Biochem. J. (1973) **135**, 203–213
Printed in Great Britain

The Alkylation of 2′-Deoxyguanosine and of Thymidine with Diazoalkanes

SOME OBSERVATIONS ON *O*-ALKYLATION

By P. B. FARMER, A. B. FOSTER, M. JARMAN and M. J. TISDALE
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London SW3 6JB, U.K.

(*Received* 12 *March* 1973)

The reaction in ether–methanol between 2′-deoxyguanosine and diazomethane or its ethyl or *n*-butyl homologue gives 1-, O^6- and 7-alkyl-2′-deoxyguanosine. N^2,O^6-Dimethyl-2′-deoxyguanosine was also detected. The hydrolysis of the methyl and the ethyl derivatives gives the corresponding alkylguanines: the O^6-alkyl-2′-deoxyguanosines were sequentially hydrolysed, first to 2-amino-6-alkoxypurines, subsequently to guanine. The mass spectra of O^6-alkyl-2′-deoxyguanosines (methyl and ethyl) and of the corresponding 2-amino-6-alkoxypurines were determined. The reaction of diazomethane with thymidine afforded O^4-methylthymidine, in addition to the previously detected 3-methylthymidine.

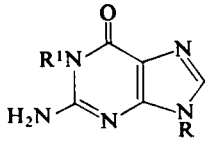
Loveless (1969) has proposed alkylation at the extranuclear O-6 atom of guanine as a possible cause of point mutations in T-even bacteriophages. He showed that, of the two alkylating agents methylmethanesulphonate and ethylmethanesulphonate, only the latter compound was mutagenic and effected O^6-alkylation of 2′-deoxyguanosine. Subsequently, Lawley & Thatcher (1970) showed that both *in vitro* and in cells DNA was alkylated at the O^6 position in guanine residues by the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

Mispairing may result from the O^6-alkylation of a guanine residue, since the alkylated base can theoretically mimic adenine in its hydrogen-bonding properties. To test this hypothesis, a homopolymer containing O^6-methylguanine residues is needed, and such a polymer, poly(O^6-methylG) has been synthesized by the enzymic polymerization of O^6-methylguanosine 5′-diphosphate (Gerchman *et al.*, 1972).

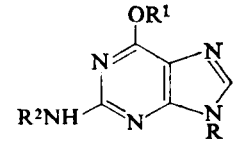
The ability of cells to repair damage to nucleic acids, in the form of alkylation of the base residues, varies according to the position of substitution of the alkyl moiety. Lawley & Orr (1970) investigated the extent of excision of alkylated bases from the DNA of *Escherichia coli* cells that had been treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Whereas 7-alkylated guanine residues were not excised to a significant extent from the DNA of either the resistant or the sensitive strains of these cells after alkylation, O^6-alkylated guanine residues were excised, but only from the resistant cells. A similar finding relating to the excision of 3-alkylated adenine residues afforded additional evidence that the quantitatively minor alkylation processes were of more significance, in their effect on cellular function, than was the major process of 7-alkylation of the guanine residues. A combination of mispairing with resistance to repair mechanisms could account fully for the role of O^6-alkylation of guanine in altering cellular processes.

In parallel with the work reported by Loveless (1969) we began a reinvestigation of the O^6-alkylation of guanine. Friedman *et al.* (1963, 1965) had shown that diazomethane reacted with 2′-deoxyguanosine in ether–methanol to give 1-(I), O^6-(II) and 7-methyl-2′-deoxyguanosine (III), and the first two compounds were isolated in crystalline form. The initial objectives of the present work were twofold: first, to determine the sensitivity of O^6-alkylguanines towards acid in relation to the conditions of acidity known to degrade DNA to apurinic acid and purines (Tamm *et al.*, 1952), and thereby define the scope of acid hydrolysis in the analysis of alkylated DNA (O^6-alkylguanines are enol ethers and, unlike *N*-alkyl derivatives, are hydrolysed by acid); in this connexion the fragmentation pattern of O^6-ethylguanine in electron-impact mass spectrometry has been reported briefly (Foster, 1969); secondly, to synthesize a series (methyl to butyl) of O^6-alkyl-2′-deoxyguanines as reference compounds in relation to the enzymic degradation of alkylated DNA. Hall (1967) has shown that DNA can be degraded enzymically to 2′-deoxy-ribonucleosides.

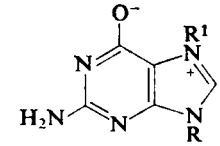
Singer (1972) has detected 1-, O^6- and 7-ethylguanosine among the products of the reaction between guanosine and various ethylating agents. Also, a minor O^6-ethylated product was observed, which was ethylated additionally on another, undefined



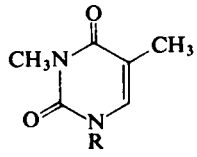
(I) $R^1 = CH_3$
(IV) $R^1 = C_2H_5$
(VII) $R^1 = nC_4H_9$



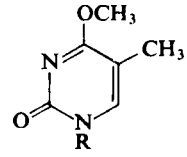
(IIa) $R^1 = CH_3$, $R^2 = H$
(IIb) $R^1 = CH_3$, $R^2 = CH_3$
(V) $R^1 = C_2H_5$, $R^2 = H$
(VIII) $R^1 = nC_4H_9$, $R^2 = H$



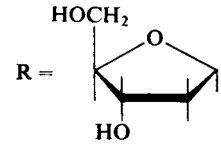
(III) $R^1 = CH_3$
(VI) $R^1 = C_2H_5$
(IX) $R^1 = nC_4H_9$



(X)



(XI)



position of the guanine residue. Since the major sites of ethylation observed by Singer corresponded to those for methylation observed by Friedman *et al.* (1963, 1965) in the reaction between 2′-deoxyguanosine and diazomethane, the possible formation of an analogue of the above-mentioned minor product became an additional consideration in the present investigations.

Friedman *et al.* (1963, 1965) also investigated the reaction of thymidine and diazomethane; 3-methylthymidine was the sole product described. The more recent discovery (Wong & Fuchs, 1971) that a similarly conducted methylation of 1-methyluracil yielded 4-methoxy-1-methyl-2-pyrimidone (1,O^4-dimethyluracil), in addition to 1,3-dimethyluracil, prompted a re-investigation of the methylation of thymidine.

