethylthioethyl)guanine (Brookes & Lawley, 1960*a*), and the other had $R_F$ values identical with those of the minor alkylation product from deoxyadenosine and is therefore tentatively identified as 3-(2-hydroxyethylthioethyl)adenine. The specific radioactivities of [$^{14}$C]ethylated nucleic acids were not sufficient to permit detailed studies of the hydrolysis products, although their acid hydrolysates were shown to contain products other than 7-ethylguanine. In ethylated yeast RNA (2·5 ethyl groups/RNA phosphate group) 7-ethylguanine accounted for 85% of the products: an ethylated purine with $R_F$ values identical with those of the major product from the ethylation of adenosine was also present (10% of total), this being tentatively identified as 1-ethyladenine; and a third product with $R_F$ values similar to those of 1-methylcytidylic acid amounted to 5% of the total. In ethylated salmon-sperm DNA and ethylated T2-phage DNA the only minor product in measurable quantity was the suspected 3-ethyladenine, in amounts of 4 and 8% of the total ethylated bases. These yields may be compared with those resulting from ethylation of an equimolar mixture of deoxyguanosine and deoxyadenosine, which showed a similar pattern to the products of methylation, i.e. 72·5% of 7-ethylguanine, 26% of suspected 1-ethyladenine and 1·5% of suspected 3-ethyladenine.

The reactions found to follow from alkylation of nucleic acids were also shown to occur with the corresponding alkylated nucleosides or nucleotides, and the variation in the rates of these reactions with pH were studied in some detail.

The rate of conversion of 1-methyladenylic acid into the corresponding nucleotide of 6-methylaminopurine, a reaction that had been shown to occur under alkaline conditions (Brookes & Lawley, 1960*b*), was shown to be decreased by decreasing pH, but was still measurable under neutral conditions at 37° (Fig. 3). The analogous conversion of 1-methyldeoxyadenylic acid was also found, but there was no evidence of acid hydrolysis to yield 1-methyladenine at pH 7 and 37°. An analogous conversion at a similar rate was found for the major product from ethylation of deoxyadenylic acid. The yield of 3-methyldeoxyadenylic acid from methylation of the nucleotide was insufficient to permit similar studies of its hydrolysis.

A comparison of the rates of hydrolysis of 7-methyl- and 7-ethyl-deoxyguanosine and of 7-methyl- and 7-ethyl-deoxyguanylic acid at

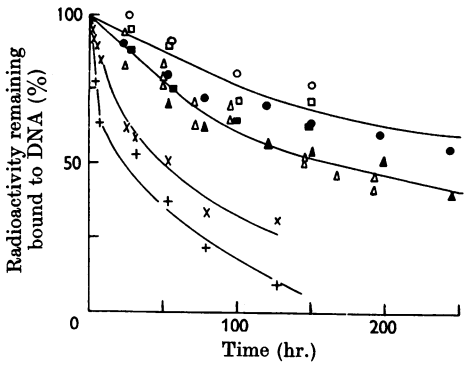



Fig. 1. Hydrolysis at pH 7 and 37° of [$^{14}$C]methylated or [$^{14}$C]ethylated DNA, and of DNA alkylated with 2-chloroethyl 2-hydroxyethyl [$^{35}$S]sulphide at 37°. Experimental details are given in the text. Source of DNA and extent of alkylation (m-moles of alkyl group/mole of nucleic acid phosphate): ○, ethylated T4-phage DNA (1·3); □, ethylated *E. coli* DNA (2·5); ●, ethylated salmon-sperm DNA (9); △, methylated T4-phage DNA (5); ■, methylated *E. coli* DNA (1·6); ▲, methylated salmon-sperm DNA (40); ×, (2-hydroxyethylthioethyl)ated T4-phage DNA at pH 7·15 (2·2); +, (2-hydroxyethylthioethyl)ated T4-phage DNA at pH 5·8 (1·5).

