Biochem. J. (1975) **145**, 73–84
Printed in Great Britain

Isolation and Identification of Products from Alkylation of Nucleic Acids: Ethyl- and Isopropyl-purines

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(*Received* 13 *June* 1974)

Ethylation and isopropylation of guanine in alkaline solution, or of adenine in formic acid, by alkyl methanesulphonates gave the following products: 1-, N^2-, 3-, O^6-, 7- and 9-alkylguanines; 1-, 3-, 7- and 9-alkyladenines. The products were identified from their characteristic u.v-absorption spectra, by comparison with either known ethyladenines or with the corresponding known methyladenines, and were also characterized by mass spectrometry. Their chromatographic properties on paper, t.l.c. and various columns were determined. DNA was alkylated in neutral solution with ^{14}C-labelled alkyl methanesulphonates and the ratios of the alkylpurines formed were obtained, and compared for alkylation by methyl, ethyl and isopropyl methanesulphonates and by *N*-methyl-*N*-nitrosourea. The extents of alkylation at O-6 of guanine relative to those at N-7 of guanine varied with the reactivity of the methylating agents according to the predictions of Swain & Scott (1953) relating nucleophilicity of the groups alkylated with the substrate constants of the alkylating agents. The relative extents of alkylation at N-3 of adenine did not follow this correlation.

Within a series of methylating agents, carcinogenic potency and ability to induce mutations by the transition mechanism have been positively correlated with S_N1 reactivity (reviewed by Lawley, 1972), or negatively correlated with the value of the Swain–Scott (1953) substrate constant *s* (Osterman-Golkar *et al.*, 1970; Veleminsky *et al.*, 1970; Ehrenberg, 1971) of the alkylating agents. The constant *s* is a measure of the sensitivity of the alkylating agent to the strength of the nucleophile with which it reacts, so that reagents of lower *s* are expected to discriminate less, i.e. to react relatively more extensively with less nucleophilic centres. In nucleic acids, likely important cellular targets of these agents, O-atom sites are expected to be less nucleophilic than ring N atoms. In agreement with these concepts, it has been found that methylating agents of lower *s* react relatively more at O-atom sites, including O-6 of guanine (Loveless, 1969; Lawley & Thatcher, 1970; Lawley & Shah, 1972*a*,*b*, 1973) and the phosphodiester groups of DNA (Bannon & Verly, 1972; Lawley, 1973) or of RNA (Shooter *et al.*, 1974*a*). With regard to the ability of these agents to cause transition mutations by alkylation of purine residues in DNA, miscoding by O^6-methylguanine in the DNA template (Loveless & Hampton, 1969) seems the most likely mechanism [cf. evidence for miscoding by this base in a polyribonucleotide template *in vitro* (Gerchman & Ludlum, 1973)].

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The variation of chemical reactivity with structure in the methylating agents examined follows from the nature of the leaving group, i.e. that part of the alkylating agent which acquires a negative charge during the alkylation reaction. Thus dimethyl sulphate and methyl methanesulphonate ($s = 0.83$) are regarded as typical S_N2 alkylating agents, whereas *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, which methylate through the highly reactive methyldiazonium ion (Lawley & Shah, 1973; Lawley & Thatcher, 1970), may be classified as tending to S_N1 reactivity, in both cases the derived value of *s* being 0.42 (Veleminsky *et al.*, 1970; Osterman-Golkar, 1974).

Further, chemical reactivity through a homologous series of alkylating agents containing the same leaving group will vary with the nature of the alkyl group. Thus S_N1 character increases through the series methyl < ethyl < isopropyl (Ingold, 1970), and the values of *s* derived for the corresponding methanesulphonates decrease from 0.83 for methyl, through 0.67 for ethyl, to 0.29 for isopropyl methanesulphonate. Two principal factors may be invoked to account for this variation, namely the inductive or hyperconjugative effect of methyl substituents at the same C atom, and increasing steric hindrance to the approach of an attacking nucleophile at the methyl-substituted C atom.

The principal effects of reactivity of methylating agents on relative extents of methylation of various sites in nucleic acids have been investigated in some detail (reviewed by Lawley, 1972) and may be

summarized as follows. The most reactive site is at N-7 of guanine, but relative reactivities at both other ring N atoms, and at extranuclear O-atom sites and at the phosphodiester group, show dependence on the nature of the methylating agent. Notably, in DNA, the ratio of extent of methylation at O-6 of guanine to that at N-7 of guanine was 0.004 for methyl methanesulphonate (here taken as typical of S_N2 agents), whereas for *N*-methyl-*N*-nitrosourea (here denoted as an S_N1 agent) this ratio was 0.1 (Lawley & Shah, 1972*a*). Conversely, the reactivity of the N-3 atom of adenine was somewhat less towards the S_N1 agent than towards the S_N2 agent. With RNA, a similar pattern emerged, with O-6 of guanine relatively more reactive towards the S_N1 agent, and N-1 of adenine and N-3 of cytosine less reactive. Clearly the relative reactivities of O-6 and N-7 of guanine appeared to conform with the Swain–Scott (1953) correlations, i.e. that the group of higher nucleophilicity (denoted *n* by Swain & Scott, 1953), in this case N-7 of guanine, should be relatively more reactive towards the alkylating agent of higher *s* factor, here methyl methanesulphonate. However, the relative reactivities of the other ring-N-atom sites mentioned did not appear to agree with the Swain–Scott (1953) concepts, since the agents of lower *s* appeared to be more, rather than as predicted, less, selective towards these sites of minor reactivity.

To test further the generality of these correlations, we studied a series of alkylating agents in which variation of the nature of the alkyl group was responsible for variation in chemical reactivity, namely ethyl and isopropyl methanesulphonates. To estimate relative reactivities of the various sites in nucleic acids towards these agents we have isolated and characterized appropriate alkylpurine derivatives. The methods used were essentially those applied to the question of hydroxypropylation of DNA purines by propylene oxide (Lawley & Jarman, 1972).

Materials and Methods

Materials

Ethyl and isopropyl methanesulphonates were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. The *alkyl*-^{14}C-labelled reagents were obtained by the method of Brookes & Lawley (1961*a*) from [^{14}C]alkyl iodides supplied by The Radiochemical Centre, Amersham, Bucks., U.K.; the specific radioactivity of [1-^{14}C]ethyl methanesulphonate was 6.5 μCi/μmol and of [1,3-^{14}C]isopropyl methanesulphonate was 10.3 μCi/μmol.

