Biochem. J. (1970) **116**, 693–707
Printed in Great Britain

Methylation of Deoxyribonucleic Acid in Cultured Mammalian Cells by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

THE INFLUENCE OF CELLULAR THIOL CONCENTRATIONS ON THE EXTENT OF METHYLATION AND THE 6-OXYGEN ATOM OF GUANINE AS A SITE OF METHYLATION

BY P. D. LAWLEY* AND CAROLYN J. THATCHER
Cancer Research Laboratory, University of Western Ontario, London, Ont., Canada.

(*Received* 3 *September* 1969)

1. In neutral aqueous solution *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) yields salts of nitrocyanamide as u.v.-absorbing products. With cysteine, as found independently by Schulz & McCalla (1969), the principal product is 2-nitramino-thiazoline-4-carboxylic acid. Both these reactions liberate the methylating species; thiols enhance the rate markedly at neutral pH values. An alternative reaction with thiols gives cystine, presumably via the unstable *S*-nitrosocysteine. 2. Thiols (glutathione or *N*-acetylcysteine) *in vitro* at about the concentration found in mammalian cells enhance the rate of methylation of DNA markedly over that in neutral solution. 3. Treatment of cultured mammalian cells with MNNG results in rapid methylation of nucleic acids, the extent being greater the higher the thiol content of the cells. Rodent embryo cells are more extensively methylated than mouse L-cells of the same thiol content. Cellular thiol concentrations are decreased by MNNG. Proteins are less methylated by MNNG than are nucleic acids. 4. Methylation of cells by dimethyl sulphate does not depend on cellular thiol content and protein is not less methylated than nucleic acids. Methylation by MNNG may therefore be thiol-stimulated in cells. 5. Both *in vitro* and in cells about 7% of the methylation of DNA by MNNG occurs at the 6-oxygen atom of guanine. The major products 7-methylguanine and 3-methyladenine are given by both MNNG and dimethyl sulphate, but dimethyl sulphate does not yield O^6-methylguanine. Possible reaction mechanisms to account for this difference between these methylating agents and its possible significance as a determinant of their biological effects are discussed.

Study of the methylation of DNA in cultured mammalian cells by the mutagen and carcinogen MNNG† was expected to yield information on two aspects of the mode of action of this compound. The first concerns the activation of the nitrosamidine to yield the methylating species. This latter was expected to be either the methyldiazonium ion or the methylcarbonium ion derived from it (Lawley, 1968). Since DNA is methylated *in vitro* by MNNG (McCalla, 1968; Craddock, 1969; Lawley, 1968) these species are presumably generated by the decomposition of this compound at neutral pH values, and also since cysteine stimulates the methylation (McCalla, 1968; Craddock, 1969) the reaction between MNNG and thiols presumably also yields these intermediates.

It was desirable therefore to study the decomposition of MNNG in neutral solution and in the presence of thiols to determine the nature of the products, which had not been reported in detail when this work was started. Since then reports by McCalla, Reuvers & Kitai (1968) and by Schulz & McCalla (1969) have appeared that are relevant to these aspects; the corresponding part of the present work is in substantial agreement with their findings, although the techniques used are somewhat different. It was also decided to compare methylations by MNNG, both *in vitro* and in the cells, with those by dimethyl sulphate, a typical simple alkylating agent, and to compare the effects of thiols on these reactions. Whereas a thiol would be expected to

* Present address: Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London S.W.3, U.K.

† Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

stimulate methylation by MNNG, it would be expected to inhibit methylation by dimethyl sulphate.

A further related question concerns the rate of methylation of cellular constituents by these agents. With the simple alkylating agents this rate approximates to the rate of hydrolysis of the compound. If methylation by MNNG were stimulated *in vivo* by thiols, as suggested by Schoental (1965), the rate of this methylation would be expected to be much higher than that of the relatively slow decomposition at neutral pH. Further, the final extent of alkylation of cellular constituents might correlate positively with the thiol content of the cells, which is known to vary with the state of growth of cells in culture (Sparkes & Walker, 1966).