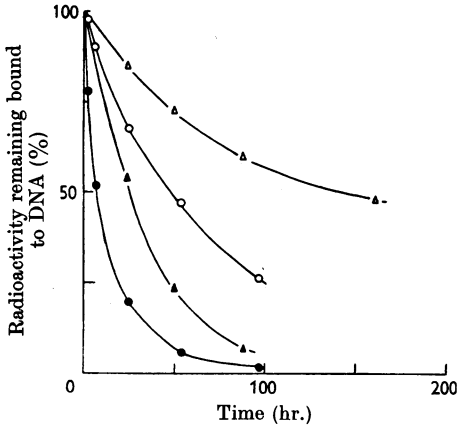



Fig. 2. Hydrolysis of T2-phage DNA alkylated with [$^{14}$C]methyl methanesulphonate for 6 hr. or 2-chloroethyl 2-hydroxyethyl [$^{35}$S]sulphide for 20 min. The alkylated DNA was dissolved in 0·05 M-sodium phosphate buffer, pH 7·2, at 37°, and the proportions of alkylated purines bound to and split off from the alkylated DNA were determined chromatographically at various times as described in the text. △, 7-Methylguanine; and ▲, 3-methyladenine; in overall ratio 17:3; ○, 7-(2-hydroxyethylthioethyl)guanine; and ●, suspected 3-(2-hydroxyethylthioethyl)adenine; in overall ratio 21:4.

neutral pH and over the range of pH 5–10 showed that the methylated nucleoside or nucleotide was less stable than the corresponding ethylated derivative under all conditions (Figs. 4 and 5). The spectroscopic method of determination of hydrolysis rates at pH 7 was compared, for the alkylated nucleotides, with a radioactive method, showing good agreement between the two methods (Fig. 4). The effect of variation in the nature of the 7- or 9-substituent on the stability of 7,9-disubstituted guanines was also studied (Table 3). Within the range of pH 5–10, two types of hydrolysis of 7-substituted deoxyguanosines or deoxyguanylic acids were found. At pH values below about 8·5 an acid-catalysed hydrolysis yielding 7-alkylguanines occurred, characterized by the change in absorption spectrum shown in Fig. 6. Above this pH an alkali-catalysed ring-fission occurred to yield substituted 5-alkylformamido-2,4-diamino-6-hydroxypyrimidines (III) with characteristic changes in absorption spectra as shown in Fig. 7. With 7-substituted guanosides the acid hydrolysis required much lower pH than for the deoxyguanosines, and within the range pH 5–10 the only detectable reaction was that of alkaline ring-fission. When both the 7- and 9-substituents were alkyl groups no acid hydrolysis was observed under the conditions studied, and more vigorous

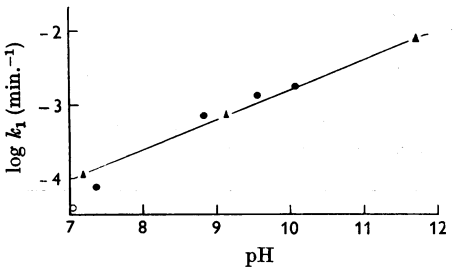



Fig. 3. Variation of rate of conversion of 1-alkyladenine nucleotides into 6-alkylaminopurine nucleotides with pH at 37°. Experimental details are given in the text. ●, 1-Methyldeoxyadenosine 5′-phosphate; ▲, 1-methyladenosine 5′-phosphate; ○, 1-ethyldeoxyadenosine 5′-phosphate.

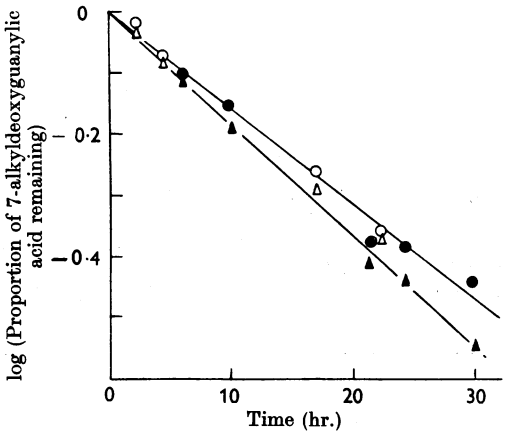



Fig. 4. Hydrolysis at pH 6·9 and 37° of 7-alkyldeoxyguanylic acids to yield 7-alkylguanines. Experimental details are given in the text. 7-Methyldeoxyguanylic acid: ▲, spectroscopically; △, by radioactive method (see the text). 7-Ethyldeoxyguanylic acid: ●, spectroscopically; ○, by radioactive method.