It may be questioned whether the reactions of diazoalkanes in an organic solvent system, methanol–ether, as used here, could be relevant to the mode of action of *N*-alkyl-*N*-nitroso compounds *in vivo*, i.e. in neutral aqueous media. Consideration of the probable mechanism of these reactions suggests that, despite the different solvent conditions, both types of alkylation may involve the same reactive intermediates, namely the alkyldiazonium ions. Thus diazomethane is a strong base, and might well be considered to react as $CH_3N_2^+OCH_3^-$ in methanol. The principal differences to be expected between reactions in the two types of system would therefore be that diazoalkanes in methanol might alkylate acidic groups in nucleosides, which are ionized in alkaline solution, such as N-1 of deoxyguanosine and N-3 of thymidine, to a greater extent than in neutral aqueous solution (see Lawley, 1966, for discussion of this point). Otherwise, in both systems basic groups alkylated by alkyldiazonium ions (non-acidic ring-N atoms and the less nucleophilic extranuclear O atoms) would be expected to react.

Materials and Methods

General

U.v.-absorption spectra were measured on a Cary recording spectrophotometer model 11. Chromatography was performed on Whatman no. 1 paper by using the descending method and development with (A) propan-2-ol–water (7:3, v/v) or (B) propan-2-ol–aq. NH_3 (sp.gr. 0.88)–water (7:1:2, by vol.). Mass spectra were obtained by the direct insertion technique by using an AEI MS-12 spectrometer operating on an ionizing potential of 70eV (source temperature 200°C). Silicic acid (Merck Kieselgel G; Anderman and Co., London S.E.1, U.K.), because of its higher capacity, was preferred to cellulose (Friedman *et al.*, 1963, 1965) for column chromatography. For t.l.c. plates (5cm × 20cm or 20cm × 20cm) were coated with silicic acid (Merck Kieselgel GF_{254}) unless otherwise stated. Melting points were corrected.

Purification of 2′-deoxyguanosine

Commercial 2′-deoxyguanosine usually contained a small percentage of guanosine. For the isolation of minor reaction products in particular, removal of guanosine before reaction was essential.

A solution of 2′-deoxyguanosine (4g) in methanol (1.2 litres) was concentrated in the presence of silicic acid (55g). The dry residue was applied to a dry column (20cm × 4cm) of silicic acid (42g), which was subsequently eluted with a 1% solution of boric acid in methanol–chloroform (1:3, v/v). Fractions containing deoxyguanosine were freed from boric acid

by repeated concentrations with methanol (Zill *et al.*, 1953), and the pure 2′-deoxyguanosine was recrystallized from water.

A further sample of 2′-deoxyguanosine (0.15g; BDH Chemicals Ltd., Poole, Dorset, U.K.) was applied similarly to a dry column (25cm×2cm) of silicic acid. Elution with a 1% solution of boric acid in methanol–chloroform (1:1, v/v) yielded pure deoxyguanosine, which was freed of boric acid as described above; subsequent elution of the column with methanol gave guanosine. The molar percentage of guanosine in the deoxyguanosine sample, as determined by spectrophotometric determination of the components [guanosine: $\log \epsilon_{max.}$ (in methanol) 4.12; 2′-deoxyguanosine: $\log \epsilon_{max.}$ (in methanol) 4.12] was 2.7.

Reaction of 2′-deoxyguanosine with diazoalkanes

Methylation of 2′-deoxyguanosine. To a solution of 2′-deoxyguanosine (0.25g) in methanol (75ml) at 0°C, ethereal diazomethane [150ml, prepared (Arndt, 1943) from 2.5g of *N*-nitrosomethylurea] was added. After 1h at 0°C a white precipitate separated which, after 2h, was collected by centrifugation and washed with methanol. It was almost pure 7-methyl-2′-deoxyguanosine (III; yield 56mg, 21%) [R_F (solvent A) 0.40 (u.v.-fluorescent), with traces of contaminants at 0.56, 0.69]. Recrystallization from methanol–water (4:1, v/v) gave the chromatographically homogeneous *monohydrate* as colourless needles 34mg), m.p. 210°C (decomp.) (Found: C, 44.0; H, 6.0; N, 23.4. $C_{11}H_{15}N_5O_4,H_2O$ requires C, 44.15; H, 5.75; N, 23.4%).

The methanolic supernatant was concentrated in the presence of silicic acid (3g) and the residue was added to a column (14cm×3cm) of the same adsorbent. Elution with methanol–chloroform (3:22, v/v; 180ml) gave impure O^6-methyl-2′-deoxyguanosine (IIa; 70mg, 27%), R_F (solvent A) 0.69 (u.v.-fluorescent), which crystallized as colourless granules, m.p. 121–123°C, from acetone (1ml) at 0°C. The mass spectrum (see the Results and Discussion section) indicated the presence of a dialkylated impurity, the isolation of which compound is described below.

Subsequent elution with methanol–chloroform (9:41, v/v; 180ml) gave 1-methyl-2′-deoxyguanosine (Friedman *et al.*, 1965) (I; 80mg, 30%), R_F (solvent A) 0.56, which crystallized from water (1.5ml) to give colourless rods (27mg), m.p. 200°C (decomp.) (Found: C, 47.2; H, 5.65; N, 25.2. $C_{11}H_{15}N_5O_4$ requires C, 46.93; H, 5.38; N, 24.90%).

The above proportions and conditions were used in the following alkylation reactions.

Ethylation of 2′-deoxyguanosine. 2′-Deoxyguanosine (1g) was made to react with diazoethane [200ml, prepared from 10g of *N*-nitrosoethylurea (Werner, 1919)]. The solution was concentrated to 50ml and left at 0°C overnight. Pure *7-ethyl-2′-deoxyguanosine* (VI; 0.15g, 14%), m.p. 210°C (decomp.), R_F (solvent A) 0.52 (u.v.-fluorescent), separated as colourless needles (Found: C, 48.6; H, 5.5; N, 23.4. $C_{12}H_{17}N_5O_4$ requires C, 48.8; H, 5.8; N, 23.7%).

The filtrate was treated as in the methylation reaction described above. The column (20cm×3cm) of silicic acid was eluted as follows. After a fore run of chloroform (100ml), elution with methanol–chloroform (3:22, v/v; 350ml) gave *O^6-ethyl-2′-deoxyguanosine* (V; 0.71g, 64%), R_F (solvent A) 0.76 (u.v.-fluorescent), which crystallized from water (2.5ml) as colourless needles (0.455g), m.p. 79–82°C (Found: C, 48.6; H, 5.6; N, 23.75%).

