



The Mechanism of Decomposition of *N*-Methyl-*N*-nitrosourea (MNU) in Water and a Study of its Reactions with 2'-Deoxyguanosine, 2'-Deoxyguanosine 5'-Monophosphate and d(GTGCAC)[⊙]

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Abstract: The carcinogenicity of *N*-methyl-*N*-nitrosourea (MNU) arises from its ability to methylate DNA. This occurs in an aqueous environment and therefore an appreciation of the mode of decomposition of MNU in water is essential to understanding the mechanism of DNA methylation and its base sequence dependence. The kinetics of MNU hydrolyses are shown to be first order in MNU with a steep rise in rate above pH 8. Using NMR for *in situ* monitoring of reaction intermediates and products from hydrolyses of [¹³CO]MNU, [¹⁵NH₂]MNU and [¹³CH₃]MNU, it is proved that base-induced hydrolysis of MNU is initiated by deprotonation at the carbamoyl group. The critical reactive species are shown to be the methyldiazonium ion (Me-N₂⁺) and cyanate (NCO⁻). Investigations of reactions of [¹³CH₃]MNU with 2'-deoxyguanosine (dGuo) and 2'-deoxyguanosine 5'-monophosphate (dGuo-5P) showed that: a) the site of methylation of dGuo is highly pH-dependent (relatively more N-1 and O⁶-methylation compared to N-7 occurs at higher pH); b) the principal site of methylation of dGuo-5P by MNU is at phosphate; c) incorporation of deuterium into methyl groups occurs in D₂O at higher pH. Methylation of the oligonucleotide d(GT[¹⁵N]GCAC) by MNU in D₂O showed partial deuteration of the N⁷-methyl groups of the guanines, whilst methylation by MNU in water indicated no significant preference for either guanine with respect to N⁷-methylation.

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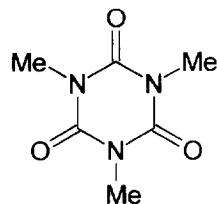
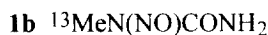
INTRODUCTION

In a programme exploring the reactivities of both mono- and bifunctional agents that modify DNA^{1,2} we have studied the aqueous chemistry of *N*-methyl-*N*-nitrosourea (MNU, **1a**). MNU is the oldest³ and simplest member of a group of compounds (alkylnitrosoureas, ANU's) that have the ability to alkylate DNA.⁴ In consequence, many of these compounds are potent mutagens and carcinogens,^{5,6} although paradoxically some have value for the treatment of cancer.⁷ MNU has been widely used as a model compound in studies of chemical carcinogenesis.⁸ The substance may be formed *in vivo* by the interaction of the nitrosonium ion (from

⊙ The writing of this paper was begun in May 1993 during a visit that the corresponding author (Bernard T Golding) made to the Australian National University, Canberra. Regrettably, this was the last time he saw Arthur Birch.

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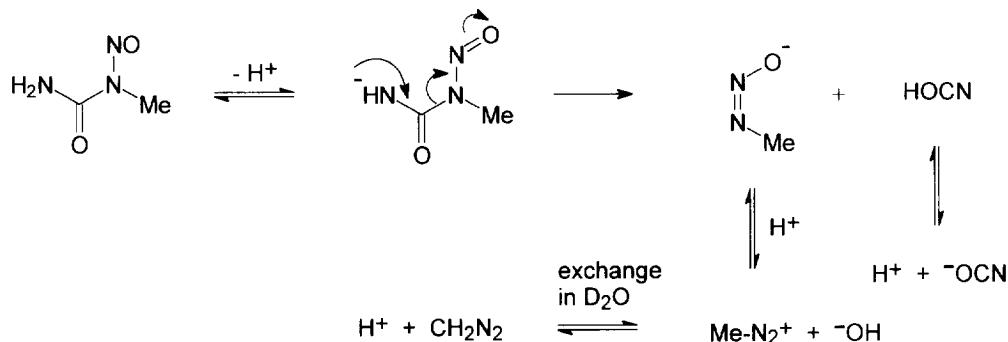
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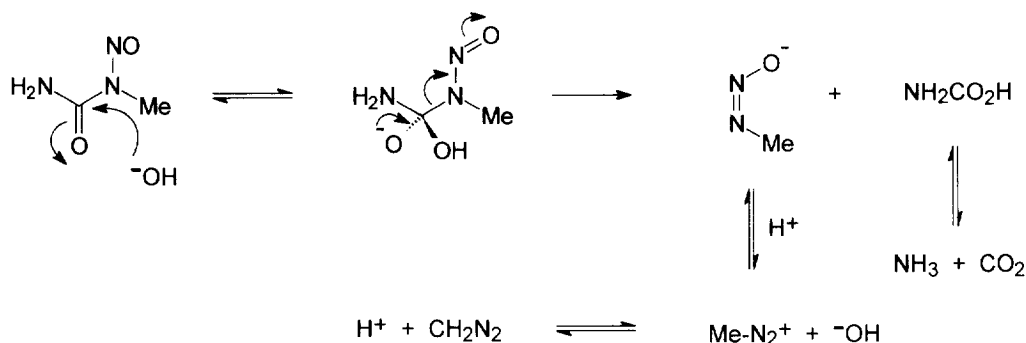
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sodium nitrite in foods containing this preservative) with endogenous *N*-methylurea.⁹ MNU was formerly used as a precursor of diazomethane¹⁰ but is now replaced by the less toxic *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide.¹¹

MNU is thermally labile under anhydrous conditions and also readily decomposes in water, especially at higher pH.^{12,13} It is important to understand the mechanisms of these decompositions because of their possible relevance to the carcinogenic and carcinostatic actions of ANU's. The thermal decomposition of MNU can occur explosively and yields methyl isocyanate (MIC) and 1,3,5-trimethylcyanuric acid **2**, the latter being presumably derived by trimerisation of MIC.¹³ Ethereal solutions of diazomethane prepared by treatment of MNU in ether with aqueous base contain MIC as a contaminant.¹⁴ It was originally proposed that MIC is formed via the shift of the nitroso group from the methyl-substituted nitrogen of MNU to the carbamoyl nitrogen.¹⁵ The resulting isomer of MNU (*N*-methyl-*N'*-nitroso-urea) fragments to MIC. However, using MNU which was ¹⁵N-labelled in either the nitroso group or the nitrogen atom connected to the nitroso group showed that the nitrogen atoms of **2**, and hence the nitrogen atom of MIC, came from the carbamoyl nitrogen of MNU.¹⁶ Based on these results, the most reasonable mechanism for the origin of MIC is by reaction of cyanate with the methyldiazonium ion, these species having been formed by the fragmentation of MNU (Scheme 1). Cyanate had already been reported in 1919 as a major product of both aqueous and anhydrous decomposition of MNU.¹⁷ That the base-induced decomposition of MNU proceeded *via* an initial deprotonation at the carbamoyl group, followed by fragmentation to cyanate and methyldiazoate, became firmly established in the literature.¹⁸⁻²⁰



Scheme 1: Fragmentation mechanism for the base-induced decomposition of MNU in water.



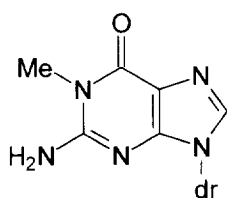
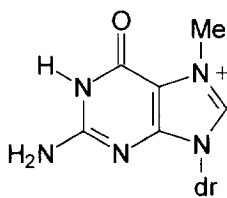
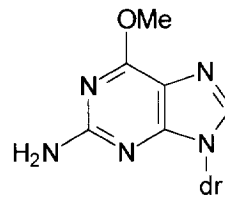
Scheme 2: Base-induced decomposition of MNU via a tetrahedral intermediate.

In 1980, notwithstanding the large body of information in support of Scheme 1, evidence was presented for an alternative mechanism of base-induced hydrolysis of MNU, see Scheme 2.^{21,22} The essence of this mechanism is an initial nucleophilic attack by hydroxide at the carbamoyl carbon of MNU to give a tetrahedral intermediate that decomposes to methyldiazoate and carbamate. This mechanism had been considered, but dismissed by Kirmse and Wachtershäuser,²³ in a study of the alkaline hydrolyses of several ANU's. They cited, in particular, the non-formation of urethanes from alkoxide-induced cleavages of ANU's as evidence against the mechanism of Scheme 2. However, since 1980, ref. 21 has been widely quoted, e.g. in a textbook of organic chemistry,²⁴ and assumed by many (e.g. ref. 25) to provide the definitive mechanism of decomposition of MNU.

The concept that base-induced decomposition of MNU is initiated by hydroxide attack on the carbamoyl group (Scheme 2) rather than deprotonation at this group (Scheme 1) has been used to explain the base-sequence dependence observed in alkylations of DNA by ANU's.²⁶ It has also been employed to explain the purported formation of *N*⁷-(2,2-dideuterio-2-fluoroethyl)deoxyguanosine from the reaction of deoxyguanosine with *N,N'*-bis-(1, 1-dideuterio-2-fluoroethyl)-*N*-nitrosourea.²⁵ Thus, the critical event was said to be nucleophilic attack by *N*⁷ or *O*⁶ of guanine on the carbamoyl carbon (or imino carbon of the imino tautomer) of an ANU rather than alkylation of *N*⁷/*O*⁶ by an alkyl diazonium ion. In support of this theory, results were cited that claimed intact methyl transfer in the methylation of DNA by MNU.²⁷ In these experiments there was no apparent tritium loss from the methyl group of [C³H₃]MNU during methylation of DNA. As the methyldiazonium ion in water is known to equilibrate with diazomethane,^{28,29} these results if true, might indicate the non-involvement of the methyldiazonium ion in the methylation of DNA by MNU (in ref 26 they were taken merely to imply the non-involvement of diazomethane in DNA methylation).