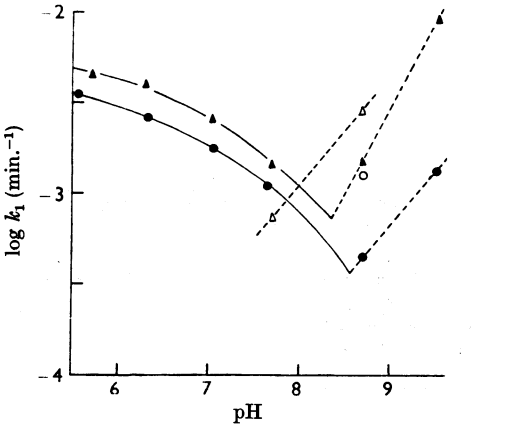



Fig. 5. Variation of rate of hydrolysis of alkylated guanine nucleosides with pH at 37°. Experimental details are given in the text. ▲, 7-Methyldeoxyguanosine; △, 7-methylguanosine; ●, 7-ethyldeoxyguanosine; ○, 7-ethylguanosine. Continuous lines denote acid hydrolysis to yield 7-alkylguanine; broken lines denote alkaline hydrolysis to yield substituted 5-alkylformamido-2,4-diamino-6-hydroxypyrimidines.

Table 3. *Rate of hydrolysis at* 37° *of 7-substituted guanine nucleosides or nucleotides* (I) *to yield the corresponding 7-substituted guanines* (II)

Experimental details are given in the text.

| R | R′ | pH | Half-life (hr.) | $10^3 k_1$ (min.$^{-1}$) |
|---|---|---|---|---|
| Methyl | Ribosyl | 0 | 7·1 | 1·6 |
| Ethyl | Ribosyl | 0 | 9·9 | 1·2 |
| Methyl | Deoxyribosyl 5′-phosphate | 6·9 | 16·4 | 0·7 |
| Ethyl | Deoxyribosyl 5′-phosphate | 6·9 | 18·9 | 0·6 |
| 2-Hydroxyethylthioethyl | Deoxyribosyl 5′-phosphate | 7 | 8 | 1·4 |

alkaline conditions were required to yield ring-fission than for the 7-alkylated nucleosides (Table 4).

The effect of change in temperature on the rate of hydrolysis of 7-methyl- and 7-ethyl-deoxyguanosine was studied at neutral pH by determination of the rate constants for these hydrolyses at 30° and 46·3°. This increase in temperature raised the value of first-order rate constant for 7-methylguanosine from $0{\cdot}98 \times 10^{-3}$ min.$^{-1}$ to $8{\cdot}0 \times 10^{-3}$ min.$^{-1}$, i.e. 8·2-fold, and for 7-ethylguanosine from $0{\cdot}6 \times 10^{-3}$ min.$^{-1}$ to $5{\cdot}3 \times 10^{-3}$ min.$^{-1}$, i.e. 8·8-fold. No significant difference in the effect of temperature due to difference in the alkyl substituent is apparent, therefore.

Study of the effects of variation in the 7- and 9-substituents on the p$K_a'$ values of 7,9-disubstituted guanines did not show any significant dependence on the nature of the substituents (Table 5). Spectrophotometric titration of 3-methyladenine over the range of pH 2–13 showed a single dissociation with a value of p$K_a'$ of 6·1.