Subsequent elution with methanol–chloroform (9:41, v/v; 200ml) gave *1-ethyl-2′-deoxyguanosine* (IV; 0.16g, 15%), R_F (solvent A) 0.66. Rechromatography (column, 17cm×0.75cm) with acetone (300ml) as eluent and concentration of the appropriate fractions to 5ml gave white crystals (75mg) m.p. 129–131°C (Found: C, 48.45; H, 5.9; N, 23.8%).

Butylation of 2′-deoxyguanosine. 2′-Deoxyguanosine (0.5g) was treated with 1-diazobutane [generated from 5g of *N-n*-butyl-*N*′-nitro-*N*-nitrosoguanidine (R. N. Emanuel Ltd., Wembley, Middx., U.K.)]. No crystals separated from the concentrated reaction solution, which was therefore processed as described above and the column (25cm×2cm) of silicic acid was eluted as follows. After a fore run of chloroform (100ml), elution with methanol–chloroform (3:47, v/v; 300ml) gave *O^6-n-butyl-2′-deoxyguanosine* (VIII; 0.24g, 40%), R_F (solvent A) 0.81 (u.v.-fluorescent), as a colourless glass (Found: C, 52.1; H, 6.6; N, 21.6. $C_{14}H_{21}N_5O_4$ requires C, 52.0; H, 6.55; N, 21.6%).

Subsequent elution with methanol–chloroform (9:41, v/v; 200ml) gave slightly impure but chromatographically homogeneous 1-*n*-butyl-2′-deoxyguanosine (VII; 55mg, 9%), R_F (solvent A) 0.73, as a colourless glass (Found: C, 51.05; H, 6.55; N, 20.2%).

After the elution of unchanged 2′-deoxyguanosine with methanol–chloroform (1:3, v/v; 200ml), a 1:1 (v/v) mixture (470ml) of these solvents eluted *7-n-butyl-2′-deoxyguanosine* [IX; 55mg, 9%; R_F (solvent A) 0.62 (u.v.-fluorescent)], which gave colourless plates (29mg), m.p. 200°C (decomp.) on crystallization from methanol–water (4:1, v/v; 5ml) (Found: C, 51.9; H, 6.3; N, 21.7%).

Hydrolyses of alkyldeoxyguanosines

7-Alkyl-2′-deoxyguanosines (III and VI). A solution of the crystalline deoxyribonucleoside (25mg) in water (1ml) was boiled under reflux for 5min. The solid that separated (10mg) was collected by filtration. The products were respectively 7-methylguanine (Found: C, 43.7; H, 4.75; N, 42.3. Calc. for $C_6H_7N_5O$: C, 43.6; H, 4.25; N, 42.4%) and 7-ethylguanine (Found: C, 46.9; H, 5.15; N, 39.6. Calc. for $C_7H_9N_5O$: C, 46.9; H, 5.05; N, 39.1%). The u.v.-absorption results

for solutions in 0.1M-HCl and 0.1M-NaOH were identical with the literature values (Brookes & Lawley, 1961).

1- *and O⁶-Alkyl-2′-deoxyguanosine.* The deoxyribonucleoside (2.5mg) was dissolved in 1M-HCl (0.5ml) at 24°C. After suitable time-intervals, samples (0.1ml) were added to aq. 2M-NH_3 (0.1ml). Samples (0.5ml) were subjected to paper chromatography in solvent B. For each hydrolysis time, the areas containing starting material and the corresponding base (1-alkylguanine and 2-amino-6-alkoxypurine) were excised and extracted overnight with 0.1M-HCl (5ml). The eluates were decanted into 1cm light-path cells and analysed spectrophotometrically against blanks eluted from an equal area of the chromatogram. The wavelength chosen was that of maximum absorption for the deoxyribonucleoside concerned (Table 1). The values, and the associated extinction coefficients, differed insignificantly from those of corresponding known bases (Haines *et al.*, 1962; Balsiger & Montgomery, 1960); 1-ethylguanine is undescribed.

The results are depicted in Fig. 1 and Table 3.

O⁶-Methyl-2′-deoxyguanosine and N^2,O⁶-dimethyl-2′-deoxyguanosine

(a) *Pure O⁶-methyl-2′-deoxyguanosine.* 2′-Deoxyguanosine (1.47g) was treated with diazomethane as described above. After removal of 7-methyl-2′-deoxyguanosine (0.38g) by filtration, the product was applied (see above) to a column (50cm×2cm) of silicic acid. Elution with methanol–chloroform (3:22, v/v; 10ml fractions) gave, in fractions 31–50, chromatographically homogeneous (on t.l.c., methanol–chloroform, 1:9, v/v) O⁶-methyl-2′-deoxyguanosine (0.3g, 23%), which gave colourless crystals (0.27g), m.p. 127–132°C, from ethyl acetate. A sample for analysis was dried for 4h at 60°C *in vacuo* over P_2O_5 (Found: C, 46.63; H, 5.58; N, 24.32%).

The mass spectrum (see the Results and Discussion section) exhibited a molecular ion at *m/e* 281 and a peak attributable to the O⁶-methylguanine residue at *m/e* 165. Peaks attributable to a dialkylated product (see below) were absent.

(b) *Isolation of a dialkyl 2′-deoxyguanosine.* Fractions 21–30 obtained in (a) contained several u.v.-absorbing components (separated by t.l.c. in methanol–chloroform, 1:9, v/v) in addition to O⁶-methyl-2′-deoxyguanosine (R_F 0.25). Of these the principal component, R_F 0.31, was selected for a detailed investigation. After t.l.c. of 5% of the total eluate this component was subjected to mass spectrometry (for elution procedure see Rix *et al.*, 1969). The mass spectrum (see the Results and Discussion section) showed a molecular ion at *m/e* 295 and a peak at *m/e* 179 attributable to a dialkylated base residue.

A further concentrate of the total eluate (19%) was heated under reflux in ^2H_2O for 1h. The freeze-dried product was dissolved in methanol and subjected to t.l.c. as described above. The mass spectrum of the component of R_F 0.31 exhibited a molecular ion at *m/e* 282 and a fragment corresponding to a dialkylated guanine moiety at *m/e* 180, indicating the net incorporation of 1 deuterium atom into the base residue.