Salmon sperm DNA, adenine, guanine and nucleosides were obtained from Sigma Chemical Co., London, U.K. For column chromatography, the sulphonic resin Dowex 50 (AG 50W, X4, less than 400 mesh, H^+ or NH_4^+ form; Bio-Rad Laboratories, Richmond, Calif., U.S.A.), the strongly basic resin Dowex 1 (AG, X8, 200–400 mesh, formate form; Bio-Rad Laboratories), and Sephadex G-10 (Pharmacia Ltd., London W.5, U.K.) were used. For paper chromatography Whatman 3MM paper was used with the following solvents: A, methanol–conc. HCl–water (7:2:1, by vol.); B, propan-2-ol–conc. NH_3 (sp.gr. 0.88)–water (7:1:2, by vol.); C, butan-1-ol–water–conc. NH_3 (sp.gr. 0.88) (84:12:2, by vol.). For t.l.c. cellulose (Polygram CEL 300$_{uv254}$; Camlab Ltd., Cambridge, U.K.) was used with solvent C, as above, or with solvent D [butan-1-ol–water (86:14, v/v)]. O^6-Ethyl- and O^6-isopropyl-guanines were obtained by the method of Balsiger & Montgomery (1960). 3- and 7-Ethyladenines were obtained from Dr. R. Denayer (see Denayer, 1962). 7-Ethylguanine was obtained by the method of Brookes & Lawley (1961*b*). N^2-Ethylguanine was obtained by heating 2-chloro-6-hydroxypurine (250mg) with aqueous ethylamine (70%, w/v; 1ml) and dimethyl sulphoxide (1ml) under reflux for 24h. The mixture was then chromatographed on a column (15cm × 3cm) of Dowex 50 (H^+ form), eluted with 1M-HCl (100 × 12ml fractions) to remove unchanged 2-chloro-6-hydroxypurine (in fractions 65–85), and then with 2M-HCl, which eluted N^2-ethylguanine in fractions 135–155; evaporation of these fractions yielded the product as a crystalline hydrochloride, with u.v. spectra identical with those reported by Shapiro *et al.* (1969).

Mass spectrometry

Mass spectra were obtained by the direct-insertion technique by using an AEI MS-12 spectrometer operating at an ionizing potential of 70eV (source temperature 200°C). Samples were eluted from paper chromatograms (with solvent B) as described by Lawley & Jarman (1972).

Ethylation of guanine

Guanine (550mg) was dissolved in 1M-NaOH (8ml) and treated with ethyl methanesulphonate (1ml) at room temperature (about 18°C) for 3h. The resultant mixture was applied to a column (18cm × 2.5cm) of Dowex 50 (H^+ form) and eluted with 1M-HCl until no further u.v. absorption was detected (160 × 17ml fractions). Examination of u.v. spectra showed five zones of elution (Table 1) indicated from constancy of the spectra throughout the peaks to contain individual products; the first peak (fractions 50–72) contained unchanged guanine. Fractions 85–91 were combined, evaporated to dryness, dissolved in ammonium formate and applied to a column (29cm × 2.5cm) of Dowex 1 (formate form) eluted with 0.04-ammonium formate pH6.5, and 60 fractions, each of volume 14.5ml,

Table 1. *U.v.-spectral characteristics and chromatographic behaviour of ethylguanines*

The solvents A, B and C for paper chromatography and t.l.c. are described in the Materials and Methods section, as are the details of the column eluents.

Compound	Fractions numbers of column peaks: Dowex 50 (H^+)	Dowex 1 (formate)	Sephadex G-10	$R_{Adenine}$: Paper A	Paper B	T.l.c. C	pH	$\lambda_{max.}$ (nm)	E_{280}/E_{260}
Guanine	50–72	—	—	0.51	0.53		1	248, 270	0.77
							7	246, 274	1.06
							13	274	1.2
1-Ethylguanine	93–112	12–14	—	0.9	1.2		1	252, 270	0.77
							7	249, 274	0.96
							13	278, 264	1.2
N^2-Ethylguanine	113–150	30–80	39–44	1.3	1.8	1.4	1	251, 281	0.58
							7	247, 280	1.1
							1	277, 254	1.3
3-Ethylguanine	85–91	10–18	18–24	1.3	0.9	0.78	1	264	0.43
							7	269	0.81
							13	273	1.4
O^6-Ethylguanine	—	—	80–90		1.6	1.75	1	284	3.3
							7	280, 235	3.1
							13	285	7
7-Ethylguanine	113–150	18–27	—	0.9	1.1	0.95	1	250, 272	0.64
							7	283, 247	1.7
							13	280, 240	1.8
9-Ethylguanine	73–84	33–50	28–37	1.1	1.3	1.0	1	253, 277	0.73
							7	270, 255	0.81
							7	270, 255	0.81
							13	270, 255	0.81

Table 2. *U.v.-spectral characteristics and chromatographic behaviour of isopropylguanines*

The solvents A, B and C for paper chromatography and t.l.c. are described in the Materials and Methods section, as are the details of the column eluents.

Compound	Fraction numbers of column peaks: Dowex 50 (H^+)	Sephadex G-10	$R_{Adenine}$: Paper A	Paper B	T.l.c. C	pH	$\lambda_{max.}$ (nm)	E_{280}/E_{260}
Guanine	65–66	—	0.61	0.53		1	249	0.72
						7	246, 272	0.85
						13	272	0.97
1-Isopropylguanine	84–86	—	0.85	1.5		1	253	0.68
						7	251, 274	0.93
						13	277	1.1
N^2-Isopropylguanine	—	46–58	1.5	1.4	1.9	1	252	0.62
						7	248, 270	0.93
						12	277	1.2
3-Isopropylguanine	75–80	22–30	1.5	1.3	1.5	1	263	0.4
						7	269,236	0.64
						13	273	1.4
O^6-Isopropylguanine	—	83–95		1.9	2.0	1	285	4.5
						7	280, 235	5.3
						13	283	4.7
7-Isopropylguanine	87–98	—		1.3	1.7	1	249, 274	0.84
						7	282, 247	1.6
						13	278	1.6
9-Isopropylguanine	69–75	33–45	1.6	1.5	0.9	1	252, 276	0.82
						7	251, 270	0.77
						13	269	0.74

were collected (Table 1). Fractions 93–112 and 113–150 were similarly pooled and subsequently applied to the same Dowex 1 column and 80 fractions of volume 14.5ml were collected.