To investigate the effect of methylation of deoxycytidine on its susceptibility to hydrolysis, the rates of change of absorption spectra of 1-methyldeoxycytidine and of deoxycytidine in N-hydrochloric acid at 100° were compared. In

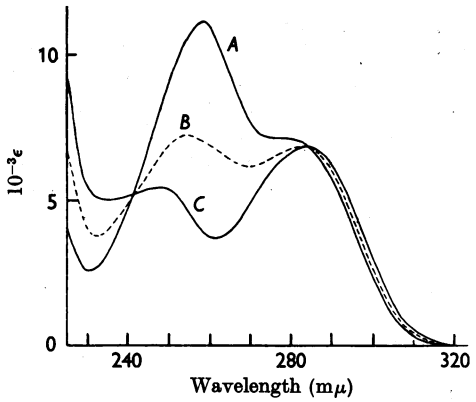



Fig. 6. Spectroscopic determination of the rate of acid hydrolysis of 7-methylguanosine to yield 7-methylguanine. A solution of 7-methylguanosine at a concentration of 0·82 mg./ml. in N-HCl was maintained at 37°, and after various times portions were diluted to give a concentration of 0·041 mg./ml. in 0·2M-sodium phosphate buffer, pH 7. Spectra at zero-time (*A*) and after 437 min. (*B*) and 2900 min. (*C*) are shown.

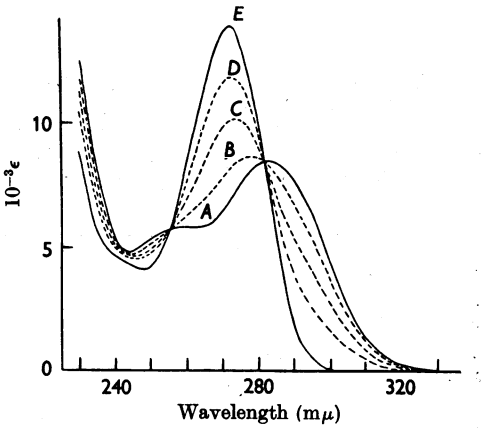



Fig. 7. Spectroscopic determination of the rate of alkaline ring-fission of 7-methylguanosine (0·032 mg./ml.) to yield 2-amino-6-hydroxy-5-methylformamido-4-ribosylaminopyrimidine at pH 8·7 and 37°. Spectra at zero-time (*A*) and after 25 min. (*B*), 74 min. (*C*), 148 min. (*D*) and 1120 min. (*E*) are shown.

Table 4. *Rate of alkaline ring-fission of* 7,9-*disubstituted guanines* (I) *to yield substituted* 5-*alkylformamido*-2,4-*diamino*-6-*hydroxypyrimidines* (III)

Experimental details are given in the text.

| R | R′ | Temp. | pH | Half-life (hr.) | $10^3 k_1$ (min.$^{-1}$) |
|---|---|---|---|---|---|
| Methyl | Ribosyl | 20° | 10·2 | 1·5 | 7·7 |
| Methyl | Ethyl | 37 | 12 | 37·5 | 0·3 |
| 2-Hydroxyethyl | 2-Hydroxyethyl | 20 | 12 | 2·3 | 5·0 |

Table 5. *Acidic dissociation constants of* 7,9-*disubstituted guanines* (I) *at* 20°

Values were determined spectroscopically with 0·1M-sodium phosphate buffers as solvent. Experimental details are given in the text.

| | | | At pH 3 | | At pH 9 | |
|---|---|---|---|---|---|---|
| R | R′ | p$K_a'$ | $\lambda_{max.}$ (mμ) | $10^{-3}\epsilon$ | $\lambda_{max.}$ (mμ) | $10^{-3}\epsilon$ |
| Methyl | Ethyl | 7·3 | 254, 281 | — | 251, 282 | — |
| 2-Hydroxyethyl | 2-Hydroxyethyl | 7·3 | 254, 281 | 10·9, 7·1 | 252, 283 | 5·6, 7·8 |
| Methyl | Ribosyl | 7·2 | 258 | 10·7 | 259, 282 | 5·8, 8·0 |
| Ethyl | Ribosyl | 7·2 | 257·5 | 12·0 | 259, 282 | 5·8, 7·8 |

both cases the absorption spectra changed to those of the corresponding pyrimidine bases at approximately equal rates with half-lives of about 0·3 hr.