In this paper we present evidence proving that the mechanism of base-induced hydrolysis of MNU is as shown in Scheme 1. This was achieved by NMR studies with MNU samples specifically enriched with ¹³C (**1b** and **1c**) and ¹⁵N (**1d**), and also by studying the kinetics of hydrolysis of MNU. A preliminary communication on some of these results has been published.³⁰ In addition, we describe a study of the methylation of deoxyguanosine by MNU, showing that the site of methylation is highly pH-dependent. The methylated deoxyguanosines formed (*N*¹-methyldeoxyguanosine **3a**; *N*⁷-methyldeoxyguanosine **3b**; *O*⁶-methyldeoxyguanosine **3c**)² show significant incorporation of deuterium when the methylation is performed in

deuterium oxide, as expected if the methyldiazonium ion is the methylating agent. We have also studied the methylation of deoxyguanosine 5'-monophosphate by MNU [unlabelled and ^{13}C -labelled (**1b**)] in which case there is extensive phosphate methylation. Finally, we have investigated methylations of the oligonucleotide d(GTGCAC) by MNU. We have shown that exchange occurs during the methylation of d(GTGCAC) by MNU and furthermore, by the use of ^{15}N -labelled d(GTG*AC) [^{15}N -label in G*], that there is no significant preference for N^7 -methylation of the terminal guanine in d(GTG*AC).

**3a****3b****3c**

The results obtained demonstrate the value of using isotopically enriched carcinogens for studies of their reactions with nucleophiles acting as models for biological macromolecules (for previous applications of this kind see refs. 2, 29, 31 and 32). Not only have we diagnosed the critically reactive species from MNU, but we have also identified methylated products formed from MNU and guanine [in dGuo, dGuo-5P and d(GTGCAC)] under physiologically relevant conditions.

MATERIALS AND METHODS

Hazardous Compounds. MNU is a potent carcinogen. All operations involving the handling of the solid compound or its solutions were carried out wearing gloves, over spillage containment trays in a fume cupboard reserved for such operations. MNU was destroyed by treatment with saturated aqueous sodium bicarbonate.^{33,34}

Instruments. NMR spectra were measured on a Bruker AC200 (^1H : 200.13 MHz, ^{13}C : 50.29 MHz), a Bruker WM300WB (^1H : 300.13 MHz, ^{13}C : 75.46 MHz, ^{15}N : 30.40 MHz) and a Bruker AMX500 (^1H : 500.14 MHz, ^{13}C : 125.72 MHz, ^{15}N : 50.66 MHz) NMR spectrometer. Unless stated otherwise, ^{13}C NMR spectra were measured with broad band proton decoupling. The spectra were determined for solutions in the solvents cited below. For ^1H and ^{13}C spectra tetramethylsilane was used as internal standard for organic solvents and 3-trimethylsilylpropionic acid for D_2O , or solvent was used as reference. Chemical shifts for ^{15}N NMR spectra were measured relative to ammonium nitrate as an external standard. Mass spectra (EI or CI or FAB mode)* were recorded with a Kratos MS80 RF or VG 7070E instrument. The pH Stat equipment

* Abbreviations: CI, chemical ionization; DMSO, dimethyl sulfoxide; EI, electron impact; FAB, fast atom bombardment; NOE, nuclear Overhauser effect; ODS, octadecylsilyl; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]); AMPSO, (3-[1,1-dimethyl-2-hydroxyethylamino]-2-hydroxypropanesulfonic acid); N^1 -Me-dGuo, N^1 -methyldeoxyguanosine; N^7 -Me-dGuo, N^7 -methyldeoxyguanosine; N^7 -Me-Gua, N^7 -methylguanine; ring-opened N^7 -Me species, formylaminopyrimidines from base-induced cleavage of N^7 -Me-dGuo at C-8; O^6 -Me-dGuo, O^6 -methyldeoxyguanosine; dGuo-5P, deoxyguanosine 5'-monophosphate; G*, deoxyguanosine labeled at N-1 or NH_2 with ^{15}N .

consisted of a Metrohm 702 SM Titrino fitted with a 10 mL Exchange Unit and a Metrohm 6.0216.100 Combined Glass Electrode. All pH values quoted are those displayed by the instrument, no correction being made for the effects of deuteriated or organic solvents (n.b. $pD = pH + 0.4$ for water). Oligonucleotide syntheses were performed using phosphoramidite methodology with an Applied Biosystems DNA Synthesiser (model 380A).

HPLC. For preparative HPLC a Gilson System (pump 303, gradient former 305 and Holochrome UV detector) was used. Analytical and semi-preparative HPLC were performed with a Kontron Instrument System consisting of a 420 Pump, a 425 Gradient Former, a 430 Dual wavelength detector and the 450 Data System (MT 450 Software). The following HPLC columns were used: Partisil M20 10/50 50 cm x 10 mm, 10 μ m (normal phase preparative work); Brownlee Aquapore RP Octyl 25 cm x 5 mm (oligonucleotide purification); Hichrom Partisil ODS-3 PXS 5/25 25 cm x 5 mm, 5 μ m; Jones Cartridge Spherisorb ODS2 25 cm x 5 mm, 5 μ m; Jones Cartridge Spherisorb Phenyl 15 cm x 5 mm, 5 μ m (all analytical work on deoxyguanosine derivatives); Hichrom Partisil ODS 50 cm x 5 mm, 10 μ m (deoxyguanosine derivatives).

Conditions 1: aliquots of 20 μ L were taken from the reaction of MNU with deoxyguanosine and analysed on the Phenyl column (see above) with monitoring at 254 nm. The eluent was pump A: 10 mM phosphate buffer pH 7.5 and pump B: 80% methanol in the phosphate buffer. The eluted fractions were characterised by on-line UV spectra (200 - 400 nm). Retention times were: ring-opened N⁷-Me species 2.5 - 4 min, dGuo 3.8 min, N¹-Me-dGuo 5.6 min, N⁷-Me-Gua 6.8 min, O⁶-Me-dGuo 8.2 min and N⁷-Me-dGuo 11.2 min.

Conditions 2: 4 x 0.8 mL of the mixture from a [¹³CH₃]MNU-dGuo reaction were analysed and fractionated on the 50 cm ODS column (see above) with gradient elution [t (min) / %B : 0/30, 15/35, 25/55, 33/80, 37/30, 45/end] using the same instrument and solvent system as Conditions 1. The product ratios were N¹-Me-dGuo : N⁷-Me-dGuo : O⁶-Me-dGuo = 1 : 2 : 1.

Chemicals. Chemicals were either AnalaR grade, which were used directly, or laboratory reagent grade purified further when appropriate. Solvents for HPLC were commercially available HPLC-grade. Isotopically labelled starting materials were purchased from Aldrich Chemical Company, Poole, U.K. or were gifts from Dr I. M. Lockhart (formerly B.O.C. Ltd., Morden, U.K.). The [¹³C]potassium cyanide contained 92 atom % ¹³C, the [¹³C]methylamine hydrochloride contained 99 atom % ¹³C and the [¹⁵N₂]urea was 96 atom % ¹⁵N. 0.5 M Hydrochloric acid and sodium hydroxide ['Convol' - a trade mark of Merck Ltd.] solutions were obtained by diluting the contents of a vial to 500 mL.

Buffer Solutions. The buffers (pH values given in Table 1 are \pm 0.01- 0.05 at 25 °C; all solutions 0.11 M ionic strength) were as follows:

- Phosphate buffers were prepared from mixtures of NaH₂PO₄·2H₂O and Na₂HPO₄·12H₂O (e.g. 30 mM NaH₂PO₄ + 20 mM Na₂HPO₄ to give 50 mM phosphate, pH 7.0).
- HEPES buffer solutions were prepared by dissolving HEPES sodium salt (2.8633 g) in distilled water (80 mL). Varying amounts of 0.5 M hydrochloric acid solution were added, followed by water (total volume = 100 mL).
- AMPSO buffers were prepared by dissolving AMPSO sodium salt (2.742 g) in distilled water (80 mL). Varying amounts of 0.5 M hydrochloric acid were added, followed by water (total volume = 100 mL).

Preparation of *N*-Methyl-*N*-nitrosourea (MNU) 1a. A solution of methylamine hydrochloride (0.5 g, 7.8 mmol) and potassium cyanate (0.75 g, 9.2 mmol) in water (3 mL) was boiled under reflux for 30 min and concentrated to dryness. To the residue was added propan-2-ol (5 mL). The mixture was refluxed for 30 min and filtered whilst hot. A further quantity of propan-2-ol (5 mL) was added to the undissolved solid and the process repeated. The combined filtrates were evaporated to dryness to give *N*-methylurea (approximately 90% yield), which was used without further purification.