A further small (0.5ml) portion of the reaction mixture was neutralized with 1M-HCl, the resultant white precipitate (mainly guanine) was removed by centrifugation, and the supernatant solution was applied to a column (75cm × 1.5cm) of Sephadex G-10 eluted with 0.05M-ammonium formate, pH6.8, and 100 fractions each of volume 6.4ml were collected. Products from the rechromatographed fractions were obtained by evaporation to dryness, redissolution in minimal volumes of 0.1M-HCl and neutralization with conc. NH_3 solution. They showed single spots on paper or t.l.c. (Table 1) and u.v. spectra characteristic of guanines alkylated at the assigned positions (Table 1).

Isopropylation of guanine

Guanine (275mg) dissolved in 1M-NaOH (4ml) was treated with isopropyl methanesulphonate (0.6ml) at room temperature with additions of 1M-NaOH to maintain the pH value above 12, the reaction being complete in 60min. The mixture was chromatographed on Dowex 50 (H^+ form) as described for ethylated guanine. Another mixture obtained by the same method was chromatographed on Sephadex G-10 as described for products from ethylated guanine.

The chromatographic profiles for the isopropylated guanine were analogous to those for ethylated guanine (see Table 2). The use of Sephadex G-10 enabled the isolation of the acid-labile O^6-isopropylguanine, as it was the last product to be eluted in this system.

Table 3. *Mass-spectral analysis of alkylguanines*

R = ethyl or isopropyl. For identification of positions of substitution by u.v. spectra, see Tables 1 and 2.

Designation of base	Molecular weight	Relative intensity of fragment (%)			
		$M^{+\cdot}$	$(M-1)^+$	$(M-CH_3)^+$	$[M-(R-H)]^{+\cdot}$
1-Ethylguanine	179	100	5	5	50
3-Ethylguanine	179	92	6	6	100
O^6-Ethylguanine	179	100	3	55	75
7-Ethylguanine	179	100	3	19	19
9-Ethylguanine	179	100	2	4	4
N^2-Isopropylguanine	193	100	10	83	90
3-Isopropylguanine	193	50	2	5	100
O^6-Isopropylguanine	193	40	—	—	100
7-Isopropylguanine	193	100	2	10	80
9-Isopropylguanine	193	100	1	6	70

Table 4. *U.v.-spectral characteristics and chromatographic behaviour of ethyladenines*

For details of chromatography and rechromatography of the products, see the Materials and Methods section.

Compound	Fraction numbers of column peaks Dowex 50 (H^+)	$R_{Adenine}$ Paper A	Paper B	T.l.c. C	pH	$\lambda_{max.}$ (nm)	E_{280}/E_{260}
Adenine	36–46	1.0	1.0	1.0	1	262	0.37
					7	260	0.14
					13	269	0.6
1-Ethyladenine	50–56	1.7	1.3	0.4	1	259	0.27
					13	271	1.0
3-Ethyladenine	28–34	1.7	1.4	1.2	1	274	1.2
					7	273	1.3
					13	273	1.3
7-Ethyladenine	50–56	1.2	1.5	1.3	1	273	1.0
					7	272	0.76
					13	272	0.74
9-Ethyladenine	60–100	1.5	1.5	1.5	1	260	0.14
					13	262	0.23

Characterization of the alkylguanines was also carried out by mass spectrometry and details of the mass spectra are given in Table 3.

Ethylation of adenine

Adenine (0.6g) was dissolved in formic acid (98–100%; 3ml) and ethyl methanesulphonate (2ml) was added. The mixture was stirred for 2 days at 37°C, and then left at 50°C for a further 2 days. After evaporation under reduced pressure, the residual mixture was dissolved in 0.1M-NH_3 and passed through a column of Dowex 1 (OH^- form) to remove unchanged adenine, as described by Brookes & Lawley (1960).

The eluate containing the ethylated adenines was concentrated by evaporation under reduced pressure and applied to a column (17cm×2.5cm) of Dowex 50 (H^+ form) eluted with 2M-HCl, and fractions each of volume 17ml were collected. The chromatographic profile of u.v. absorption resembled that found for analogous mixtures from methylated or hydroxypropylated adenine (Lawley & Jarman, 1972). The mono-substituted adenines, identified by their characteristic u.v. spectra (Table 4), were eluted as follows: 3-ethyladenine, after passing 500–600ml of eluent through the column; 1-ethyladenine and 7-ethyladenine, not separated from some residual adenine, after 800–1000ml; and 9-ethyladenine after 1200–1800ml. The fractions eluted from 800 to 1000ml were concentrated by evaporation, neutralized and applied in 4ml to a column (29cm×2.5cm) of Dowex 1 (formate form), eluted with 0.1M-NH_3 to fraction 44, and 0.1M-ammonium formate, pH6, to fraction 100 (24ml fractions). 7-Ethyladenine was recovered in fractions 7–12, 1-ethyladenine in fractions 49–51 and adenine in fractions 53–86. After evaporation of appropriate pooled fractions under reduced pressure, dissolution of the residues in methanol, and addition of ethyl acetate, crystalline samples of the hydrochlorides of the ethyladenines were obtained and identified by comparison with the authentic bases by paper chromatography, t.l.c., u.v. and mass spectra (Tables 4 and 6).