1-Methyldeoxycytidine was, however, unstable in neutral aqueous solution at 37°, a slow change in the ultraviolet-absorption spectrum being observed, with a decrease in extinction and wavelength of the maximum. Paper chromatography of portions of solutions of 1-methyldeoxycytidine at pH 7·4 and at pH 10·9 after various times at 37° showed its conversion into a product of higher $R_F$ (0·57) than that of 1-methyldeoxycytidine ($R_F$ 0·15), and with $\lambda_{max.}$ = 262 mμ at pH 2, 7 or 12. The half-life of the conversion was about 400 hr. at pH 7·4 and 28 hr. at pH 10·9. A similar study with 1-methylcytidine at pH 7·4 and 37° showed that the product had $R_F$ values and absorption spectra at pH 2–12 identical with those of 1-methyluridine [$R_F$ 0·37 in solvent (5); $\lambda_{max.}$ at pH 2 or pH 12 = 262 mμ; $\epsilon_{280\,m\mu}/\epsilon_{260\,m\mu}$ = 0·34]. The half-life of the conversion was about 350 hr. At longer times, small amounts of an unidentified product ($\lambda_{max.}$ = approx. 350 mμ) in addition to 1-methyluridine were detectable. As with the conversion of 1-methyladenine moieties, the rate of conversion of 1-methylcytosine moieties in RNA was somewhat less than that for 1-methylcytidine at the same pH.

## DISCUSSION

Previous studies suggested that at low extents of reaction (0·001–0·1 m-mole/mole of nucleic acid phosphate) in the range which could result from alkylations *in vivo*, the sole site of alkylation of nucleic acids was at N-7 of guanine moieties. However, alkylation with $^{35}$S-labelled mustard gas of high specific radioactivity at levels of 0·1–10 m-moles/mole of nucleic acid phosphate gave radioautographs with weak but consistent spots not associated with 7-alkylguanines. The ready oxidation of the sulphur atom of the mustard gas side chain and the absence of authentic reference compounds made the identification of these minor products impracticable. With the preparation of [$^{14}$C]methyl methanesulphonate and [$^{14}$C]ethyl methanesulphonate at relatively high specific radioactivities it became possible to investigate minor alkylation products, by comparison with the expected methylated adenine and cytosine derivatives that had previously been prepared and characterized (Brookes & Lawley, 1960*b*, 1962).

From this previous work it was expected that the principal minor products from methylation of nucleic acids would be 1-methyladenine and 1-methylcytosine, together with smaller amounts of 3-methyladenine. This expectation was confirmed with RNA but not with undenatured DNA, where the yield of 3-methyladenine was much greater than anticipated from the low relative reactivity of N-3 in adenine nucleotides, whereas the yield of 1-methyladenine was low and 1-methylcytosine was detectable only at the higher extents of methylation used. The predominance of 7-methylguanine was greater in the methylation products from undenatured DNA than in those from RNA or denatured DNA. These differences in relative yields of alkylation products reflect the differences in macromolecular structure of the nucleic acids. In undenatured DNA the reactive groups of adenine and cytosine moieties at N-1 are involved in hydrogen-bonding according to the model of Watson & Crick (1953*a*), whereas N-3 of adenine and N-7 of guanine moieties are sterically available for reaction. The apparently enhanced reactivity of N-3 of adenine moieties in DNA suggests that the electronic distribution within the bases is altered when these are embodied in the DNA structure, a finding in agreement with the theoretical studies of Pullman & Pullman (1959). The observation that small amounts of 1-methyladenine are found in undenatured DNA methylated at low levels at which 1-methylcytosine was not detectable suggests that the hydrogen-bonding between adenine and thymine moieties may be weaker than that between guanine and cytosine. This interpretation parallels that of Litman (1961), who found that the amino groups of adenine moieties in undenatured DNA were more available for reaction with nitrous acid than were those of cytosine moieties.