To a solution of the *N*-methylurea (0.52 g) in water (5 mL) was added sodium nitrite (1.1 g, 15 mmol), with stirring and cooling in an ice-bath. Concentrated hydrochloric acid (1.6 mL, *ca.* 18 mmol) was added dropwise over about 25 min. A foamy white material formed on top of the solution. After stirring for a further 15 min, the solid was filtered off and washed quickly with a little water. The crude product was dried in high vacuum (0.55 g, 68 %). Recrystallization of the crude product was effected by dissolving it in the minimum volume of dichloromethane (*ca.* 1 mL per 10 mg), filtering, adding an equal volume of 60-80 petrol and cooling in ice for 1-2 hours. The fine white crystals were filtered off and dried under vacuum to give the title compound (0.38 g, 47 %) which was stored at -20 °C: no sharp m.p. (n.b. literature values span a 20 °C range); δ_{H} (D₂O) 3.16 (3H, s, CH₃), 5.71 (1H, br s, NH) and 6.90 (1H, br s, NH); δ_{C} 26.49 (CH₃).

Preparation of [¹³CH₃]*N*-Methyl-*N*-nitrosourea, ([¹³CH₃]MNU), 1b. The procedure described for the preparation of MNU was followed, starting with [¹³C]methylamine hydrochloride (0.50 g, 7.30 mmol) and potassium cyanate (0.75 g, 9.2 mmol) in water (3.0 mL) to give the title compound (0.55 g, 73%) after recrystallization: δ_{H} (D₂O) 3.19 (3H, d, $J^{13}\text{C-H}$ 141 Hz, ¹³CH₃) 5.55 (1H, br s, NH) and 6.90 (1H, br s, NH); δ_{C} 26.46 (¹³CH₃); *m/z* (EI) *M*⁺ 104.

Preparation of [¹³CO]*N*-Methyl-*N*-nitrosourea, ([¹³CO]MNU), 1c. The procedure described for the preparation of MNU was followed, starting with methylamine hydrochloride (0.35 g, 5.18 mmol) and potassium [¹³C]cyanate {prepared by oxidising potassium cyanide (0.34 g, 5.1 mmol) with potassium permanganate^{35,36}} in water (1.5 mL) to give the title compound (0.187 g, 35% based on potassium [¹³C]cyanide) after recrystallization: δ_{C} 26.8 (natural abundance Me), 156.9 (¹³CO).

Preparation of [¹⁵N]Potassium Cyanate. Potassium carbonate (345 mg, 2.5 mmol) and [¹⁵N]urea (350 mg, 5.6 mmol) were ground together in a porcelain crystallising dish. The mixture was heated with a Bunsen flame to give a dirty brown melt, with evolution of gas. Heating was continued until the melt began to foam again and became colourless. As the bubbling lessened the flame was removed. After cooling, the residual solid was dissolved in water (1 mL) and the solution was filtered. Ethanol (5 mL) was added to the filtrate and the resulting cloudy solution was stored in the freezer. The crystals were filtered off and the filtrate was evaporated to dryness. The residue was recrystallised from water(0.9 mL)-ethanol (2 mL) to give a crystalline solid (262 mg, 57%); IR (KBr disc); 3326 (m), 3224 (m), 3600 - 2800, 2153 (s, ¹⁵NCO⁻), 1453 (m), 1383 (m), 1290 (m), 1192 (m), 634 (m); ¹³C NMR (D₂O) δ 129.1 (d, 23 Hz, very broad); ¹⁵N NMR(D₂O) δ 57 (very broad). Later experiments with unlabelled material on the same scale using a platinum crucible gave a crude product containing much less carbonate (IR 1453 cm⁻¹) and in a higher yield (80%). It was crucial that the heating was stopped as soon as the melt became colourless.

Preparation of [¹⁵NH₂]*N*-Methyl-*N*-nitrosourea, ([¹⁵NH₂]MNU), 1d. A solution of [¹⁵NH₂]*N*-methylurea (0.39 g, 5.2 mmol) in water (3.5 mL) was added to sodium nitrite (0.85 g, 12.2 mmol). To the resulting solution, stirred and cooled in an ice-salt bath, conc. hydrochloric acid (1.2 mL, 14 mmol) was added

dropwise over 5-10 min. The mixture was stirred at ca. 0 °C for a further 15-20 min. The precipitate was filtered off, washed with ice-cold water and dried under reduced pressure to give the title compound (0.30 g, 55%), δ_N 61.5.

Decomposition of [$^{15}\text{NH}_2$]MNU. [$^{15}\text{NH}_2$]MNU was dissolved in dry methanol (0.25 mL) to give a 0.5 M solution and mixed with 0.625 M phosphate buffer in D_2O (1 mL), pH 7.2. The mixture was filtered into a NMR tube (sonicated with detergent and rinsed with water and methanol before drying). ^{15}N NMR data were acquired continuously, but stored every 2 h 45 min to give 6 individual spectra. Shift values for individual species were (*versus* 5 M NH_4Cl): 61.5 (br s, MNU), 59.0 (s, carbamoyl phosphate), 55.6 (br s, NCO^-), -0.9 (s, NH_4^+). After 2 weeks only the signal at -0.9 ppm was observed. Reference samples: MNU (d_4 -methanol): 58.9 (quintet, 14 Hz); 0.1M $^{15}\text{NH}_4\text{Cl}$ in 0.625 M phosphate pH 7.2/20% methanol: -0.9 (s); K^{15}NCO 0.5 M in 0.625 M phosphate pH 7.2/20% MeOH: ca. 55.5 (very broad with spike at 59 ppm).

Stoichiometry of MNU Decomposition in Aqueous Solution. One volume of 0.5 M MNU in methanol was added to 4 volumes of 25 mM phosphate buffer, pH 7.4; the final reaction mixture was thus 10% v/v in methanol, 20 mM in phosphate and 0.1 M in MNU. As the reaction proceeded, 0.5 M NaOH was added by the pH-stat; the pH was kept within ± 0.1 units. Consumption of approximately 1 equivalent of hydroxide was observed, indicating 1:1 stoichiometry rather than catalysis by hydroxide. The presence of the phosphate buffer was necessary because the pH-stat could not respond quickly enough in unbuffered solution, and added too much hydroxide initially, resulting in a large increase in pH and decomposition of the MNU within a few seconds. Since MNU was initially in 5-fold excess over the buffer, the buffer capacity of the reaction mixture was not enough to maintain constant pH by itself, hence addition of hydroxide by the pH-stat was required.

Reactions of MNU with Deoxyguanosine. 20 mM Deoxyguanosine in 25 mM phosphate buffer (1.5 mL) of the appropriate pH was thermostatted at 37 °C in a 3 mL vial containing a magnetic follower, and fitted with the burette and electrode of the pH Stat. The burette was filled with 0.5 N NaOH and the pH Stat set to 0.03 units below the desired pH. MNU (0.5 M in methanol, 0.6 mL) was added at such a rate that the pH was maintained within ± 0.05 units. As the reaction slowed down the pH Stat could be set to the exact pH value, which was maintained by the burette to ± 0.01 units. In reactions at lower pH, the pH started to drift upwards after ca. 12 h (owing to hydrolysis of KNCO) and was adjusted manually by occasional addition of dilute HCl. The total NaOH consumption was: at pH 9.5, 0.533 mL (after 15 min), at pH 7.5, 0.50 mL (after 105 min) and at pH 6.5, 0.338 mL (after ca. 20 h). The reaction at pH 6.5 was started at pH 5.5, but as hardly any reaction occurred it was adjusted to pH 6.5 after 3 h. The reactions were analysed by HPLC (conditions 1, see above).

Reaction of [$^{13}\text{CH}_3$]MNU with Deoxyguanosine. The reaction was carried out at pH 9.5 using 20 mM deoxyguanosine in 25 mM borate in D_2O (3 mL) and 0.5 M [$^{13}\text{CH}_3$]MNU in methanol (1.5 mL). The burette cylinder was removed from the pH Stat and a 2 mL plastic syringe filled with 0.5 N NaOH in D_2O was connected to the burette tubing and positioned so that the burette piston would depress the syringe plunger. At the end of the reaction (consumption of NaOD was 1.5 mL) the pH was adjusted to 8.6 and the methanol removed by a stream of nitrogen. The reaction products were analysed by HPLC (Conditions 2) and products were isolated using the same system. For NMR analysis the samples were extracted with D_2O (0.7 mL) and filtered (Acrodisc 45 μm) into a NMR tube. All three products were seen to contain the same impurities by ^1H NMR: 1.1 and 3.6 (multiplets); $\text{N}^1\text{-Me-dGuo}$: δ_{H} 2.46 (1H, m), 2.77 (1H, m), 3.40 (3H, d, J 142.1 Hz, $^{13}\text{CH}_3$, with small s, $^{12}\text{CH}_3$), 3.55-3.77 (2H + imp., m), 4.06 (1H, m), 4.56 (1H, m), 6.23 (1H, t, J 6.9 Hz), 7.92 (1H, s);

δ_C 28.85 (t, J 21.8 Hz, CH_2D), 29.05 (s, CH_3); δ_C (not decoupled): 30.94 (quartet, J 142.1 Hz). N^7 -Me-dGuo: δ_H 2.61 (2H, m), 3.58-3.80 (2H + imp., m), 4.02 (d, J 145.3 Hz and small s, 3H), 4.12 (1H, m), 4.54 (1H, m), 6.31 (1H, t); δ_C 35.74 (t, J 22.1 Hz, CH_2D), 35.94 (s, CH_3). O^6 -Me-dGuo: δ_H 2.47 (1H, m), 2.75 (1H, m), 3.42-3.79 (m), 4.02 (3 H, d, J 148.1 Hz and small s), 4.10 (1H, m), 4.58 (1H, m), 6.30 (1H, t), 8.02 (1H, s); δ_C 39.2, 54.70 (t, J 22.7 Hz, CH_2D), 54.94 (s, CH_3), 71.8 (s), 84.7 (s), 87.7 (s).