Ethylation of 2′-deoxyadenosine

2′-Deoxyadenosine (250mg) was dissolved in *NN*-dimethylformamide (5.0ml), ethyl iodide (0.4ml) was added, and the mixture was stirred at 37°C for 2 days. The residue after evaporation under reduced pressure was treated with 0.1M-HCl (5.0ml) at 70°C for 30min, cooled and neutralized with conc. NH_3 solution, concentrated by evaporation under reduced pressure, and applied to a column (75cm×1.5cm) of Sephadex G-10 eluted with 0.05M-ammonium formate, pH6.8, and fractions each of volume 6.4ml were collected. Two major u.v.-absorbing peaks were obtained, the second containing adenine. Fractions 11–19, containing a product with u.v. spectra of a 1-alkyladenine, were evaporated under reduced pressure and applied in a small volume of methanol to a preparative t.l.c. plate of silica gel (Merck 7734; Anderman and Co., London S.E.1, U.K.) developed with methanol–chloroform (1:3, v/v). Three u.v.-absorbing bands were obtained, which were eluted with 0.1M-HCl. The bands having u.v. spectra of 1-alkyladenine was evaporated to dryness, and redissolved in a small volume of methanol, which yielded a crystalline precipitate on addition of ethyl acetate. This was identified as the hydrochloride of 1-ethyladenine from its u.v. spectra (Table 4) and mass spectrum (Table 6).

Table 5. *U.v.-spectral characteristics and chromatographic behaviour of isopropyladenines*

For details of chromatography and rechromatography of the products, see the Materials and Methods section.

Compound	Designation of column peak on Dowex 1 (formate) Fraction no.	$R_{Adenine}$ Paper A	Paper B	T.l.c. C	pH	$\lambda_{max.}$ (nm)	E_{280}/E_{260}
Adenine	After 70	1.0	1.0	1.0	1	262	0.37
					13	269	0.54
1-Isopropyladenine	60–70	1.0	1.0	0.86	1	259	0.27
					13	269	0.92
3-Isopropyladenine	7–13	1.8	1.5	1.6	1	274	1.2
					7	273	1.4
					13	273	1.4
7-Isopropyladenine	5–7	1.9	1.5	1.3	1	272	0.93
					7	272	0.78
					13	272	0.76
9-Isopropyladenine	13–24	1.7	1.6	1.9	1	260	0.24
					13	262	0.16

Table 6. *Mass-spectral analysis of alkyladenines*

R = ethyl or isopropyl. For identification of positions of substitution of alkyl groups, see Tables 4 and 5.

Designation of base	Molecular weight	Relative intensity of fragment (%)			
		$M^{+\cdot}$	$(M-1)^+$	$(M-CH_3)^+$	$[M-(R-H)]^{+\cdot}$
1-Ethyladenine	163	31	10	7	100
9-Ethyladenine	163	100	8	4	90
3-Isopropyladenine	177	24	1	1	100
7-Isopropyladenine	177	100	3	36	70
9-Isopropyladenine	177	65	3	10	100

Isopropylation of adenine

Adenine was treated with isopropyl methanesulphonate as described for the ethylation of adenine. The residual mixture after evaporation was dissolved in 0.1M-NH_3 solution and applied to a column (23cm×2.5cm) of Dowex 1 (formate form), eluted with 0.1M-NH_3 solution (25 fractions each of volume 24ml), and then with 0.1M-ammonium formate, pH6. Six u.v.-absorbing peaks were found (Table 5) of which the first had no specific u.v. absorption, and the remainder were characterized by their u.v. spectra as shown in Table 5. The suspected 1-, 3- and 7-alkyladenines were further purified by rechromatography on a column (23cm× 1.5cm) of Dowex 50 (NH_4^+ form) eluted with 0.3M-ammonium formate, pH6, and fractions each of volume 6.4ml were collected. After evaporation of appropriate fractions the products were identified by mass spectra (Table 6) as giving molecular ions of isopropyladenines, and from comparisons of their characteristic u.v. spectra with those of the known methyladenines (Lawley & Jarman, 1972) the positions of substitution were assigned (Table 5). The relative yields are shown in Table 7.

Ethylation of DNA

Salmon sperm DNA (10mg) in a buffer solution (2.1ml) containing sodium acetate (0.08M) and the sodium salt of EDTA (0.03M) (pH7.1) was treated with [^{14}C]ethyl methanesulphonate (39μmol; 255μCi) at 37°C for 6 or 16h. The fibrous DNA was then precipitated with 2-ethoxyethanol (1.5vol.), redissolved in sodium acetate (0.25M) and reprecipitated with ethanol (2vol.), washed with ethanol and ether and then dried. The purines from the alkylated DNA were liberated by hydrolysis in 0.1M-HCl at 70°C (Lawley & Thatcher, 1970), and the hydrolysate was chromatographed on a column (80cm×1.5cm) of Dowex 50 (NH_4^+ form) eluted with 0.3M-ammonium formate, pH6.65 or 8.9 (6.4ml fractions), or on a column (80cm×1.5cm) of Sephadex G-10 eluted with 0.05M-ammonium formate, pH6.8 (6.4ml fractions).

Table 7. *Estimated yields of alkyladenines*

The yield of each product is expressed as the percentage of the total yield of alkyladenine from each reaction mixture. To obtain these estimates the column fractions containing each product were pooled and the extinction of this solution was measured at the wavelength of maximal absorption of the base. Approx. 20% of the available adenine was alkylated by ethyl methanesulphonate and 2% by isopropyl methanesulphonate.

Position of substitution	Ethyl substituted	Isopropyl substituted
1	2	1
3	38	46
7	6	3
9	54	50

Isopropylation of DNA

Salmon sperm DNA (25mg) in a buffer solution (8.25ml) containing sodium acetate (0.08M) and the sodium salt of EDTA (0.03M) (pH7.1) was treated with [^{14}C]isopropyl methanesulphonate (3.2μmol; 33μCi) for 19h at 37°C, during which time the pH did not change. The fibrous DNA was treated with ethanol and ether as described above. The hydrolysate, obtained as described above, was applied to a column (27cm×1.5cm) of Dowex 50 (NH_4^+ form) and eluted with 0.3M-ammonium formate, pH6.65.

Analyses of alkylated DNA

The extents of alkylation were determined by counting for radioactivity a portion of the acid hydrolysate, the concentration of DNA being obtained from u.v. absorption of the solution assuming a molar extinction coefficient/nucleotide unit $\varepsilon_P = 8950$. The positions of elution of appropriate alkylpurines added as markers were obtained by monitoring the u.v. absorption of the column eluate (Uvicord; LKB Instruments, S. Croydon, Surrey, U.K.) at 254nm. Radioactivity was assayed as described by Lawley & Shah (1972*a*). For both alkylating agents, preliminary chromatograms were

Table 8. *Relative positions of elution of ethyl- and isopropyl-purines in column chromatography*

These positions are expressed as peak fractions of elution of the product relative to that of adenine = 100. Adenine was eluted from a column (80cm × 1.5cm) after about 400ml of eluent had been passed at pH8.9 and after about 330ml at pH6.65. In elution at pH6.65, 1M-ammonium formate was used after elution of O^6-alkylpurines to elute 1- and 3-alkyladenines.