Brookes & Lawley (1961*a*) have shown that, after alkylation of DNA, 7-alkylguanines split off at neutral pH. The influence of the nature of the alkyl group on this hydrolysis has now been studied in more detail. The rate of loss of alkylated bases decreased in passing from DNA alkylated with 2-chloroethyl 2-hydroxyethyl sulphide through methylated DNA to ethylated DNA (Fig. 1), and the same order of hydrolysis rates is found for the alkylated deoxyguanosines or deoxyguanylic acids (Table 3 and Fig. 5). This order is that expected if the increasing electron-donating power of the alkyl groups tends to oppose the destabilizing action of the withdrawal of electrons from the purine-ring system resulting from the quaternization of the ring nitrogen atom.

A similar influence of the substituents in the 7- and 9-positions on the ease of ring-fission by alkali of 7,9-disubstituted guanines is apparent (Fig. 5 and Table 4). This process is facilitated by the presence of hydroxy substituents in the alkyl groups, and again a methyl substituent is slightly more destabilizing than an ethyl substituent.

It was consistently found that the rates of hydrolysis at pH 7 decreased in the order: 7-alkyldeoxyguanosine, 7-alkyldeoxyguanylic acid, alkylated DNA. This suggests that the presence of a

negative charge associated with the deoxyribose substituent inhibits the hydrolysis.

Since it is known that both adenine and guanine deoxyribosides are readily hydrolysed under mild acid conditions, and that the guanine deoxyribosides are further destabilized by alkylation enabling their hydrolysis at neutral pH (Lawley, 1957*a*), it was desirable to investigate the stability of alkylated adenine deoxyribosides under neutral conditions.

The principal methylation product from deoxyadenylic acid, 1-methyldeoxyadenylic acid (IV, R = deoxyribosyl 5′-phosphate) was appreciably unstable at neutral pH. However, the conversion product was not 1-methyladenine, as expected by analogy with the alkylated deoxyguanosines, but 6-methylaminopurine deoxyriboside phosphate (V, R = deoxyribosyl 5′-phosphate), resulting from net migration of the methyl group from the ring nitrogen atom to the extranuclear amino group, as previously found to occur in alkaline conditions (Brookes & Lawley, 1960*b*). This same reaction proceeded at a slow rate at pH 5·6, but at pH 3·8 slow acid hydrolysis predominated. Similar results to those found with the 1-methyldeoxyadenylic acid were found with 1-methyladenylic acid over the range of pH 7–12. The conversion of 1-methyladenine moieties in methylated RNA and denatured DNA into 6-methylaminopurine moieties also occurred at neutral pH at rates somewhat less but of the same order as those found for the corresponding nucleotides. The corresponding conversion of 1-ethyldeoxyadenylic acid at pH 7 and 37° was somewhat slower than that of 1-methyldeoxyadenylic acid.

Since it was shown that alkylation of DNA occurred to a considerable extent at N-3 of adenine moieties it was decided to investigate whether this reaction would have an effect on the stability of the deoxyriboside bond. Owing to the low reactivity of this position in the free nucleoside it was not possible to isolate sufficient 3-methyldeoxyadenosine for detailed studies. However, it was shown that 3-methyladenine was split off from methylated DNA at neutral pH, and that the rate of this hydrolysis was more rapid than that of 7-methyldeoxyguanosine moieties, the ratio of the half-lives of these reactions being 1:6. An analogous hydrolysis was found with DNA alkylated with 2-chloroethyl 2-hydroxyethyl sulphide, and here again the ratio of the half-lives of the splitting off of the 3-alkyladenine to that of the corresponding 7-alkylguanine was 1:6.

The time-courses of the splitting off of the 3-alkyladenines and 7-alkylguanines determined individually approximated to first-order kinetics. The curves previously reported for the loss of alkylated bases from DNA which showed deviation from such kinetics (Brookes & Lawley, 1961*a*) can therefore be explained as the summation of the two reactions of different rate.

No evidence was found that methylation of deoxycytidine at N-1 caused any increased lability of the deoxyriboside bond to acid hydrolysis. However, instability of both 1-methylcytidine (VII, R = ribosyl) and 1-methyldeoxycytidine (VII, R = deoxyribosyl) in neutral aqueous solution was found, and it was shown for 1-methylcytidine that this conversion at neutral pH was the same as that previously found in alkali (Brookes & Lawley, 1962) yielding 1-methyluridine (VIII, R = ribosyl). The close similarity in the kinetics and nature of the product from 1-methyldeoxycytidine indicates the analogous conversion into 1-methyldeoxyuridine (VIII, R = deoxyribosyl). Slow conversion of 1-methylcytosine moieties in methylated RNA into 1-methyluracil moieties was also shown.