The remainder of the total eluate (76%) was subjected to t.l.c. on silicic acid and the component of R_F 0.31 was recovered from the silicic acid in the appropriate areas by elution with methanol. The total yield [from 1.47g (5.5mmol) of 2′-deoxyguanosine] was 31 extinction units, measured at the $\lambda_{max.}$ in methanol solution. Assuming an ϵ value of approx. 10000, this represents an overall yield of about 0.05%. The u.v.-absorption results (compound IIb, Table 3) were indicative of O⁶-substitution (cf. compound IIa, Table 1).

(c) *Hydrolysis of the dialkyl-2′-deoxyguanosine.* (i) 0.1M-HCl. A sample of the product (6% of the total) in 0.1M-HCl (0.1ml) was kept at room temperature for 45min. The solution was neutralized (aq. 0.1M-NH_3) and concentrated to dryness. T.l.c. on silicic acid as described above revealed a single u.v.-absorbing component, R_F 0.34, which on mass spectrometry gave a molecular ion at *m/e* 179. The u.v. spectrum (Table 3) and mass spectrum (Fig. 3*a*) were consistent with retention of the O⁶-substituent (see the Results and Discussion section). An additional component at R_F 0.14 was detected by the cysteine–H_2SO_4 spray reagent (Buchanan, 1951) and was attributable to 2′-deoxyribose.

(ii) Dowex 50 (H^+ form). Some 59% of the isolated dimethyl-2′-deoxyguanosine was dissolved in water (2ml) and the solution heated with Dowex 50 (H^+ form) resin (AG 50W; X8; 200–400 mesh) for 50min on a steam bath. The resin was collected, washed with water (10ml) and the product was eluted with aq. 2M-NH_3 (10ml). The NH_3 soln. eluate was concentrated to dryness. T.l.c. of a sample on a plate coated with fluorescent cellulose (144 LS 254; Schleicher and Schüll, Dassel, Germany) developed in system B revealed a major u.v.-absorbing component at R_F 0.46, and a trace at R_F 0.34 (cf. N^2-methylguanine, 0.46; guanine, 0.33; 1-methylguanine, 0.45; 3-methylguanine, 0.39; 7-methylguanine, 0.42). In butan-1-ol–formic acid–water (77:10:12, by vol.) the product had R_F 0.43 (cf. N^2-methylguanine, 0.42; 1-methylguanine, 0.32; 7-methylguanine, 0.35). The mass spectrum (Fig. 3*b*) of the product (without further purification), although it revealed traces of guanine (M^+ at *m/e* 151) and dimethylguanine (*m/e* 179), was substantially identical with that of an authentic sample of N^2-methylguanine (Fig. 3*c*) prepared by hydrolysis of N^2-methylguanosine (Cyclochemicals; Micro-Bio Laboratories Ltd., London W.11, U.K.) by the above procedure.

U.v.-absorption data (Table 3) for the product also supported this structural assignment (see Shapiro, 1968).

Reaction between thymidine and diazomethane

To a solution of thymidine (0.5g) in methanol (250ml) cooled to 0°C was added ethereal diazomethane (100ml; from 5g of *N*-methyl-*N*-nitrosourea). After 30min, the solution was concentrated and the concentrate applied (for procedure see corresponding reaction with 2′-deoxyguanosine) to a column (26cm × 2cm) of silicic acid, which was eluted with acetone (10ml fractions). Fractions 11–20 contained 3-methylthymidine (X; R_F 0.57 in acetone, cf. 0.50 for thymidine) (0.49g, 93%), which gave colourless crystals, m.p. 130–132°C [lit. 131–133°C (Friedman *et al.*, 1965)] from ethyl acetate. U.v. spectra at pH1, 7 and 13 showed $\lambda_{max.}$ 268nm.

After the mixed fractions, *O^4-methylthymidine* (XI; R_F 0.30 in acetone) was eluted in fractions 23–36. Recrystallization from ethyl acetate gave colourless prisms (30mg, 5%), m.p. 172–174°C (Found: C, 51.3; H, 6.15; N, 11.0. $C_{11}H_{16}N_2O_5$ requires C, 51.55; H, 6.3; N, 10.95%). The mixed m.p., u.v. spectra and mass spectrum were identical with those reported for an authentic sample of compound (XI) prepared by an unambiguous route (see Lawley *et al.*, 1973).

Results and Discussion

Preparation of 1-, *O^6-* and 7-*alkyl-2′-deoxyguanosines*

A re-examination of the reaction of 2′-deoxyguanosine with diazomethane previously described by Friedman *et al.* (1963, 1965) gave the same three products, namely the 1-, *O^6-* and 7-methyl derivatives. The 7-methyl derivative (III), not hitherto obtained crystalline, was isolated in an almost pure state from the reaction mixture. The product of R_F 0.67 reported by Friedman *et al.* (1965) as contaminating the 7-methyl derivative, but which was not further characterized, was not encountered in the present work. However, an additional *O^6*-alkylated product, apparently not detected by Friedman *et al.* (1965), was detected and is described below. The crystalline 7-methyl derivative was characterized by a comparison of its u.v.-absorption spectra with those of the most closely related crystalline analogue, 7-methylguanosine (Haines *et al.*, 1962), and by hydrolysis to 7-methylguanine. The *O^6*-methyl structure, previously assigned by Friedman *et al.* (1965) on the basis of methoxyl group analysis and vigorous acidic hydrolysis to guanine, was here additionally confirmed by mild acid-catalysed hydrolysis, which gave 2-amino-6-methoxypurine. Interestingly, a minor product previously observed from a two-phase reaction between aqueous 2′-deoxyguanosine and ethereal diazomethane (Haines *et al.*, 1962; R_F 0.12, fluorescent in the solvent D of that publication) was chromatographically identical, in the reported solvent and in the solvents A and B of the present work, with *O^6*-methyl-2′-deoxyguanosine (II*a*).

Haines *et al.* (1962) had therefore unknowingly observed the *O^6*-alkylation of 2′-deoxyguanosine in an aqueous medium before the first recognition, by Loveless (1969), of this phenomenon, and its potentially important biological implications.

The reactions of 2′-deoxyguanosine with diazoethane and with 1-diazobutane gave the corresponding ethyl (IV, V and VI) and *n*-butyl (VII, VIII and IX) derivatives. These were characterized by a comparison of their u.v. spectra (Table 1) with those of the corresponding methyl derivatives (I, IIa and III).