The second major aim of this investigation was to examine in detail the nature of the sites of methylation in cellular DNA reactive towards the nitrosamidine. This question has now become of the first importance with respect to the known differences between the mutagenic action of nitrosamides and nitrosamidines and the simple alkylating agents. For induction of point mutations in bacteriophage T2, Loveless & Hampton (1969) have shown that *N*-methyl-*N*-nitrosourea is a highly effective mutagen, but methyl methanesulphonate is inactive. Since the major site of alkylation of DNA by both compounds is the 7-nitrogen atom of guanine, this important finding effectively rules out the previously proposed molecular mechanisms for mutagenesis involving anomalous base-pairing of 7-methylguanine (Lawley & Brookes, 1961; Nagata, Imamura, Saito & Fukui, 1963).

The nature of the site or sites of methylation in DNA essential for induction of this type of mutation (presumably 'transitions' in the nomenclature of Freese, 1959) has thus become an open question. Olson & Baird (1969) found that DNA in *Escherichia coli* was degraded rapidly in alkali after treatment of cells with MNNG and suggested that chain breaks in DNA were caused by formation of phosphotriester groups in DNA. This reaction was also suggested to account for mutagenesis by MNNG. A perhaps more attractive proposal has been made by Loveless (1969), that alkylation of guanine residues in DNA at the extranuclear 6-oxygen atom would be a highly effective cause of mispairing of alkylguanine with thymine when alkylated DNA was replicated. Evidence for reaction between deoxyguanosine and *N*-methyl-*N*-nitrosourea at this position has been found by Loveless (1969).

It seemed likely therefore that the essential difference between MNNG and a simple methylating agent such as dimethyl sulphate, with respect to methylation of DNA, would be found to result from their different modes of activation. Whereas dimethyl sulphate would form a transition complex with a nucleophilic group in DNA and react almost exclusively by Ingold's (1953) S_N2 mechanism, the alkyl-nitrosamides and -nitrosamidines would be expected to react either through the diazoalkyl cations by the S_N2 mechanism, or by the S_N1 mechanism involving generation of the carbonium ion.

This might well explain the ability of this latter group of agents to alkylate sites not attacked by dimethyl sulphate, since the S_N1 type of reagent attacks groups at random independently of their nucleophilicity, whereas the S_N2 reagents are specifically reactive towards more nucleophilic groups.

It therefore appears that alkylation of only the known groups of high nucleophilicity in DNA, namely the 7-nitrogen atom of guanine and the 3-nitrogen atom of adenine, with minor sites of reaction at the 3-nitrogen atom of cytosine and the 1- and 7-nitrogen atoms of adenine (cf. Lawley, 1966) might well be diagnostic for the S_N2 type of reagent. On the other hand, *O*-alkylation, with possibly the 6-oxygen atom of guanine being a reactive site, might indicate reaction by the S_N1 mechanism.

It was therefore decided to compare the alkylation products in DNA derived from MNNG and dimethyl sulphate both *in vitro* and in cells. The formation in cells of the product presumed to cause anomalous base-pairing in replicating DNA, namely 2-amino-6-methoxypurine (O^6-methylguanine) might well, as pointed out by Loveless (1969), be of significance in the induction of the hypothetical somatic mutations postulated as a factor in chemical carcinogenesis.

MATERIALS AND METHODS

Materials. [^{14}C]Methyl methanesulphonate (45.2mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) and di[^{14}C]methyl sulphate (2.13mCi/mmol) (New England Nuclear Corp., Boston, Mass., U.S.A.) were kept in ether solution at −20°C. *N*[^{14}C]-Methyl-*N'*-nitro-*N*-nitrosoguanidine (0.29mCi/mmol, m.p. 117°C) was prepared from [^{14}C]methylamine (The Radiochemical Centre) by a modification of the methods of McKay (1949) and McKay & Wright (1947); the intermediate *N*[^{14}C]-methyl-*N'*-nitroguanidine was obtained by evaporation of the reaction mixture of [^{14}C]methylamine and MNNG in the ether–water two-phase system.

MNNG and nitroguanidine were obtained from K & K Laboratories, Plainview, N.Y., U.S.A.; *N*-methyl-*N'*-nitroguanidine was prepared from MNNG (McKay, 1949). 7-Methylguanine was prepared from guanosine, and 1-methyladenine from adenosine, by the methods of Jones & Robins (1963). 6-Methoxy-2-aminopurine was prepared by the method of Balsiger & Montgomery (1960) and O^6-methyldeoxyguanosine by the method of Friedman, Mahapatra, Dash & Stevenson (1965). 3-Methyladenine was obtained as the hydrochloride by methylation of

adenine (Pal, 1962) in neutral aq. sodium acetate with dimethyl sulphate followed by chromatography on Dowex 50 (H^+ form) eluted with M-HCl. The second major u.v.-absorbing peak (Brookes & Lawley, 1960) yielded the desired product on evaporation and recrystallization of the residue from methanol. 3-Methylcytosine was obtained by methylation of cytidine (Brookes & Lawley, 1962). Salmon sperm DNA and yeast RNA (Mann Research Laboratories, New York, N.Y., U.S.A.) were used for reactions *in vitro*.