Column material ...	Dowex 50 (NH_4^+)	
Eluent ...	0.3M- and 1M-ammonium formate	
	pH8.9	pH6.65
1-Ethyladenine	111	330
3-Ethyladenine	93	290
7-Ethyladenine	140	125
3-Ethylguanine	86	98
O^6-Ethylguanine	220	195
7-Ethylguanine	123	132
1-Isopropyladenine	—	400
3-Isopropyladenine	100	312
7-Isopropyladenine	138	135
3-Isopropylguanine	118	136
O^6-Isopropylguanine	246	244
7-Isopropylguanine	150	164

obtained by running alkylpurines singly or from mixtures of appropriate alkylpurines, and u.v. spectra of various peaks were used to identify their positions of elution as shown in Table 8.

Results and Discussion

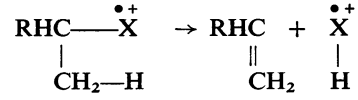
Ethylation of guanine by ethyl methanesulphonate at pH12–13 gave six products identified by u.v. and mass spectra as 1-, N^2-, 3-, O^6-, 7- and 9-ethylguanine (Tables 1 and 3). The results may be compared with those found by Litwack & Weissmann (1966) on methylation of guanine, where alkylation at the 1-, N^2-, 7- and 9-positions was observed.

In parallel experiments on isopropylation of guanine, the analogous isopropylguanines were obtained, and their chromatographic and spectral properties were closely similar to those of the ethylguanines (Tables 2 and 4).

Ethylation and isopropylation of adenine gave 1-, 3-, 7- and 9-alkyladenines, and again the respective chromatographic and spectral properties were closely similar (Tables 4, 5 and 6). Ethylation of 2′-deoxyadenosine gave 1-ethyladenine after acid hydrolysis of the products, in line with the methylation of this deoxyribonucleoside at this position (Jones & Robins, 1963), but isopropylation by the same procedure failed to give the corresponding isopropyl derivative. The results for ethylation of adenine confirm and extend those of Pal (1962).

In all cases, the u.v.-absorption spectra of the alkylpurines were closely similar to those of the corresponding methylpurines. The identification of the products where no authentic alkylpurines were available rests, first, on obtaining a molecular ion of the appropriate *m/e* ratio to establish that the product was a monoalkyl-substituted purine, and, secondly, on the close similarity of the u.v.-absorption spectra with those of the analogous methylpurines, where the positions of alkyl substitution are rigorously established. It should be noted in this regard that the present data for isopropylpurines do not accord with u.v.- and mass-spectral data attributed to such products by Walles (1970). In particular, the data of Walles (1970) do not appear to agree with the concept that the u.v. absorption of homologous methyl-, ethyl- and isopropyl-purines should be closely similar, since the position of substitution in the heterocyclic ring should be the main determinant of these spectra, not the nature of the alkyl substituent.

The mass spectra given by representative examples of each of the four categories of derivative (ethyl- and isopropyl-adenines; ethyl- and isopropyl-guanines) are depicted in Figs. 1 and 2. In contrast with the situation with the variously substituted methylguanines (Rice & Dudek, 1967), the mass-spectral fragmentation pathways of the ethyl- and isopropyl-guanines are relatively little influenced by the position of substitution on the guanine moiety. This is because the principal fragmentation process of the molecular ion in the higher alkylguanines (and in the adenine derivatives) is a rearrangement, whereby the alkyl residue is eliminated, with simultaneous transference of a proton from the alkyl moiety to the base, resulting in a net loss of (alkyl-H) and formation of the ions $(adenine)^{+\cdot}$ or $(guanine)^{+\cdot}$, e.g.:



where R = H or CH_3 and X is a heteroatom (N or O) of the purine. Subsequent fragmentation is, as would be expected, very similar to that observed for adenine and guanine (for examples, see Figs. 1 and 2) (Rice & Dudek, 1967) and is thus relatively independent of the initial site of alkylation. No similar comparison between the fragmentation pathways of the alkyladenines can be made at present, since data on the methyladenines are limited.

Other fragments derived from the molecular ion are those formed by the loss of H or CH_3 (Figs. 1 and 2, and Tables 3 and 6). The ion $(M–H)^+$ has a high relative abundance in the mass spectra of 7-methylguanine (Rice & Dudek, 1967) and of O^6-methylguanine (Farmer *et al.*, 1973). This high intensity