Hydrolysis of methyl and ethyl derivatives of 2′-deoxyguanosine

Boiling water sufficed to convert the 7-alkyl-2′-deoxyguanosines (III and VI) into the corresponding 7-alkylguanines. The hydrolyses in HCl of the 1- and *O^6*-alkyl-2′-deoxyguanosines (I, IIa, IV, V) followed first-order kinetics (Fig. 1). The half-times (Table 2) for the conversions into the corresponding alkylguanines under these conditions (1 M-HCl, 24°C) may be compared with that of approx. 12min computed from the rate constant ($k = 9.72 \times 10^4 s^{-1}$) at pH0.97 and 37°C for the hydrolysis of 2′-deoxyguanosine to guanine (Venner, 1964). The comparison clearly shows that the conditions (Tamm *et al.*, 1952) that will liberate guanine from DNA should also liberate the 1- and the *O^6*-alkylguanine. Indeed, Lawley & Thatcher (1970) have shown that 2-amino-6-methoxypurine was liberated from alkylated DNA during incubation for 16h at pH1 and 37°C.

Mass spectrometry of O^6-alkylguanines

Recently, mass spectrometry has been used as an aid to the characterization of alkylguanines obtained from hydrolysates of alkylated nucleic acids (Lee & Lijinsky, 1966; Lijinsky & Ross, 1969). Experiments with deuterated alkylating agents have also helped to elucidate the mechanism of alkylation at N-7 of guanine in nucleic acids both *in vivo* by dimethylnitrosamine (Lijinsky *et al.*, 1968) and *in vitro* and in *E. coli* by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (Süssmuth *et al.*, 1972). In these reactions, when trideuteromethyl derivatives were used, 7-trideuteromethylguanine (molecular ion, M^+, at *m/e* 168) was isolated from the alkylated nucleic acids. Thus the methyl group was transferred intact, and hence a diazomethane type of intermediate could not be involved. These conclusions do not necessarily extend to other alkylating agents, or to alkylations by the above-mentioned agents at sites other than N-7 of guanine.

The mass spectrum of 2-amino-6-methoxypurine and its trideuteromethyl analogue were determined

Table 1. *U.v.-absorption spectra of alkyl-2′-deoxyguanosines*

The products were obtained from reactions of 2′-deoxyguanosine with diazoalkanes as described in detail in the text; (I)–(III) are methyl-substituted, (IV)–(VI) are ethyl-substituted and (VII)–(IX) are *n*-butyl-substituted.

Compound	Position of alkyl group	pH	$\lambda_{max.}$ (nm)	$\log \epsilon_{max.}$	$\lambda_{min.}$ (nm)	$\log \epsilon_{min.}$	$\lambda_{infl.}$ (nm)	$\log \epsilon_{infl.}$
(I)	1-Me	1	257	3.96			284	3.79
		7	256	4.13			276	3.98
		13	256	4.14			276	3.98
(IIa)	O^6-Me	1	285	4.02	253	3.26		
		7	247	3.93	260	3.72		
			278	3.91				
		13	247	3.94	260	3.72		
			279	3.92				
(III)	7-Me	1	258	4.02			280	3.84
		7	257	3.97	271	3.86		
			281	3.89				
		13	266	4.06	245	3.76		
(IV)	1-Et	1	256	4.04			282	3.87
		7	256	4.12			276	3.97
		13	257	4.12			276	3.96
(V)	O^6-Et	1	286	4.09	252	3.34		
		7	248	3.97	261	3.71		
			281	3.96				
		13	248	3.93	261	3.68		
			280	3.94				
(VI)	7-Et	1	253	4.02			280	3.83
		7	257	3.96	270	3.87		
			281	3.90				
		13	266	4.06	244	3.74		
(VII)	1-*n*-Bu	1	258	*			282	*
		7	255	*			276	*
		13	257	*			280	*
(VIII)	O^6-*n*-Bu	1	246	3.69	260	3.51		
			287	4.01				
		7	248	3.98	261	3.73		
			280	3.99				
		13	248	3.91	261	3.58		
			280	3.99				
(IX)	7-*n*-Bu	1	257	4.03			280	3.86
		7	257	3.79	266	3.76		
			282	3.86				
		13	267	4.00	241	3.76		

* Not determined: compound not analytically pure.

Table 2. *Half-times for the hydrolysis in* 1 M-*HCl at* 24°C *of* 1- *and* O^6-*alkyl-2′-deoxyguanosines*

For details see the text. R_F values in system B are given in parentheses.

Nucleoside	Base	Half-time
(I) (0.57)	1-Methylguanine (0.40)	5 min
(IIa) (0.69)	2-Amino-6-methoxypurine (0.59)	20 s
(IV) (0.65)	1-Ethylguanine (0.54)	3 min
(V) (0.76)	2-Amino-6-ethoxypurine (0.69)	17 s

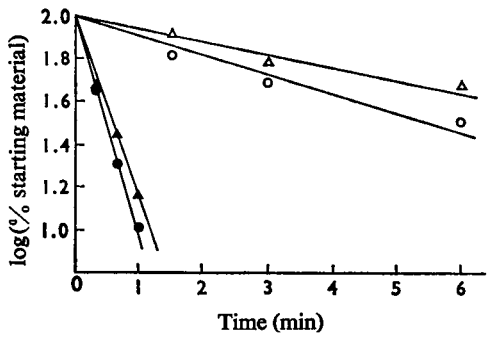


Fig. 1. *Hydrolysis of alkyldeoxyguanosines in* 1 M-*HCl at* 24°*C*

For experimental details see the text. △, 1-Methyl-2′-deoxyguanosine (I); ○, 1-ethyl-2′-deoxyguanosine (IV); ▲, O^6-methyl-2′-deoxyguanosine (IIa); ●, O^6-ethyl-2′-deoxyguanosine (V).