Reactions of [$^{13}CH_3$]MNU with Deoxyguanosine-5'-monophosphate.

a) *Reaction of [$^{13}CH_3$]MNU with dGuo-5P in water.* A solution of dGuo-5P (5.1 mg, 0.012 mmol) in water (1 mL), pH 8.12, was thermostatted at 37 °C in a 3 mL vial containing a magnetic follower and fitted with the electrode of the pH Stat. A drop of a solution of [^{13}C]MNU (26.5 mg, 0.26 mmol) in HPLC grade methanol (0.5 mL) at 37 °C was added *via* syringe, followed a solution of 0.5 N 'Convol' NaOH added dropwise *via* a microsyringe to maintain the pH of the reaction mixture at *ca.* 8.0. A total volume of NaOH solution (0.420 mL) was used. The reaction mixture was stirred at 37 °C for 2 h. The water was removed by freeze-drying to give a white solid (20 mg): 1H NMR spectrum (500 MHz, D_2O) similar to that described under c) below, but with all methyl singlets showing as doublets; δ_C 25.95, 26.28, 26.72, 27.90, 35.86 and 35.93, 53.62 and 53.62 and 53.06, 54.70 and 71.49; δ_P 1.7 (d, $J_{13C-31P}$ 5 Hz), 4.1 (br s).

b) *Reaction of [^{13}C]MNU with dGuo-5P in D_2O .* The procedure was as described for the corresponding reaction in water: dGuo-5P (5.4 mg, 0.014 mmol) in D_2O (1 mL) at 37 °C, pH 8.30 and [^{13}C]MNU (26.8 mg, 0.26 mmol) in HPLC grade methanol (0.5 mL). A total volume of 0.5 M NaOH in D_2O (0.480 mL) was added. The pH of the reaction mixture was 9.0 - 7.9, and finally 8.78 ± 0.10 . The product was a white solid (10 mg) showing similar NMR spectra to those described under a) above, but with CH_2D doublets in addition to methyl singlets.

c) *Reaction of MNU with dGuo-5P in water:* The procedure was similar to that described above under a) [dGuo-5P (5.5 mg, 0.014 mmol) in water (1 mL) at 37 °C; MNU (26.8 mg, 0.26 mmol) in HPLC grade methanol (0.5 mL); 0.5 M NaOH in water added (0.375 mL)]. The pH of the reaction mixture was 9.24 - 7.90, and finally 9.24 ± 0.10 . The product was a white solid (10 mg): δ_H (500 MHz, D_2O) 2.55 (m), 2.67 (s), 2.80 (m), 2.77 (s), 2.81 (s), 3.48 (d, J 10.8 Hz, $^{13}CH_3$ -OP), 3.50 (d), 3.75 (s), 3.77 (s), 3.82 (t), 4.02 (t), 4.09 (s), 4.10 (s), 4.20 (m), 6.31 (2 \times t), 6.38 (t), 8.05 (s, imidazole H), 8.15 (s, imidazole H).

d) *Reaction of MNU with dGuo-5P in D_2O .* The procedure was similar to that described above under a) [dGuo-5P (5.5 mg, 0.014 mmol) in D_2O (1 mL) at 37 °C; MNU (26.8 mg, 0.26 mmol) in HPLC grade methanol (0.5 mL); 0.5 M NaOH in D_2O added (0.50 mL)]. The pH of the reaction mixture was 8.56 - 7.90, and finally 8.30 ± 0.10 . The product was a white solid (10 mg) showing similar NMR spectra to those described under c) but with CH_2D doublets in addition to methyl singlets.

Reaction of Sodium Phosphate with [^{13}C]MNU in Water. To a solution of $Na_2HPO_4 \cdot 12H_2O$ (9.2 mg, 0.26 mmol) in water (1 mL), which was incubated at 37 °C, a solution of [^{13}C]MNU (26.8 mg, 0.26 mmol) in methanol (0.5 mL) was added dropwise. The pH of the reaction mixture was maintained in the range 8.2 - 9.25 by slowly adding 0.5 M NaOH solution (0.5 mL) during addition of MNU. The reaction was stirred at 37 °C for 2 h. The water was removed by freeze-drying to give a white solid (25 mg): δ_H 2.25 and 3.0 (3H, d, J_{H-13C} 138 Hz, CH_3), 3.05-3.10 - 3.78-3.81 (3H, dd, J_{H-13C} 144 Hz, J_{P-H} 10 Hz, $^{13}CH_3$ -OP), 3.19-3.21 - 3.90-3.95 (3H, dd, J_{H-13C} 147 Hz, J_{P-H} 10.7 Hz, $^{13}CH_3$ -OP); δ_C 25.0 (weak), 51.9 ($^{13}CH_3$) and 53.4 ($^{13}CH_3$); δ_P 3.75 (br), 3.98 (t) and 5.90 (d).

Synthesis of 5'-(dGTGCAC)-3' by Phosphoramidite Methodology. The column size was 1 μmol and for larger scale work multiple runs were performed. The crude oligonucleotides were purified in batches of six by reverse phase HPLC (Brownlee Aquapore RP-octyl, 0.1 M ammonium acetate, 0 - 20% acetonitrile), each batch yielding 3-4 mg of the hexamer as determined by UV absorption at 260 nm. The purified oligonucleotides were desalted either by repeated freeze-drying or by chromatography on Sephadex G10, followed by cation exchange on Dowex 50W (Na^+ -form) to replace ammonium with sodium ion. The ^1H NMR of the resultant oligonucleotide was in good agreement with literature data.³⁷ Samples contained variable amounts of acetate and isobutyramide, which were assumed not to affect subsequent reactions.

Preparation of [^{15}N]5'-O-Dimethoxytrityl-N²-isobutyryl-2'-deoxyguanosine **4c.** ^{15}N -labelled deoxyguanosine monohydrate (mainly **4a**) was dried by dissolving in DMF and removal of the solvent. The labelled guanosine was converted into the N²-isobutyryl derivative **4b** in the standard manner.³⁸ N²-Isobutyryl-[^{15}N -1]deoxyguanosine (101.5 mg, 0.3 mmol) was dried by dissolution in dry pyridine (2 mL) and removal of the solvent. The residue was taken up in dry pyridine (2 mL) and 4,4'-dimethoxytrityl tetrafluoroborate (234 mg, 0.6 mmol)³⁹ was added. The orange solution was stirred at room temperature under nitrogen for 3 h. The resulting yellow mixture was poured into ice-cold saturated sodium bicarbonate and the product was extracted with dichloromethane (3 \times 25 mL). The combined extracts were washed with brine, dried with sodium sulphate and concentrated to give a dark yellow oil (0.43 g). Flash chromatography on silica (30 g) eluting with initially 5%, then 10% methanol in dichloromethane containing a trace of triethylamine, gave pure **4c** as a white solid (106 mg, 55%): δ_{H} (CDCl_3) 0.94 (3 H, d, J 6.8 Hz), 1.02 (3 H, d, J 6.8 Hz), 1.61 (3 H, s, OH), 2.22 (1H, septet, J 6.8 Hz), 2.39 (1 H, m), 3.30 (2 H, m), 3.67 (6 H, 2, s), 4.08 (1 H, m), 4.65 (1H, m), 6.14 (1 H, m), 6.69 (4 H, m), 7.09-7.36 (9 H, m), 7.72 (1H, s), 8.74 (0.74 H, s, NH_2), 8.74 (0.26 H, d, J 92 Hz), $^{15}\text{NH}_2$), 12.06 (0.31 H, s, HN-1), 12.06 (0.69 H, d J 100.8 Hz, H ^{15}N -1).

Preparation of Phosphoramidite **4d.** [^{15}N -1]5'-O-Dimethoxytrityl-N²-isobutyryl-2'-deoxyguanosine (320 mg, 0.5 mmol) was dissolved in dry acetonitrile (4 mL) and the solution was concentrated. The residue was taken up in dry CDCl_3 (1.25 mL) under argon. A solution of tetrazole in acetonitrile (0.5 M, 0.80 mL, 0.4 mmol) was added followed by neat cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.205 mL, 0.68 mmol) and dry acetonitrile (0.45 mL). After a few minutes a precipitate had formed. An aliquot was taken for NMR analysis, whilst the remainder was stirred under argon overnight. The reaction mixture was filtered (Acrodisc 45 μ) under argon, and diluted with dry acetonitrile (4.4 mL) to give a *ca.* 0.05 M solution of **4d** which was blanketed with argon and used directly on the DNA synthesiser: δ_{P} ($\text{CH}_3\text{CN}/\text{CDCl}_3$) at 0 h reaction time 149.2/149.4 (80%, 2 s, **4d**), 139.7 (3.5%, s, 3',3'-dimer), 14.8 (14%, s, hydrolysed reagent); 16 h reaction time 149.2/149.4 (71%), 139.7 (2%), 14.8 (17%), 0.44 (3.6%).

Synthesis of 5'-d(GTG*CAC)-3'. The solution of phosphoramidite **4d** was filtered prior to being placed on the synthesiser at 'position X'. The best coupling yields obtained were 70%. The labelled oligonucleotides were cleaved and deprotected in the usual way with crude yields (as A_{260}) decreasing from 58% to 40% for the last columns. The oligonucleotides were purified by reverse phase HPLC, desalted by passing through Sephadex G10 twice, converted into the sodium salt and freeze-dried. The yield from six columns of GTG*CAC was 2.16 mg (36% by measurement of A_{260}). The ^1H NMR was identical to that of unlabelled GTGCAC. The ^{15}N NMR, accumulated for 48 h with inverse gated decoupling (in D_2O , vs NH_4Cl) showed δ 126 (s, N-1) and *ca.* 54 (weak, NH_2) ppm.