In addition to processes involving fission on rearrangement of covalent bonds, immediate effects of alkylation of nucleic acids on the secondary structure of the macromolecules can be envisaged. In the Watson & Crick (1953*a*) model for DNA, hydrogen bonds between adenine and thymine bases and between guanine and cytosine bases are important for the specificity of the structure in that they establish the complementarity of the twin strands. In the scheme for transmission of genetic information in terms of this model (Watson & Crick, 1953*b*) it was proposed that mutation could result if a base occurred in one of its less likely tautomeric forms, thus causing it to pair with an anomalous base. It has been pointed out that changes in the state of ionization of guanine or thymine moieties could also affect the specificity of base-pairing (Lawley & Brookes, 1961, 1962). In particular, the enhanced acidic ionization of 7-alkyldeoxyguanosine at pH 7 compared with the low extent of this ionization of deoxyguanosine could increase the probability of anomalous base-pairing between alkylated guanine in DNA and thymine (Lawley & Brookes, 1961).

In this connexion, values of $pK'_a$ for the acidic dissociation of some 7,9-disubstituted guanines were determined, and these were found to be 2·0–2·1 units lower than the value of 9·2 for guanosine. This increased tendency to acidic dissociation showed negligibly small dependence on the nature of the substituents, in agreement with the concept that it results from quaternization of a ring nitrogen atom on the imidazole ring, and is not influenced to an appreciable extent by the inductive effect of the substituents.

The finding that an appreciable proportion of the alkyl groups is attached to adenine moieties at N-3 after alkylation of DNA even at low extents of alkylation raises the question whether the base-

pairing properties of adenine would be affected by alkylation. Spectrophotometric titration of 3-methyladenine in aqueous solution showed one $pK'_a$ value of 6·1, indicating that this compound would exist mainly as an uncharged base at neutral pH. No acidic dissociation was found up to pH 13. This confirms the similar finding of Leonard & Deyrup (1962), who found a single $pK'_a$ value of 5·3 for 3-methyladenine in aq. 50% (v/v) dimethylformamide. These authors suggested that the lack of an acidic $pK'_a$ indicates that the proton dissociates from the imidazole ring of the 3-methyladenine cation.

This cannot occur for a 3-alkyladenine moiety in alkylated DNA since N-9 is substituted by a sugar moiety, and the corresponding dissociation would necessarily involve loss of a proton from the extranuclear amino group. The $pK'_a$ of such a group in DNA is not known, but if it were similar to that of 3-methyladenine it would exist at pH 7 largely in the imino form (VI'). This might be expected to weaken the hydrogen bond linking the extranuclear nitrogen atom of the alkylated adenine moiety and the oxygen atom attached to position 6 of thymine according to the Watson–Crick model. However, there appears to be no reason why this ionization should facilitate the anomalous base-pairing of the alkylated adenine with cytosine. This contrasts with the case of alkylated guanine moieties in DNA where the increased ionization of the proton from N-1 would permit the formation of two hydrogen bonds with thymine, thus facilitating anomalous base-pairing (Lawley & Brookes, 1961). It appears much more likely therefore that mutagenesis due to differences in the ionization of bases in DNA resulting from alkylation would be of the type involving conversion of a guanine–cytosine base-pair into an adenine–thymine base-pair, rather than the opposite.

The loss of alkylated bases from DNA could also be envisaged as a possible factor in mutagenesis. The duplication, according to the scheme of Watson & Crick (1953*b*), of DNA in which the sugar–phosphate chain was intact but a basic moiety was missing could occur in two ways. First, the position in the complementary strand opposite to the gap might be filled at random. Alternatively, the effect of loss of a basic moiety from a DNA strand acting as a template for further DNA synthesis could be envisaged as a deletion of the complementary base in the newly formed strand. The model for a possible mutagenic effect due to the deletion would then be that discussed by Brenner, Barnett, Crick & Orgel (1961) as a possible mechanism for the mutagenic action of proflavin. The base deleted as a result of alkylation could be either guanine, after its alkylation at N-7, or adenine, after its alkylation at N-3, the loss of the latter occurring more rapidly after alkylation but being less in overall extent.