(Fig. 2) to provide an additional parameter for the identification of this base in hydrolysates of alkylated DNA. The bases were preferred to the deoxyribosides for this purpose, since the volatilization of the latter compounds in the mass spectrometer is sometimes accompanied by thermal decomposition. Also the mass spectra of the mono-alkylated derivatives of purines and pyrimidines, particularly guanine (Rice & Dudek, 1967), is comparatively well documented. The mass spectrum of 2-amino-6-ethoxypurine has been recorded previously (Foster, 1969). 2-Amino-6-trideuteromethoxypurine was prepared from 25mg of 2-amino-6-chloropurine by the method of Balsiger & Montgomery (1960) with the substitution of $C^2H_3O^2H$ for methanol. The mass spectrum (Fig. 2) of the deuterated compound afforded insight into the fragmentation pathways of the methoxy derivative. Thus a noteworthy difference between the mass spectrum of 2-amino-6-methoxypurine and those of the 1- and the 3-methylated analogues (Rice & Dudek,

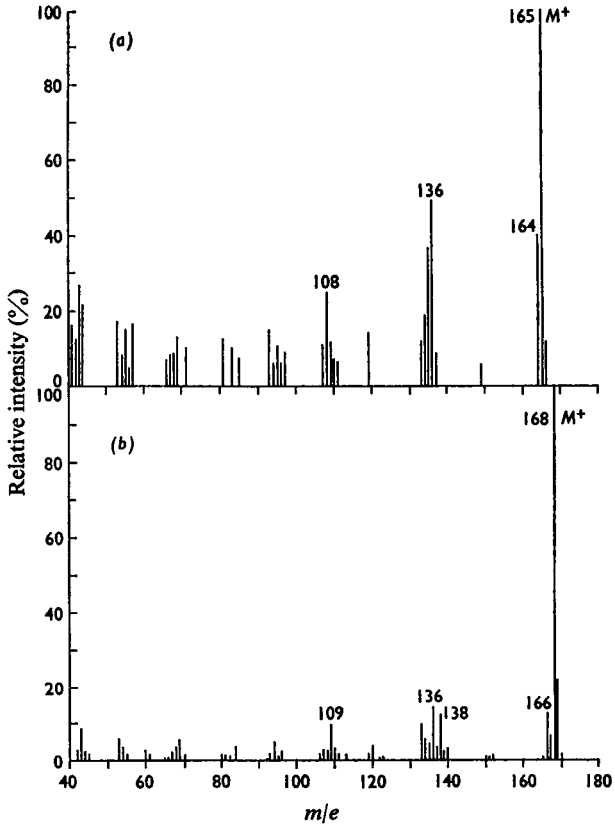


Fig. 2. *Mass spectra of methoxypurines*

(*a*) 2-Amino-6-methoxypurine. (*b*) 2-Amino-6-trideuteromethoxypurine. For experimental details see the text.

1967) is the much greater intensity of the $(M-1)^+$ peak (*m/e* 164) for the alkoxy derivative. In the spectrum of the trideuteromethoxy derivative the peak at *m/e* 166 $(M-2)^+$ is more intense than that at *m/e* 167 $(M-1)^+$, showing that the major proton loss is from the alkoxy group in the spectrum of the methoxy derivative. The formation of an ion containing the grouping shown in Scheme 1(*a*) with a methylene bridge between N-7 and a heteroatom at C-6 has been postulated, and similarly supported by appropriate labelling experiments, for other appropriately substituted alkylpurines (Deutsch *et al.*, 1970; Yeo & Williams, 1970).

Otherwise, the spectrum of 2-amino-6-methoxypurine contained many features in common with that of 1-methylguanine. This fact suggested an explanation for the presence, in the spectrum of the methoxy derivative, of a strong signal at *m/e* 136. Rearrangement in the molecular ion of this compound by Scheme 1(*b*) would give a molecular ion corresponding to that of 1-methylguanine, which is known to expel HCO or CH_3N (29 mass units). It is difficult to envisage a mechanism for the direct expulsion of these fragments from the unrearranged molecular ion of the methoxy derivative. This latter conclusion is supported by the presence in the mass spectrum of the trideuteromethoxy derivative of prominent peaks both at *m/e* 138 ($M^+ - {}^2HCO$) and *m/e* 136 ($M^+ - C^2H_3N$), and of the appropriate metastable peaks at *m/e* 113.4 (168→138) and *m/e* 110.1 (168→136) (see Beynon *et al.*, 1968; for the method of derivation of these assignments).

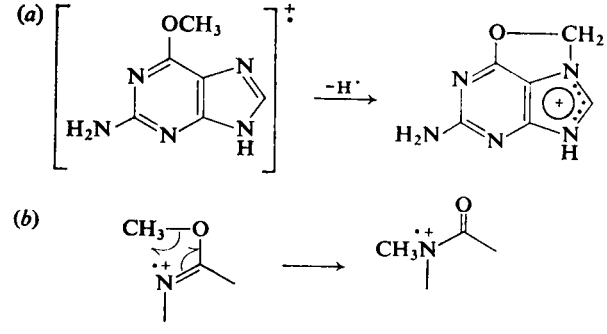
Identification of N^2,O^6-dimethyl-2′-deoxyguanosine

A mass-spectral examination of the fractions containing O^6-methyl-2′-deoxyguanosine in an initial experiment (see the Materials and Methods section) revealed an impurity of mol. wt. 295. Thus, in addition to the signals at *m/e* 281 and 165 appropriate to the monomethyl derivative, there were corresponding signals, of about 10% of these intensities, at *m/e* 295 and 179. A careful chromatographic resolution was carried out, and the major component responsible for this higher-mass molecular ion was examined.

Since the fragment corresponding to the base residue had an *m/e* value of 179, the additional methyl group was on this residue, rather than the sugar moiety. The u.v.-spectroscopic data (Table 3) suggested the 6-O atom for one of the alkylation sites, and this was subsequently confirmed by sequential hydrolysis. Before this, the 8-position was excluded as a possible additional site. The dialkyldeoxyguanosine was shown, by mass spectrometry, to incorporate a single deuterium atom under conditions known to promote the insertion of such an atom at the C-8 position in purines (Schweizer *et al.*, 1964). Therefore this position was not substituted in the dimethylated deoxyguanosine.

Mild acidic hydrolysis gave the dialkylated base. The mass spectrum (Fig. 3*a*) contained the relatively intense peak at *m/e* 178 $(M-1)^+$, which would be expected (see above) for an O^6-methylated guanine. More vigorous acidic hydrolysis gave a monomethylguanine, shown by its chromatographic properties to be N^2-methylguanine, a result additionally confirmed by the u.v. (Table 3) and mass-spectrometric data (Figs. 3*b* and 3*c*). The dialkylated base was therefore N^2,O^6-dimethylguanine, and the original dialkylated deoxynucleoside was N^2,O^6-dimethyl-2′-deoxyguanosine (IIb).