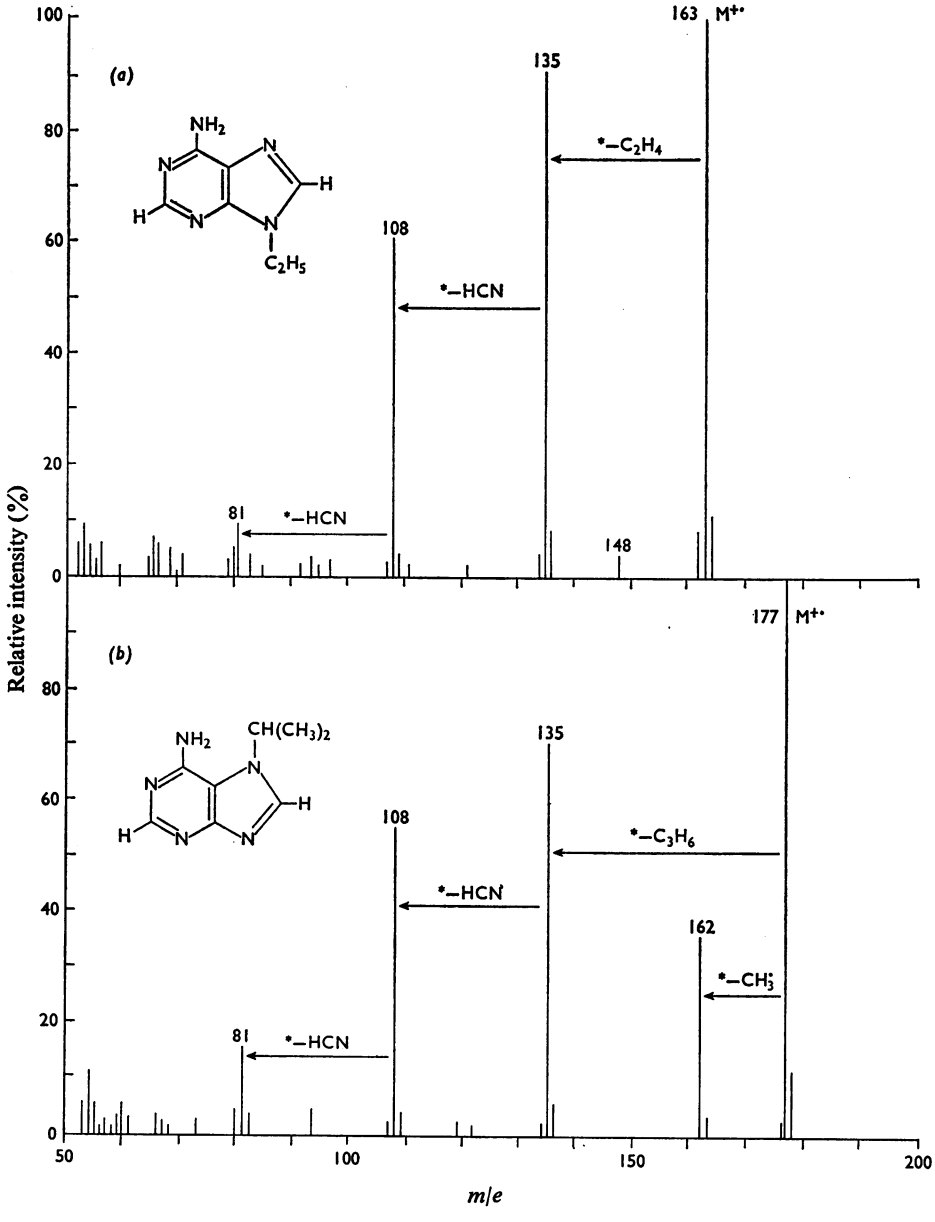


Fig. 1. *Mass spectra of alkyladenines*

(*a*) 9-Ethyladenine; (*b*) 7-isopropyladenine. * denotes that the appropriate metastable peak was observed for the loss of the fragment indicated. Spectra were obtained with an AEI MS-12 instrument by the direct-insertion technique at an ionizing potential of 70eV and source temperature of 200°C.

has been predicted to occur when the 6- and 7-substituents are such that a methylene bridge can form between the pyrimidine and imidazole residues (Deutsch *et al.*, 1972). That $(M–H)^+$ is weak in the mass spectra of the O^6- and 7-substituted ethyl- and isopropyl-guanines must therefore be ascribed either to the inability of substituted methylene groups to participate in the bridged structure, or to competition with an energetically more favourable fragmentation pathway, such as the rearrangement