Reactions of MNU with d(GTGCAC). The oligonucleotide d(GTGCAC) (2–4 mg) in water or D₂O (0.6–1.5 mL) was alkylated with 0.5 M MNU in methanol (0.33–0.754 mL, 130-fold excess) at pH 8 in a similar manner to that described for the MNU-deoxyguanosine reaction (NaOH or NaOD as appropriate). After the reaction, methanol was removed, and the pH was adjusted to *ca.* 7.5. The solutions were filtered and freeze-dried. Samples for NMR were redissolved in 1.5 mL water, filtered through 0.5 mL Duolite ES-466 (rinsing twice with water) and the pH of the filtrate (> 10) adjusted to 7.5 before freeze-drying.

a) *MNU and d(GTGCAC) in H₂O.* 3 mg oligonucleotide in 0.6 mL water was treated with 0.33 mL MNU.

b) *MNU and d(GTGCAC) in D₂O.* 3.7 mg oligonucleotide in 0.8 mL D₂O was treated with 0.4 mL MNU: δ_{H} (D₂O) the following singlets were observed amongst the unresolved oligo signals: 2.71 (main signal, possibly Me-NH₃⁺), 2.69, 2.79, 2.84, 3.21, 3.79, 3.81, 3.88.

c) *[¹³CH₃]MNU and d(GTGCAC) in D₂O.* 4.5 mg oligonucleotide in 1.5 mL D₂O was treated with 0.75 mL [¹³CH₃]MNU: δ_{H} (D₂O) 1.95 (s), 2.71 (main signal, d, *J* 139 Hz, with a cluster of smaller doublets around it), 3.21 (d, *J* 143 Hz), 3.77 (s), 3.81 (s); δ_{C} 129 (very broad, cyanate), 82, 55.42, 54.49, 52.90, 35.81, 28.07, 27.79, 26.56, 26.12, 25.96, 25.81 (main peak), 25.59, 25.42.

Acid hydrolysis of methylated oligonucleotides with Dowex 50 resin (H⁺ form) followed by elution from the resin with water and treatment with aqueous ammonia gave a mixture of purine and pyrimidine bases which were analysed by mass spectrometry.

Kinetic Studies. The rates of MNU decompositions in aqueous solution were determined using a Kontron Instrument Uvikon 810 spectrophotometer with a thermostat set at 25 °C. Phosphate, HEPES and AMPSO buffers (ionic strength 0.11 M) in water were employed. A 10 mM stock solution of MNU (5.1 mg) in dry methanol (5 mL) was prepared and stored at -20 °C, inside a larger closed tube containing silica gel, under which conditions the solution was stable for several days. In a typical experiment, 3 mL of the diluted phosphate buffer (1/100 dilution of a mixture of 78.5 mM NaH₂PO₄ and 10.7 mM Na₂HPO₄ solution containing 0.11 M NaCl, pH 5.63) was placed in a 5 mL plastic syringe and the MNU stock solution (50 μ L) was added. The resulting solution (2.0 mL) was quickly mixed in the syringe with the buffer solution (2.0 mL) of required pH and immediately placed in a 1 cm width Quartz cuvette in the UV spectrophotometer. The absorbance of MNU was monitored at λ_{max} 235 nm at 6 or 12 second intervals (80–130 readings). For experiments 1–4, phosphate buffer in the pH range 6.5–7.8 was alone used. For experiments 5–13, the above procedure was employed (i.e. pre-mixing of MNU in phosphate followed by addition to HEPES or AMPSO). For experiments 14 and 15, AMPSO buffer in the pH range 9.5–10.0 alone was used. All experiments were done in triplicate and the mean values (pH, k_{obs} , [OH⁻]) were tabulated in Table 1. The pH of each mixture was determined at the end of an experiment.

RESULTS AND DISCUSSION

Synthesis of Isotopically Labelled MNU Samples (1b–1d). These were prepared using the same procedure as that employed to make unlabelled MNU, but from an appropriately labelled starting material. Thus, a specifically labelled *N*-methylurea was obtained from methylamine hydrochloride and potassium cyanate,^{40–42} one of these being labelled with either ¹³C or ¹⁵N. For these syntheses, the [¹³C]methylamine hydrochloride was obtained commercially, [¹³C]potassium cyanate was prepared by oxidizing [¹³C]potassium

cyanide with potassium permanganate,^{35,36} whilst [¹⁵N]potassium cyanate was obtained by fusing [¹⁵N₂]urea with potassium carbonate.⁴³ Nitrosation of each of the *N*-methylureas gave the corresponding specifically labelled MNU (**1b-1d**), which was characterised by NMR spectroscopy.

Kinetic Studies of Decompositions of MNU in Aqueous Buffers. To enable reactions of MNU and its decomposition products with nucleosides and nucleotides to be studied under optimum conditions, the instability of MNU under aqueous conditions was investigated. Kinetic studies in aq. phosphate buffer (50 mM, pH 7.0) gave k_{obs} (25 °C) = $7.8 \times 10^{-5} \text{ s}^{-1}$, in agreement with a previous value.²¹ However, preliminary NMR observations on unbuffered solutions had indicated a slower, non-first order rate of decomposition, accompanied by a significant drop in pH. This is not in accord with the mechanism of Scheme 2, in which the rôle of hydroxide is purely catalytic. The stoichiometry of the reaction was therefore investigated by allowing MNU to decompose in a pH-stat and monitoring the consumption of hydroxide. Methanol-aq. phosphate buffer [20% (v/v), 20 mM in phosphate) was found to be suitable for achieving relatively high concentrations of MNU (cf. ref. 44), and the pH could be kept within ± 0.1 units by using a pH-stat. With a starting pH of 7.4 an initial uptake of ca. 1 equivalent of hydroxide occurred, an observation which is incompatible with the mechanism of Scheme 2, in which ammonium bicarbonate is generated [pH = 7.8 for a 0.1 M solution⁴⁵]. However, cyanic acid produced by the mechanism of Scheme 1 should cause the pH of the medium to fall. Thus, for the pH of the medium to remain constant under pH-stat conditions, the neutralization of the cyanic acid [pK_a 3.9⁴⁶] by addition of base is required, as was observed.

Rates of decomposition of MNU in aqueous buffers were determined as a function of pH (range 6.5-7.8 in phosphate; 7.4-7.7 in HEPES; 8.2-9.9 in AMPSO). The data were analysed in terms of Scheme 1, where the ionization of MNU is a pre-equilibrium and the decomposition of the MNU anion (MNU⁻) is rate limiting.

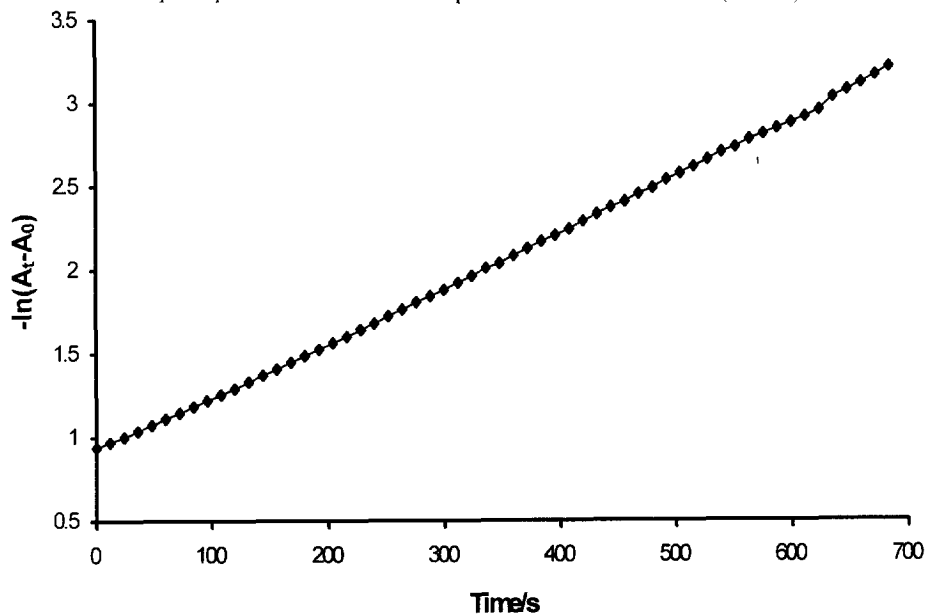


Figure 1: Plot of $-\ln(A_t - A_0)$ against time t for decomposition of MNU in AMPSO buffer (pH 8.47), where A_t and A_0 are MNU absorbance at time t and time 0, respectively.

Table 1: Kinetic Data for MNU Decomposition in Buffered Aqueous Solution.