Since deletions of this type are expected to cause alteration of a whole polypeptide chain corresponding to the altered gene (Crick, Barnett, Brenner & Watts-Tobin, 1961), whereas the mutation due to anomalous base-pairing would lead to alteration of a single amino acid only, and since, furthermore, a large number of base deletions from a DNA molecule might be expected to lead ultimately to fission of the molecule, it seems possible that purine deletion would be more likely to be a lethal process than the initial alkylation process, the biological expression of the latter being more likely to be mutagenesis. Such considerations would agree with observations that inactivation of bacteriophage by monofunctional alkylating agents generally increases when the alkylated bacteriophage is left in suspension after alkylation (Loveless, 1959). They are also in qualitative agreement with the generally observed higher toxicity of methylating agents as compared with ethylating agents, whereas the latter have higher mutagenic power, since it has been found that purine deletion occurs more rapidly after methylation of DNA than after ethylation. The relative amount of the more readily hydrolysed 3-alkyladenine was also found to be somewhat less after ethylation of DNA than after methylation.

Alkylation of DNA at N-1 of adenine and cytosine would prevent the hydrogen-bonding of these bases in DNA required by the Watson–Crick model, and therefore lead to interference with the specificity of base-pairing. However, the extent of reaction at these atoms in DNA is small, that of cytosine being detectable only at relatively high extents of alkylation.

However, 1-methyladenine has been found in small amounts in mammalian RNA and yeast RNA (Dunn, 1961). Since the present work has shown the instability of the 1-methyladenosine moiety in RNA under neutral conditions, it is indicated that, *in vivo*, these moieties would be slowly converted into 6-methylaminopurine moieties.

## SUMMARY

1. Sites in the nucleic acids reactive towards alkylating agents are shown to be, in order of decreasing reactivity: for RNA, N-7 of guanine, N-1 of adenine, N-1 of cytosine and N-3 of adenine; for DNA, N-7 of guanine, N-3 of adenine, N-1 of adenine and N-1 of cytosine. Denatured DNA behaves in this respect like RNA.

2. The observed differences between DNA and RNA are ascribed to the involvement of N-1 of adenine and of cytosine in hydrogen-bond formation in DNA.

3. In all cases alkylation results in destabilization of the nucleosides or the corresponding moieties in the nucleic acids. At neutral pH, with DNA, 7-alkylguanines and 3-alkyladenines are slowly liberated by hydrolysis, the latter at the greater rate, whereas with RNA slow rearrangements occur, 1-alkyladenine moieties yielding 6-methylaminopurine moieties and 1-alkylcytosines giving the corresponding 1-alkyluracils.

4. The influences of the nature of the 7- and 9-substituents on the rate of hydrolysis or ring-fission of 7,9-disubstituted guanines in the range of pH 5–10 was studied and their $pK'_a$ values were determined.

5. Possible correlations between these reactions and the biological properties of the alkylating agents are discussed.

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# Effects of Alkylating Agents on T2 and T4 Bacteriophages

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Loveless (1959*a*) studied the inactivation of phage T2 by alkylating agents and showed that, with bifunctional agents, inactivation required the reaction of both functional groups. With monofunctional agents inactivation appeared to be due to a dose-dependent and acid-catalysed breakdown of the alkylated phage particles. Studies on the inactivation of tobacco-mosaic virus by alkylating agents (Fraenkel-Conrat, 1961) showed that with several reagents the introduction of one to three alkyl residues/virus RNA molecule caused 73% inactivation. For both phage and virus it was considered that inactivation resulted from alkylation of the nucleic acid component rather than of the protein.

Brookes & Lawley (1961) have shown that alkylation of nucleic acid occurred at N-7 of guanine moieties, monofunctional agents yielding