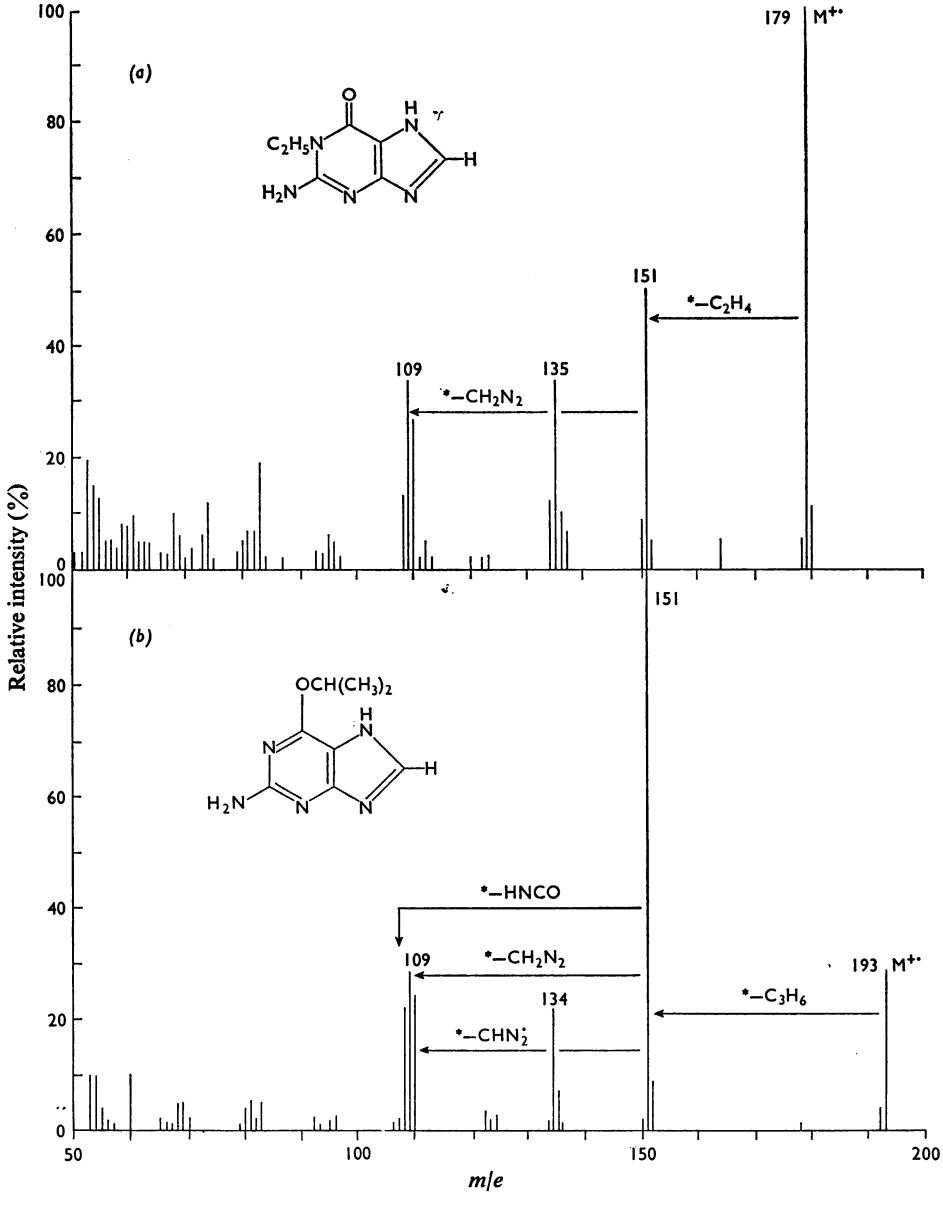


Fig. 2. *Mass spectra of alkylguanines*

(*a*) 1-Ethylguanine; (*b*) O^6-Isopropylguanine. * denotes that the appropriate metastable peak was observed for loss of the fragment indicated. Spectra were obtained with an AEI MS-12 instrument by the direct insertion technique at an ionizing potential of 70eV and source temperature of 200°C.

mentioned above. In contrast, some relatively high $(M\text{–H})^+$ intensities (e.g. N^2-isopropylguanine, Table 3 and 1-ethyladenine, Table 6) cannot be attributed to the formation of five-membered rings via methylene bridges. The highest relative intensities for $(M\text{–CH}_3)^+$ (O^6-ethylguanine and N^2-isopropylguanine, Table 3) are given by compounds for which the double bond resulting from the process:

$$CH_3\text{—}CHR\text{—}N^+ \rightarrow CHR{=}N^+ + CH_3^{\cdot}$$

is not associated with a ring N-atom, and is thus presumably minimally strained. However, the

relative intensity of this ion in the mass spectrum of another such compound, O^6-isopropylguanine (Table 3) is very low, and those of the 7-substituted derivatives, for which the $(M–CH_3)^+$ ions do not satisfy this condition, are all relatively high (Tables 3 and 6).

In summary, the principal features of the mass spectra of these derivatives can only be partially rationalized in terms of their structures. Nevertheless, the relative intensities of the peaks listed in Tables 3 and 6 enable the individual members of the four groups of compounds to be distinguished, and mass spectrometry thus provides an additional parameter for characterizing such products, particularly for amounts too small for u.v. spectra to be measured.

The mass spectra reported here for the isopropylguanines (Table 3 and Figs. 1 and 2) differ markedly from that of a product claimed (Walles, 1970) to be an isopropylguanine, and which was isolated after the reaction between guanosine or deoxyguanosine and isopropyl methanesulphonate. This product gave no molecular ion at *m/e* 193, but gave, *inter alia*, a fragment at *m/e* 163 ascribed to the loss of two methyl groups from the isopropyl moiety. In view of the consistently high molecular-ion intensities recorded for the isopropylguanines in the present study, and the absence of any peaks at *m/e* 163, it seems unlikely that the mass-spectral data given by Walles (1970) could be ascribed to an isopropylguanine.

The chromatographic properties of the alkylpurines further confirm the similar behaviour of the homologues. Thus the order of elution of the principal guanine derivatives from Dowex 50 (H^+ form) was 9-, 3-, 1- and 7-alkylguanines for both ethyl- and isopropyl-guanines, whereas the order of elution from Sephadex G-10 was 3-, 9-, N^2- and O^6-alkylguanines. Ethyladenines were eluted from Dowex 50 (H^+ form) in the order 3-, 1- and 9-, i.e. the same as for methyl- and hydroxypropyl-adenines (Lawley & Jarman, 1972). The order of elution of ethyl- and isopropyl-adenines from Dowex 1 (formate form) was 7-, 3-, 9- and 1-alkyladenines.

For analyses of alkylated DNA hydrolysed to yield purine bases, elution from Dowex 50 (NH_4^+ form) at pH 6.65 proved the most useful single method (Table 8). The order of elution for methyl-, ethyl- or isopropyl-purines was 3-alkylguanine, 7-alkylguanine, O^6-alkylguanine, 3-alkyladenine and 1-alkyladenine.

Analysis of the ethylated purines present in DNA alkylated by reaction with ethyl methanesulphonate was carried out by the methods described above. A comparison of the results obtained is given in Table 9. There was reasonable agreement on the proportions of the minor products of reaction which were obtained by the various methods. The differences which were observed probably reflect the uncertainty of measuring relatively small amounts of radioactivity. There was also some difference in the amount of radioactivity present in unidentified products, but this does not affect the calculation of the relative proportions of the alkylpurines isolated.

By using these procedures, the molar ratios of alkylpurines in DNA alkylated by [*alkyl*-^{14}C]-labelled methanesulphonates and by *N*-[^{14}C]methyl-*N*-nitrosourea (Lawley & Shah, 1973) were compared (Table 10).

The combined results showed that the ratios of products varied with the nature of the alkylating agents. The most abundant alkylpurine was in all cases 7-alkylguanine. The ratio of O^6-alkylguanine to 7-alkylguanine was lowest for methyl methanesulphonate and progressively greater for ethyl methanesulphonate, *N*-methyl-*N*-nitrosourea and isopropyl methanesulphonate. The ratio of 3-alkyladenine to 7-alkylguanine varied in the opposite sense through the series of alkyl methanesulphonates.

Table 9. *Ethylation of DNA purines by [^{14}C]ethyl methanesulphonate and low extents of reaction*

Details of ethylation of DNA and chromatographic procedures are given in the Materials and Methods section. 'n.s.' denotes not separated.

Extent of ethylation (mmol of ethyl/mol of DNA phosphorus) ...	1.7	1.7	1.7	0.95	1.7
Method of chromatography ...	Dowex 50 (NH_4^+)				Sephadex G-10
Eluent ...	0.3 M- and 1 M-ammonium formate at stated pH				0.05 M-Ammonium formate, pH 6.8
	6.65	8.9	8.9	8.9	
1-Ethyladenine	0.2	1.2	1.2	1.5	0.9
3-Ethyladenine	4.8	4.3	5.7	6.9	6.5
7-Ethyladenine	n.s.	2.3	n.s.	1.2	0.8
3-Ethylguanine	1.9	n.s.	n.s.	n.s.	n.s.
O^6-Ethylguanine	1.0	1.8	1.1	2.7	3.1
7-Ethylguanine	70	78.1	71.6	62.8	61.1
Unidentified peaks	21.7	12.3	19.8	25	27.6

Table 10. *Comparison of the relative extents of reaction on the purine bases of DNA by alkylating agents*

The data refer to treatments at neutral pH and 37°C (for details of methods, see the text). The values denoted in columns B were obtained by taking the ratio of the values in column denoted A against the figure for 7-alkylguanine in the same column. Values for methyl methanesulphonate are from Lawley & Shah (1972*b*). n.d. denotes not detected.