Expt. No.	Buffer	pH \pm 0.1	$10^3 k_{\text{obs}} / \text{s}^{-1}$	$10^7 [\text{OH}^-] / \text{M}$
1	Phosphate	6.45	0.02	
2	Phosphate	6.98	0.08	
3	Phosphate	7.30	0.14	
4	Phosphate	7.59	0.25	
5	Phosphate	7.79	0.43	
6	HEPES	7.35	0.33	
7	HEPES	7.73	0.83	
8	AMPSO	8.19	2.38	15.5
9	AMPSO	8.38	3.47	24.0
10	AMPSO	8.68	5.54	47.9
11	AMPSO	8.83	6.39	67.6
12	AMPSO	9.08	11.81	120
13	AMPSO	9.30	15.94	200
14	AMPSO	9.52	19.94	331
15	AMPSO	9.68	21.71	479
16	AMPSO	9.95	24.06	891

The kinetic behaviour is therefore analogous to that of the classic system in which diacetone alcohol $[\text{Me}_2\text{C}(\text{OH})\text{CH}_2\text{COME}]$ undergoes a base-induced fragmentation *via* its anion $[\text{Me}_2\text{C}(\text{O}^-)\text{CH}_2\text{COME}]$ to two species - acetone and the anion of acetone.⁴⁷ As required by the analysis given, linear plots were obtained for the logarithm of MNU concentration against time (see Figure 1 for a representative plot) and gave the k_{obs} values in Table 1. These data are in reasonable agreement with the literature²¹ [data for the pH range in borate buffer were presented].

The rate law associated with Scheme 1 is as given in eqn. 1, according to which a plot of k_{obs} *versus* $[\text{OH}^-]$ should appear linear at low $[\text{OH}^-]$, but should curve at higher values, reaching a maximum limiting value at which $k_{\text{obs}} = k$. By contrast, Scheme 2 predicts a linear dependence on $[\text{OH}^-]$ ($k_{\text{obs}} = k'[\text{OH}^-]$). It is noteworthy that Snyder and Stock²¹ report that $k_{\text{obs}}/[\text{OH}^-]$ (which should be a constant according to Scheme 2) decreases "by a factor of 2 between pH 7 and 10".

$$k_{\text{obs}} = kK[\text{OH}^-]/(1 + K[\text{OH}^-]) \quad (\text{eqn. 1})$$

$$1/k_{\text{obs}} = 1/kK[\text{OH}^-] + 1/k \quad (\text{eqn. 2})$$

Eqn. 1 can also be expressed in a linear form (eqn. 2). A plot of $1/k_{\text{obs}}$ *versus* $1/[\text{OH}^-]$ for the data in Table 1 at pH > 8 is shown in Figure 2. The data give a good straight line fit from which values of $k = (0.025 \pm 0.004) \text{ s}^{-1}$ and $K = (6.66 \pm 0.2) \times 10^4 \text{ M}^{-1}$ can be calculated.* The value of K corresponds to a $\text{p}K_{\text{a}}$ for MNU of 9.2. Scheme 1 implies that the decomposition of the methyldiazoate anion should be fast compared to the fragmentation of the MNU anion. The kinetics of decomposition of (*E*)-methyldiazoate in aqueous solution have been reported.⁴⁸ From the data given in ref. 48 the observed rate constant for decomposition of the

* The data of ref. 21 also give a good straight line fit when analyzed according to eqn. 2. The calculated parameters are then $k = 0.13 \text{ s}^{-1}$, $K = 7.7 \times 10^3 \text{ M}^{-1}$ and $\text{p}K_{\text{a}} = 10.1$. The differences from the results obtained in the present work can in part be attributed to the use of borate buffer in the work of ref. 21.

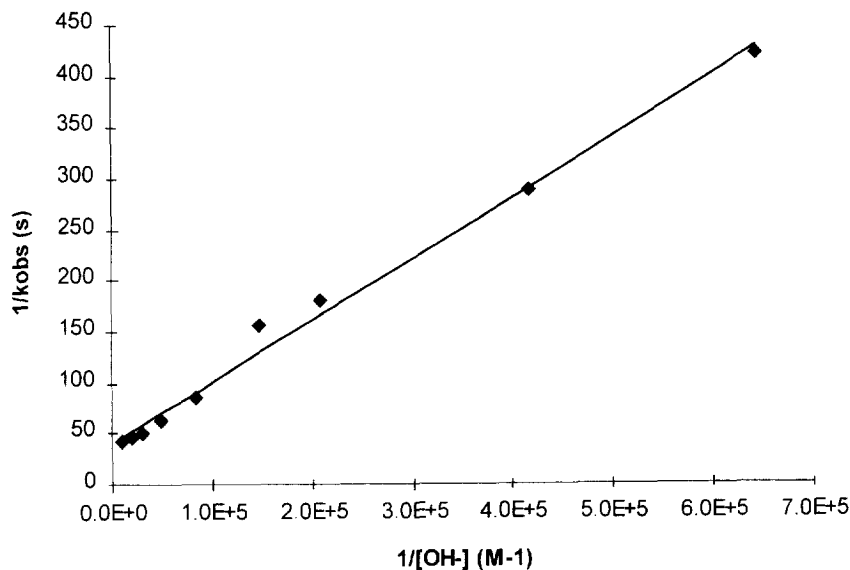


Figure 2: Kinetic data from Table 1 (pH > 8) plotted according to eqn. 2.

diazotate is $> 0.1 \text{ s}^{-1}$ at pH < 10 and ionic strength 1.0 M, faster than the value of k calculated above by a factor of 4.

NMR Analyses of Decompositions of MNU Samples (1a - 1d) in Aqueous Phosphate and Borate Buffers. The presence of only a limited amount of information in ^1H NMR spectra of MNU made it necessary to enrich MNU isotopically with ^{13}C and ^{15}N , in order for its aqueous chemistry to be monitored. That cyanate is an initial product of the decomposition of MNU in water was shown using $[^{13}\text{CO}]\text{MNU}$ and ^{13}C NMR. Thus, a 0.1 M solution of $[^{13}\text{CO}]\text{MNU}$ in 20% methanol-0.5 M aq. phosphate buffer (pH 7) at 22 °C showed the formation of cyanate (broad triplet, δ 128.9) immediately (monitoring by 125 MHz ^{13}C NMR). Up to *ca.* 4 hours reaction time this resonance gradually increased, but then decreased. Following the production of cyanate there was observed a doublet at δ 157.0 ($^2J_{\text{C-O-P}}$ 5 Hz), assigned to carbamoyl phosphate. It is known that cyanate reacts with phosphate to give carbamoyl phosphate.⁴⁹ Eventually this resonance diminished whilst resonances from bicarbonate/carbon dioxide (δ 160.6 and 125, respectively) and methylcarbonate (δ 160.5) increased. In an experiment conducted at 35 °C, after 10 hours both cyanate and carbamoyl phosphate had disappeared, leaving bicarbonate/carbon dioxide, methylcarbonate and a minor species showing δ 163 [assigned to urea, reported δ 161.2⁵⁰] as the only detectable products.

The results described were confirmed by ^{15}N NMR which showed the disappearance of the resonance at δ 61.5 from $[^{15}\text{NH}_2]\text{MNU}$ in parallel with the appearance of a resonance from cyanate at δ 55.6 in agreement

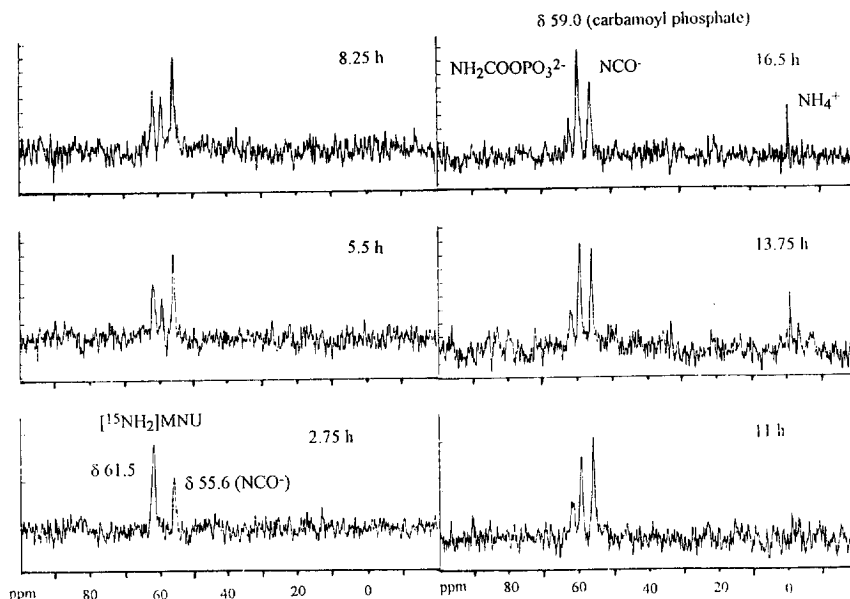


Figure 3: ^{15}N NMR spectra of the decomposition of $[\text{}^{15}\text{NH}_2]\text{MNU}$ **1d** in 20% methanol - 0.5 M phosphate buffer (pH 7) over 16.5 hours (n.b. the peak at δ 81.5 is MNU, at 55.6 CNO^- , at 59.0 $\text{NH}_2\text{CO}_2\text{PO}_3^{2-}$, and at -1.0 NH_4^+).

with the value obtained for an authentic sample of potassium $[\text{}^{15}\text{N}]$ cyanate. At 22 °C, the latter increased up to 8 hours reaction time, but then decreased whilst a resonance at δ 59.0 due to carbamoyl phosphate increased. Figure 3 shows the composition of the reaction at 16.5 h, when MNU, carbamoyl phosphate, cyanate and ammonium ion are all evident. After 2 weeks only a single resonance at δ -1.0, from the ammonium ion, was observed.

A mechanism consistent with the results described involves deprotonation of MNU at its amido function to give an anion of MNU that fragments to cyanic acid and methylidiazotate (see Introduction and Scheme 1).