Mass spectrometry provides an additional parameter for excluding substitution at the 1-, 3- or 7-positions, since the mass spectra of these derivatives have all been determined (Rice & Dudek, 1967) and differ in detail from that of N^2-methylguanine. The loss, in part or wholly, of the methylamino function would appear, by analogy with 6-methylaminopurine (Rice & Dudek, 1967), to be a major fragmentation pathway for N^2-methylguanine. Appropriate metastable peaks for the partial losses were observed at *m/e* 113.9 (165→137) and *m/e* 112.1 (165→136), both for authentic N^2-methylguanine and for the sample



Scheme 1. *Fragmentations of the molecular ion of* 2-*amino*-6-*methoxypurine*

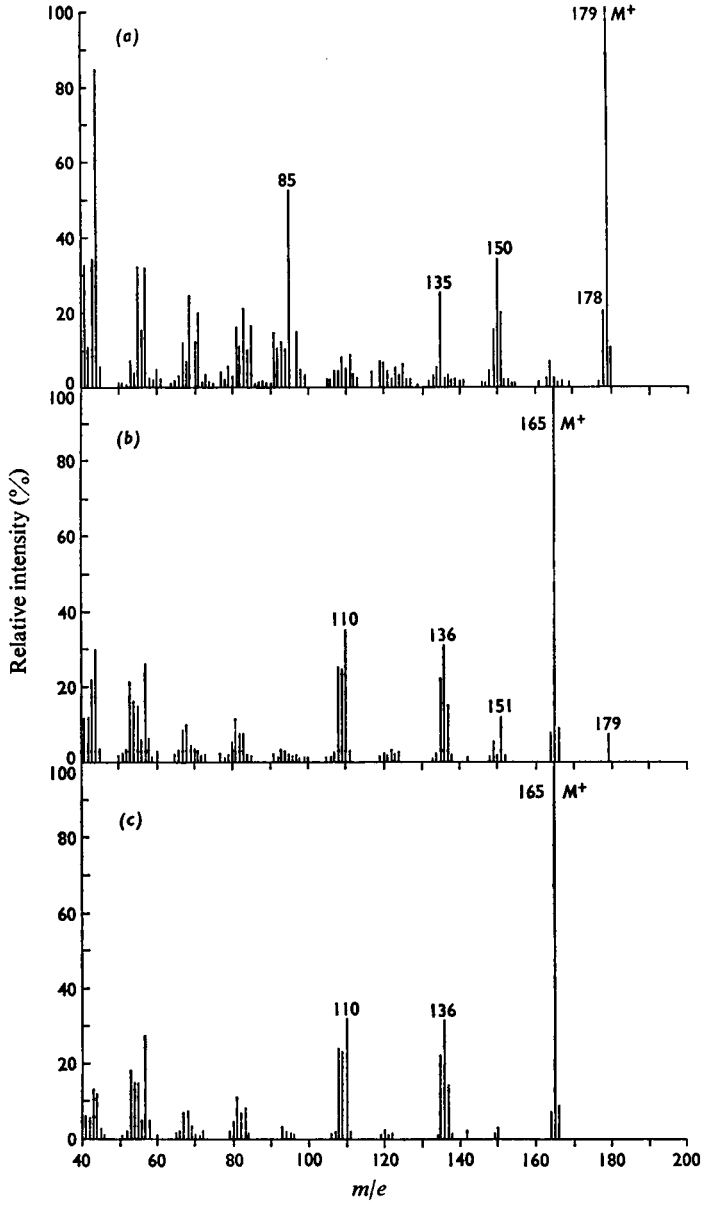


Fig. 3. *Mass spectra of alkylguanines*

(*a*) N^2,O^6-Dimethylguanine; (*b*) N^2-methylguanine, derived from compound (IIb) by acidic hydrolysis; (*c*) N^2-methylguanine, similarly derived from N^2-methylguanosine. For experimental details see the text.

derived from vigorous acidic hydrolysis of compound (IIb). The origin of the analogous fragments of *m/e* 151, 150 and 149 in the spectrum (Fig. 3*a*) of the dialkylguanine is ambiguous in the absence of high-resolution measurements or deuterium-labelling experiments, since the alkoxy group can also participate in the loss of fragments of appropriate mass (see Fig. 2). Metastable peaks were likewise present, at *m/e* 127.4 (179→151), *m/e* 125.7 (179→150) and *m/e* 124.7 (178→149).

Table 3. *U.v.-absorption spectrum of N^2,O^6-dimethyl-2′-deoxyguanosine and its hydrolysis products*

This product (IIb) was obtained by methylation of deoxyguanosine with diazomethane, as described in detail in the text.

Compound	Solvent	$\lambda_{max.}$ (nm)	$\lambda_{min.}$ (nm)	$E_{max.}/E_{min.}$
(IIb)	H_2O	252	268	2.10
		287		1.42
	MeOH	252	268	2.33
		287.5		1.54
	0.1 M-HCl	288	266	*
	0.1 M-NaOH	249	270	*
		284		*
N^2,O^6-Dimethylguanine	MeOH	245	269	3.04
		287		1.81
N^2-Methylguanine†	H_2O	247	266	1.83
		287		1.81
	0.1 M-HCl	252	273	2.03
		278		1.03
	2 M-NH_3	277	263	1.16
		($\lambda_{infl.}$ 244)		
N^2-Methylguanine‡	2 M-NH_3	277	263	1.24
		($\lambda_{infl.}$ 243)		

* End-absorption too high for accurate assessment.
† Obtained by hydrolysis of (IIb): contains some N^2,O^6-dimethylguanine and some guanine.
‡ Obtained by hydrolysis of N^2-methylguanosine. Values for other solvents given by Shapiro (1968).

The original N^2,O^6-dialkyl-2′-deoxyguanosine (IIb) could have derived from an initially formed N^2-monomethyl derivative, since O^6-methyl-2′-deoxyguanosine (IIa) was not further alkylated on the base residue by diazomethane in methanol–ether. 2′-Deoxyguanosine was completely methylated under the reaction conditions and it is likely that N^2-methyl-2′-deoxyguanosine would yield a similar range of products, but the corresponding 1,N^2- and N^2,7-dialkyl-2′-deoxyguanosine have not yet been sought. No evidence for a significant dialkylated impurity was obtained from the mass spectra of 1-methyl-2′-deoxyguanosine (I) and its 1-ethyl analogue (IV). However, the mass spectra of the homologous O^6-alkylated analogues [ethyl (V) and *n*-butyl (VIII)] contained, in addition to the molecular ions at *m/e* 295 and 323 respectively, minor peaks at *m/e* 323 and 379, corresponding to dialkylated impurities, the intensities relative to the molecular ions of the major constituents being 5 and 2.5% respectively. These percentages undoubtedly reflect the greater volatility, and hence greater concentration in the vapour phase, of the dialkyl derivatives rather than the true percentage of impurity.