Alkylating agent	Methyl methanesulphonate		Ethyl methanesulphonate		Isopropyl methanesulphonate		*N*-Methyl-*N*-nitrosourea	
Extent of alkylation (mmol of alkyl/mol of DNA phosphorus) ...	0.7		0.95 and 1.7		0.1		6.2 and 21	
Position of substitution	Proportional extent of reaction at the stated position							
	A	B	A	B	A	B	A	B
1-Alkyladenine	1.1	0.013	0.8	0.011	n.d.		2.7	0.041
3-Alkyladenine	9.8	0.121	5.6	0.08	n.d.		8.2	0.125
7-Alkyladenine	0.3	0.004	1.6	0.023	n.d.		1.2	0.018
3-Alkylguanine	0.7	0.009	1.9	0.027	0.7	0.9	0.6	0.009
O^6-Alkylguanine	0.3	0.004	2.0	0.029	2.5	0.3	6.7	0.103
7-Alkylguanine	82	1.0	69.6	1.0	7.6	1.0	65.6	1.0
Unidentified peaks	4.4	0.054	19.9	0.28	89	11.4	17.6	0.27

Table 11. *Relative extents of alkylation of sites in nucleic acids in relation to reactivity of alkylating agents*

Data for alkylation of DNA are from Table 10. Data for RNA are from Shooter *et al.* (1974*a*,*b*). Values for s are from Osterman-Golkar *et al.* (1970) and Veleminsky *et al.* (1970). The values for nucleophilicity refer to the equation of Swain & Scott (1953) as discussed in the text; $\log(k_{N\text{-}7}/k_{O\text{-}6}) = s(n_{N\text{-}7}-n_{O\text{-}6})$, where k denotes relative extent of alkylation, s the substrate constant of the alkylating agent and n the nucleophilicity of the site alkylated. n.d. denotes that 3-isopropyladenine was not detected as a reaction product.

Agent	s	Ratio O-6/N-7 alkylation of DNA guanine	Ratio N-3 alkylation of DNA adenine/N-7 alkylation of DNA guanine	Proportion of RNA strand breaks at mean lethal dose for R17 bacteriophage	Difference in nucleophilicity of sites in DNA guanine ($n_{N\text{-}7}-n_{O\text{-}6}$)
Methyl methanesulphonate	0.83	0.004	0.12	0.0	2.9
Ethyl methanesulphonate	0.67	0.03	0.08	0.15	2.3
N-Methyl-*N*-nitrosourea	0.42	0.10	0.13	0.18	2.4
Isopropyl methanesulphonate	0.29	0.30	n.d.	0.34	1.8

Differences between other products were not considered significant, owing to the small relative yields of products.

It will be noted that this observed significant ranking of the alkylating agents is also the order of their Swain–Scott (1953) s factors (Table 11). According to the correlations of Swain & Scott (1953) relative yields of products Y and Z, denoted k_Y, k_Z, should depend on the nucleophilicity of the groups alkylated, denoted n_Y, n_Z, and on the substrate constants of the alkylating agents, s, by the relationship:

$$\log(k_Y/k_Z) = s(n_Y - n_Z)$$

For two alkylating agents, denoted A and B, the ratios of two products would then be given by:

$$\log(k_Y/k_Z)_A - \log(k_Y/k_Z)_B = (s_A - s_B)(n_Y - n_Z)$$

This correlation appears to fit the data for relative yields of O^6- and 7-alkylguanines quite well. However, it clearly cannot fit the data for the relative yields of 3-alkyladenines and 7-alkylguanines.

It therefore appears that the relative yields of O^6-alkylguanine and 7-alkylguanine are principally determined by the relative nucleophilicity of the O-6 and N-7 sites in guanine residues of DNA. The present data suggest a difference in n of about 2.3 for these sites. It may be speculated that other factors are important in determining the relative yields at N-3 of adenine as the nature of the alkyl group varies. In this connection it may be noted that the O-6 and N-7 atoms of guanine are situated in the wide groove of the Crick & Watson (1954) double helix, whereas N-3 of adenine lies in the narrow groove, so that a steric factor may play some part in determining the relative reactivity of N-3 of adenine, in particular tending to oppose alkylation at this site as the size of the attached alkyl group increases.

Another feature of these results is the higher yield

of unidentified products in passing from methyl methanesulphonate to the agents of lower *s* factor. These were mainly eluted early from the columns and are expected to contain alkylpyrimidine nucleotide material and phosphotriesters. Identification of these products was not attempted. It should be noted, however, that an empirical estimate of the extents of phosphotriester formation by these agents in RNA has been obtained by comparing their lethality towards the RNA-containing bacteriophage R17 with their ability to yield chain breaks in the treated bacteriophage (Shooter *et al.*, 1974*a*,*b*), and that these estimates show increasing relative ability of these agents to break the RNA chain with decreasing value of the *s* factor (Table 10).

With regard to the negative correlation which has been discerned between mutagenic efficiency and Swain–Scott (1953) *s* factor of alkylating mutagens (Ehrenberg, 1971), which also fits the findings of Loveless & Hampton (1969), the present results provide a possible interpretation. This follows from the negative correlation between *s* of alkylating agents and relative ability to yield the potentially miscoding base O^6-alkylguanine (Loveless, 1969) in DNA.

This work was supported by grants to the Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, from the Medical Research Council and the Cancer Research Campaign. D. J. O. was recipient of a studentship from the Medical Research Council, and M. J. holds a Ludwig Fellowship awarded by the Ludwig Institute for Cancer Research. We thank Mr. M. H. Baker for skilled technical assistance. The AEI MS12 mass spectrometer was purchased on grant G969/189/C from the Medical Research Council.

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