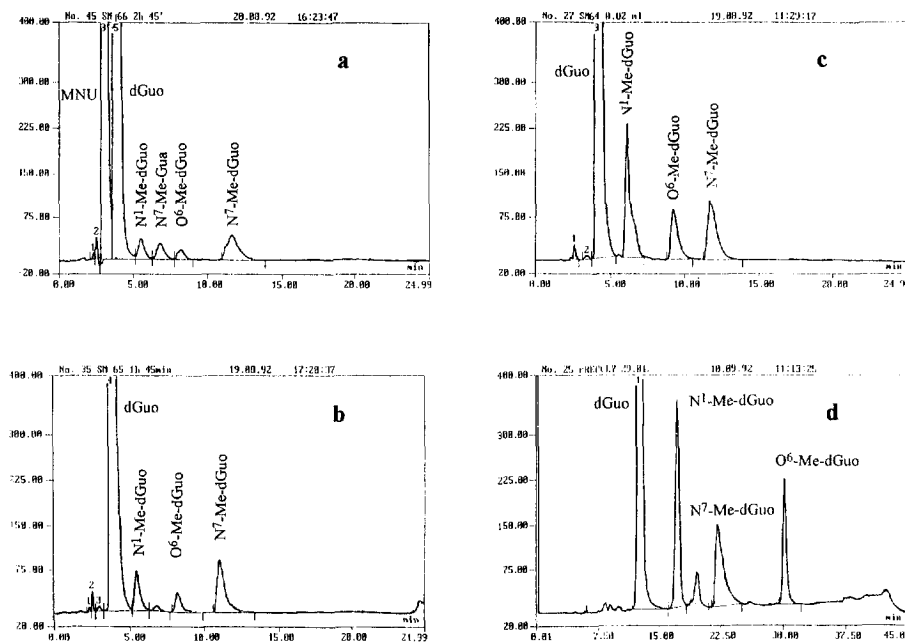
Reactions of Deoxyguanosine with MNU: HPLC Analyses and Isolation of Methylated Deoxyguanosines. The reaction of deoxyguanosine with MNU was investigated at different pH values. To achieve sufficient levels (5-10%) of methylation of deoxyguanosine it was necessary to use a large excess (10 molar equivalents) of MNU because most of the MNU was hydrolysed to methanol. The extent of methylation was pH-dependent and higher yields of methylated deoxyguanosines were obtained at higher pH (see Table 2). At pH 9.5, no MNU could be detected after 10 minutes, whereas at pH 7.5 it persisted for several hours. In phosphate buffer the main products were methyl and dimethyl phosphate, together with products of deoxyguanosine methylation. Monitoring reactions in the pH range 6.5-9.5 by RP-HPLC (Phenyl or ODS column) showed the formation of four principal products with elution times greater than that of deoxyguanosine (see Figures 4a-d). The peaks were identified as N^1 -Me-dGuo, N^7 -Me-Gua, N^7 -Me-dGuo and O^6 -Me-dGuo,

Table 2: Effect of pH on the Methylations of dGuo by MNU.

pH	time for MNU disappearance/h	ratio of <i>N</i> ¹ -Me-dGuo: <i>N</i> ⁷ -Me-dGuo: <i>O</i> ⁶ -Me-dGuo	estimated yield (%) of <i>N</i> ¹ -Me-dGuo: <i>N</i> ⁷ -Me-dGuo: <i>O</i> ⁶ -Me-dGuo
6.5	28	1.1 : 8.9 : 1	1.1 : 9.6 : 1
7.5	1.75	1.0 : 5.1 : 1	2.1 : 10.7 : 2.1
9.5	0.25	1.1 : 1.8 : 1	6.1 : 9.9 : 5.5

respectively, by comparison of their NMR [e.g. δ_{C} 55.9 (Me) for *O*⁶-Me-dGuo; δ_{C} 37.0 (Me) for *N*¹-Me-dGuo] and UV data, and stability to aqueous acid and base, with information for authentic standards.⁵¹ The overall extent of methylation and the ratio of N-1/*O*⁶ to N-7 methylated products, was significantly greater at higher pH (9.5 > 7.5 > 6.5): see Table 2. These results can be explained as a consequence of the deprotonation at N-1 ($\text{p}K_{\text{a}}$ 9.5), which selectively increases the reactivity of N-1 and *O*⁶.

*N*⁷-Me-dGuo is expected to undergo base-induced opening of the imidazole ring.⁵² In reactions at pH 9.5 several peaks which eluted before deoxyguanosine, each having the same UV spectrum (λ_{max} at 215 - 218 nm with a second maximum at *ca.* 272 nm), appeared at longer reaction times. These absorption values are in good agreement with those reported⁵³ for ring-opened *N*⁷-Me-dGuo. The latter has been shown to undergo isomerization of the sugar moiety⁵⁴ and this could account for the number of peaks observed.

**Figure 4:** HPLC chromatograms [conditions 1 for a) - c); conditions 2 for d)] for the reaction of MNU with dGuo at different pH's.

a) pH 6.5 at 2.75 h; b) pH 7.5 at 1.25 h; c) pH 9.5 at 0.25 h; d) pH 9.5, preparative scale reaction.

NMR Analysis of Reactions between MNU Samples (1a and 1b) and Phosphate, Deoxyguanosine and Deoxyguanosine 5'-Monophosphate. To enable methylation products from MNU to be more easily identified by ^{13}C NMR, we have prepared $[^{13}\text{CH}_3]\text{MNU}$ **1b** and studied its reactions with phosphate, deoxyguanosine, deoxyguanosine 5'-monophosphate, and d(GTGCAC). With phosphate in methanolic D_2O , the main product was methanol (*ie* $^{13}\text{CH}_3\text{OH}$), with additional products characterised by ^{13}C NMR as methyl phosphate (δ 51.7, d, $J_{\text{C-O-P}}$ 5 Hz), dimethyl phosphate (δ 52.7, d, $J_{\text{C-O-P}}$ 5.5 Hz) and methyl carbonate (δ 59.8), respectively. The methyl groups in the methyl phosphates were extensively deuterated, owing to rapid equilibration of the methyl diazonium ion with diazomethane (28). With deoxyguanosine in D_2O at pH 9.5, the important signals from ^1H NMR are the ones arising from the $^{13}\text{CH}_3$ group: $\text{N}^1\text{-Me-dGuo}$ showed a doublet at 3.40 ppm (J 142.1 Hz), $\text{N}^7\text{-Me-dGuo}$ a doublet at 4.02 ppm (J 145.3 Hz) and $\text{O}^6\text{-Me-dGuo}$ a doublet also at 4.02 ppm (J 148.1 Hz) with partial deuteration at the methyl group. The ^{13}C NMR also showed the main methyl signals for these compounds: δ 29.05 for $\text{N}^1\text{-Me-dGuo}$, 35.94 for $\text{N}^7\text{-Me-dGuo}$ and 54.94 for $\text{O}^6\text{-Me-dGuo}$. With deoxyguanosine 5'-monophosphate, methylation at phosphate group is a major process. ^1H NMR showed the main signal at δ 3.45 as a double doublet ($J_{\text{P-H}}$ 11 Hz and $J_{^{13}\text{C-H}}$ 147 Hz) assigned to a methylphosphate group (see Figure 5).

Synthesis and Characterization of d(GTGCAC) and d(GTG*ACAC). The self-complementary oligonucleotide 5'-d(GTGCAC)-3' contains two G residues which allow the effect of environment on G reactivity to be explored. It was synthesised by phosphoramidite methodology and characterised by mass

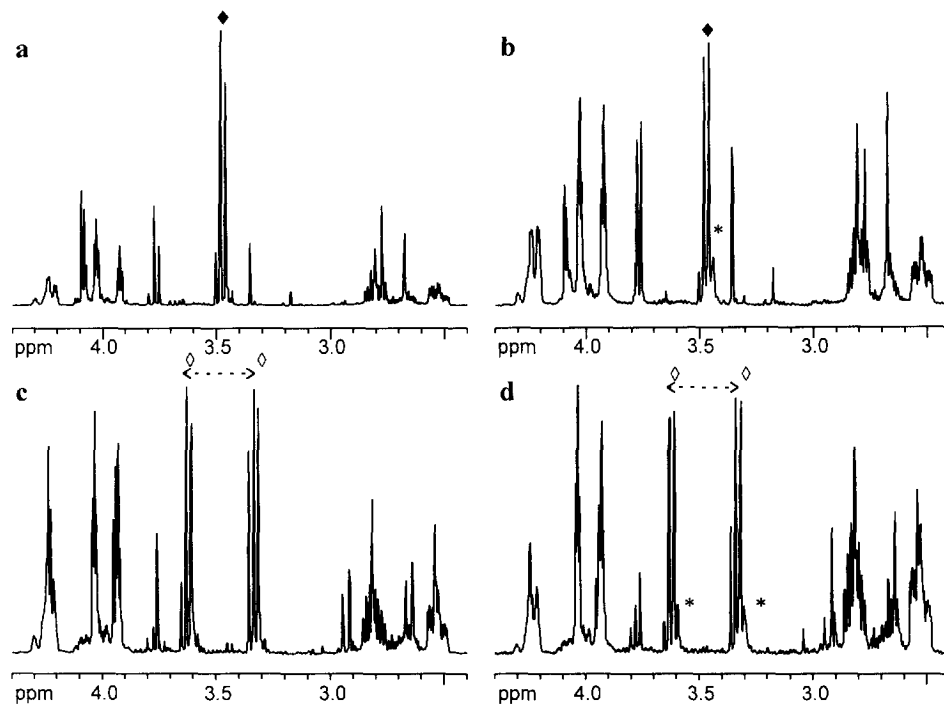


Figure 5: ^1H NMR spectra for reactions of MNU with dGuo-5P. a) **1a** + dGuo-5P in H_2O ; b) **1a** + dGuo-5P in D_2O ; c) **1b** + dGuo-5P in H_2O ; d) **1b** + dGuo-5P in D_2O [\blacklozenge = $^{12}\text{CH}_3\text{-OP}$, \circ = $^{13}\text{CH}_3\text{-OP}$, $*$ = $\text{CH}_2\text{D-OP}$, <-----> = $^1J(^{13}\text{C-H})$].