Thus N^2,O^6-dimethyl-2′-deoxyguanosine, the molecular ion of which made a 10% contribution compared with that of the O^6-monoalkyl analogue in the mass spectrum of the unseparated mixture, actually constituted only approx. one-five-hundredth of the total O^6-alkylated material on a molar basis.

Reported instances of chemical alkylation at the extranuclear N atom of guanine are few. Litwack & Weissmann (1966) described N^2-methylguanine among the products of methylation of guanine with methyl chloride in aqueous ethanolic NaOH. According to Dipple *et al.* (1971) the extranuclear amino group was the principal site of alkylation of guanine in 2′-deoxyguanosine and in DNA by 7-bromomethylbenz[*a*]anthracene in aqueous solution.

The u.v.-spectral characteristics for N^2,O^6-dimethyl-2′-deoxyguanosine (Table 3) resemble those for the O^6,X-dimethylguanosine described as a minor ethylation product of guanosine by Singer (1972) (where X denoted an undefined position of substitution). Conclusive proof that her product was N^2,O^6-substituted would similarly require the detection of the N^2-alkylguanine as a product of vigorous acidic hydrolysis of the O^6,X-substituted guanosine.

Reaction of thymidine with diazomethane

The re-examination of the reaction between thymidine and diazomethane confirmed the presence of a significant product as well as 3-methylthymidine (X). The identification of this product as O^4-methylthymidine (XI) was based on a comparison of its

physical properties with those of an authentic sample prepared by an unambiguous but lengthier route [see Lawley *et al.* (1973), in which the possible biological significance of O^4-alkylation is discussed]. The ratio of O^4- to 3-alkylation was similar to that observed for the methylation products of 1-methyluracil (Wong & Fuchs, 1971). The direct procedure clearly affords a convenient method whereby small quantities of other O^4-alkylthymidines might be prepared by using the appropriate diazo derivatives.

This investigation 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. The AEI MS-12 spectrometer was purchased on a special grant (G969/189/C) from the Medical Research Council. The work was carried out during the tenure (by P.B.F.) of the William Shepherd Fellowship of this Institute, and, in part (by M. J.) of a Ludwig Fellowship awarded by Cancer Research Institute. We thank Dr. A. Loveless for prompting our interest in this work, and Dr. P. D. Lawley for helpful discussions. The skilled technical assistance of Mr. M. H. Baker is gratefully acknowledged. The u.v. spectra in Table 1 were determined by Dr. A. Knowles.

References

Arndt, F. (1943) *Org. Synth. Coll.* vol. 2, pp. 165–167, Wiley, New York

Balsiger, R. W. & Montgomery, J. A. (1960) *J. Org. Chem.* **25**, 1573–1575

Beynon, J. H., Saunders, R. A. & Williams, A. E. (1968) *Mass Spectra of Organic Molecules*, pp. 44–63, Elsevier, Amsterdam

Brookes, P. & Lawley, P. D. (1961) *J. Chem. Soc.* 3923–3928

Buchanan, J. G. (1951) *Nature (London)* **168**, 1091

Deutsch, J., Neimann, Z. & Bergmann, F. (1970) *Org. Mass Spectrom.* **3**, 1219–1221

Dipple, A., Brookes, P., Mackintosh, D. S. & Rayman, M. P. (1971) *Biochemistry* **10**, 4323–4330

Foster, A. B. (1969) *Lab. Pract.* **18**, 743–748

Friedman, O. M., Mahapatra, G. N. & Stevenson, R. (1963) *Biochim. Biophys. Acta* **68**, 144–146

Friedman, O. M., Mahapatra, G. N., Dash, B. & Stevenson, R. (1965) *Biochim. Biophys. Acta* **103**, 286–297

Gerchman, L. L., Dombrowski, J. & Ludlum, D. B. (1972) *Biochim. Biophys. Acta* **272**, 672–675

Haines, J. A., Reese, C. B. & Todd, Lord (1962) *J. Chem. Soc.* 5281–5288

Hall, R. H. (1967) *Methods Enzymol.* **12**, 305–315

Lawley, P. D. (1966) *Progr. Nucl. Acid Res. Mol. Biol.* **5**, 89–131

Lawley, P. D. & Orr, D. J. (1970) *Chem.-Biol. Interactions* **2**, 154–157

Lawley, P. D. & Thatcher, C. J. (1970) *Biochem. J.* **116**, 693–707

Lawley, P. D., Orr, D. J., Shah, S. A., Farmer, P. B. & Jarman, M. (1973) *Biochem. J.* **135**, 193–201

Lee, K. Y. & Lijinsky, W. (1966) *J. Nat. Cancer Inst.* **37**, 401–407

Lijinsky, W. & Ross, A. E. (1969) *J. Nat. Cancer Inst.* **42**, 1095–1100

Lijinsky, W., Loo, J. & Ross, A. E. (1968) *Nature (London)* **218**, 1174–1175

Litwack, M. D. & Weissmann, B. (1966) *Biochemistry* **5**, 3007–3012

Loveless, A. (1969) *Nature (London)* **223**, 206–207

Rice, J. M. & Dudek, G. O. (1967) *J. Amer. Chem. Soc.* **89**, 2719–2725

Rix, M. J., Webster, B. R. & Wright, I. C. (1969) *Chem. Ind. (London)* 452

Schweizer, M. P., Chan, S. I., Helmkamp, G. K. & T'so, P. O. P. (1964) *J. Amer. Chem. Soc.* **86**, 696–700

Shapiro, R. (1968) *Progr. Nucl. Acid Res. Mol. Biol.* **8**, 73–112

Singer, B. (1972) *Biochemistry* **11**, 3939–3947

Süssmuth, R., Haerlin, R. & Lingens, F. (1972) *Biochim. Biophys. Acta* **269**, 276–286

Tamm, C., Hodes, M. E. & Chargaff, E. (1952) *J. Biol. Chem.* **195**, 49–63

Venner, H. (1964) *Hoppe-Seyler's Z. Physiol. Chem.* **339**, 14–27

Werner, E. A. (1919) *J. Chem. Soc.* 1093–1102

Wong, J. L. & Fuchs, D. S. (1971) *J. Org. Chem.* **36**, 848–850

Yeo, A. N. H. & Williams, D. H. (1970) *J. Amer. Chem. Soc.* **92**, 3984–3990

Zill, L. P., Khym, J. X. & Cheniae, G. M. (1953) *J. Amer. Chem. Soc.* **75**, 1339–1342