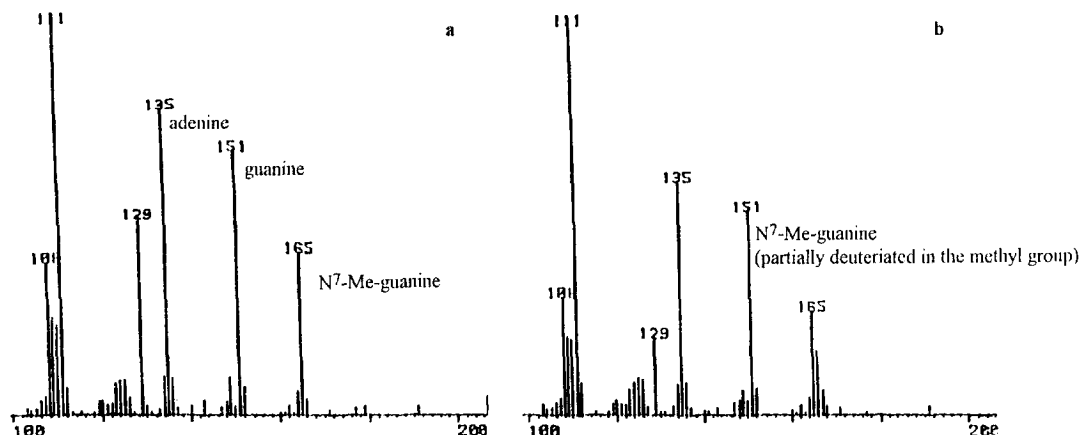


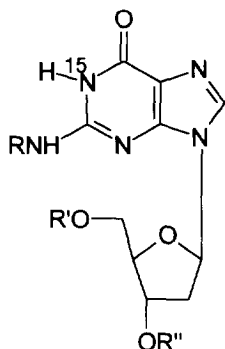
Figure 6: EI mass spectra of *N*⁷-methylguanines from a hydrolysate of the methylated oligonucleotides obtained by reacting d(GTGCAC) with MNU. a) in H₂O; b) in D₂O.

spectrometry and NMR. The positive ion FAB spectrum (in *p*-toluenesulfonic acid/glycerol matrix) showed a cluster of peaks around 1793 ($MH^+ = 1792$) and little fragmentation. The negative ion spectrum (in aminoglycerol) showed a molecular ion cluster and fragments as follows: *ca.* 1582 (GTGCA-P), 1268 (GTGC-P), 979 (GTG-P) *ca.* 1542 (P-TGCAC), 1238 (P-GCAC) and 908 (P-CAC). The d(GTGCAC) was hydrolysed in water (100 °C/ 45 min) using Dowex 50 resin (H⁺ form) as catalyst. The resin was washed with water and then ammonia. The EI mass spectrum of the aqueous extract showed a strong ion at 126 (T). The EI mass spectrum of the concentrated ammonia extract was very clean and showed ions at 151 (G), 135 (A) and 111 (C).

[¹⁵N]Deoxyguanosine **4a** [containing *ca.* 75% [1-¹⁵N]- and 20% [*amino*-¹⁵N]-deoxyguanosine⁵⁵] was converted into *N*²-isobutyryldeoxyguanosine **4b**. ¹H NMR analysis of **4b** showed 69% ¹⁵N at N-1 and 26% ¹⁵N at 2-NH₂, with a total ¹⁵N enrichment of 95% (corresponding to the isotopic content of the precursor deoxyguanosine: see ref 55). Reaction of **4b** with dimethoxytrityl tetrafluoroborate³⁹ in pyridine gave the protected deoxyguanosine **4c**, which was transformed into the cyanoethyl phosphoramidite **4d**. GTG**CAC* labelled with ¹⁵N at the central G (designated G*) was prepared from **4d** placed in the 'X' position of the DNA synthesiser. The ¹H NMR of the labelled oligonucleotide was identical to that of unlabelled material.

Analysis of Reactions of d(GTGCAC) and d(GTG**CAC*) with MNU Samples (1a and 1b).

Reactions of MNU and GTGCAC were performed in unbuffered water or D₂O at 37 °C using *ca.* 130 equivalents of MNU (0.5 M in methanol). Under these conditions the only buffering capacity was provided by the phosphate groups of the oligonucleotide (0.8-1.25 mM) and control of the pH was difficult, especially for small scale reactions. A minimal initial volume of 0.6 mL was required to ensure adequate performance using the pH-stat. The methylation of the hexamer d(GTGCAC) by [¹³CH₃]MNU **1b** was monitored directly by ¹³C NMR. This approach has been used with DNA⁵⁶ and RNA.⁵⁷ However, the ¹H and ¹³C NMR spectra of the freeze-dried reaction mixture were both complex indicating the presence of several base- and phosphate-methylated species, as well as methylamine. The oligonucleotide used was an ammonium salt, so ammonia



4a $R = R' = R'' = H$

4b $R = Me_2CHCO, R' = R'' = H$

4c $R = Me_2CHCO,$
 $R' = 4,4'$ -dimethoxytrityl, $R'' = H$

4d $R = Me_2CHCO,$
 $R' = 4,4'$ -dimethoxytrityl,
 $R'' = P(OCH_2CH_2CN)N(CHMe_2)_2$

was present as a competing nucleophile. The 1H NMR spectrum of the reaction mixture in D_2O showed a dominant doublet (J 139 Hz) at δ 2.71, which is assigned to $^{13}CH_3NH_2$ [the chemical shift observed is between those recorded for methylamine (δ 2.3) and methylammonium ion (δ 3.3)].

The methylated oligonucleotides were also analysed by mass spectrometry. The initial reactions described above were carried out in D_2O . A third reaction of the oligonucleotide with MNU was performed in H_2O to aid the interpretation of deuterium exchange effects. The EI mass spectrum of the hydrolysate of the methylated oligonucleotides obtained by reacting d(GTGCAC) with MNU in water is shown in Figure 6a. M^+ ions were observed for the parent bases cytosine (111), adenine (135), guanine (151) together with the adducted base Me-Gua (165). Figure 6b shows the spectrum obtained from a sample prepared in D_2O and confirms that partial deuteration of Me-Gua had occurred. Peaks at 166, 167, and 168 correspond to the d_1 - (31%), d_2 - (10%) and d_3 - (5%) species, respectively. For comparison, the corresponding values for the deuteration of methanol formed by hydrolysis of MNU in deuteriated phosphate buffer pH 7.4 are d_1 - (34%), d_2 - (13%) and d_3 - (2%). These results are in agreement with the premise that MNU decomposes to $Me-N_2^+$, the rate of equilibration of which with diazomethane^{28,29,58} is competitive with its rate of methylation of d(GTGCAC).

A preliminary experiment with MNU and d(GTG*AC) in water was carried out. As the oligonucleotide was known to contain acetate the excess of MNU was increased to 220 equivalents. Acetate would be expected to be methylated to volatile methyl acetate which is removed by evaporation. The freeze-dried reaction mixture was analysed after hydrolysis by MS giving a 165/166 ratio of *ca.* 1:1, indicating little selectivity in this methylation.

CONCLUSIONS

By using isotopic labelling and kinetic studies we have shown that the mechanism of decomposition of MNU in basic water features a rate-limiting deprotonation of a carbamoyl NH, followed by fragmentation of the derived anion to methyl diazoate and cyanic acid. Recent kinetic studies of the basic hydrolyses of 1,3-dimethyl-1-nitroso-urea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-urea reached similar conclusions to ours.⁵⁹ The results obtained demonstrate the value of using isotopically enriched carcinogens for studies of their reactions with nucleophiles acting as models for biological macromolecules (for previous applications of this kind see

refs 2, 29 and 31). Not only have we diagnosed the critically reactive species from MNU, but we have also identified methylated products formed from MNU and guanine [in dGuo, dGuo-5P and d(GTGCAC)] under physiologically relevant conditions. Definition of the mechanism of decomposition MNU in aqueous solution resolves a controversy concerning the mechanism of sequence selectivity in alkylations of DNA by MNU. We have confirmed Werner's report¹⁷ that cyanate is a major product from the aqueous decomposition of MNU and this raises the question of the carbamylation of DNA as a significant event in addition to methylation.⁶⁰ We have employed a new analytical method (using mass spectrometric analysis in conjunction with a ¹⁵N-labelled oligonucleotide) for determining sequence selectivity in reactions of oligonucleotides (further examples are needed to demonstrate its utility and scope). We believe that the methods that we have developed will be important for future studies of oligonucleotide chemistry. A thorough understanding of the mechanism of formation of DNA adducts, and their stability and repair, are crucial aspects in the development of procedures to assess risks posed by human exposure to genotoxic chemicals.

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