Paper chromatography. Whatman no. 1 or 3MM papers were used. The solvents were: (1), propan-2-ol–conc. HCl–water (170:41:39, by vol.); (2), butan-1-ol–acetic acid–water (2:1:1, by vol.); (3), butan-1-ol–aq. NH_3 (sp.gr.0.88)–water (43:2:5, by vol.); (4), propan-1-ol–water (7:3, v/v); (5), butan-1-ol–water (43:7, v/v); (6), propan-2-ol–aq.NH_3 (sp.gr.0.88)–water (7:1:2, by vol.). Papers were examined under u.v. light (254nm); for detection of amino acids, papers were dipped in 0.25% (w/v) ninhydrin solution in acetone and dried; for detection of sulphur a spray of 1% (w/v) potassium iodoplatinate was used.

Paper electrophoresis. The apparatus supplied by Gelman Instrument Co., Ann Arbor, Mich., U.S.A., was used, with Whatman no. 3 paper strips (17cm×2.5cm); the buffers (I 0.05) were glycine–HCl, pH2.4, sodium phosphate, pH6, or Na_2CO_3–$NaHCO_3$, pH11.9, and the current was 2mA.

Measurement of radioactivity. For liquid-scintillation counting of radioactivity of solutions, a sample (0.1ml) was assayed in 10ml of a phosphor containing naphthalene (100g), 2,5-diphenyloxazole (4g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (100mg), methanol (100ml) and dioxan (to 1 litre). For counting of paper chromatograms segments (1 or 2cm) of 3cm-wide strips were placed in scintillation vials and 0.5ml of phosphor containing 2,5-diphenyloxazole (4g) and 1,4-bis-(5-phenyloxazol-2-yl)-benzene (50mg)l of toluene was added. Packard Tri-Carb 375 or Nuclear–Chicago 720 counters were used. Efficiency of counting of liquid samples was about 75% for ^{14}C as determined with internal standards of toluene. DNA was dissolved in aq. 5% (w/v) trichloroacetic acid at 100°C for measurement of radioactivity, RNA in water, and protein in aq. 10% (w/v) tetraethylammonium hydroxide. The amounts assayed in 0.1ml samples were 0.1–0.4mg. Concentrations of nucleic acids were determined from the u.v. absorption of the solutions diluted 60-fold in water; a value $E_{260}=26$ was assumed for a solution containing 1mg of DNA (2.97μmol of P)/ml.

Rate of decomposition of MNNG. Stock solutions of MNNG (about 100mM in ethanol) were kept in the dark without loss of visible-light absorption. For kinetic studies the stock solution (0.1vol.) was added to the other components of the reaction mixture in a thermostatically controlled cell housing (Unicam SP.800 or Cary15 spectrophotometer) and spectra in the range 460nm to about 360nm were rapidly scanned at intervals.

The following conditions for decomposition of MNNG were examined: aqueous solutions from pH1–12, at 26–37°C; aq. trichloroacetic acid (5%, w/v); equimolar neutral aq. solutions of $Na_2S_2O_3$, 2-mercaptoethanol, cysteine, *N*-acetylcysteine, GSH, *S*-methylcysteine, glycine, histidine and glutamic acid; growth medium for cells, with and without serum.

Hydrolysis of MNNG in dilute alkali to yield salts of nitrocyanamide. MNNG (2.5mmol) was dissolved in ethanol (15ml) and water (15ml) was added. To the stirred solution at ambient temperature 0.2M-NaOH or 0.2M-KOH was added gradually. Gas was evolved and the yellow colour disappeared after 12.5ml of alkali had been added. For the sodium derivative, the solution was evaporated and the residue dissolved in ethanol (2ml); excess of ether was added to yield a colourless crystalline precipitate, m.p. 196°C (Found: C, 9.3; H, 1.1; N, 32.1%). Harris (1958) reported for sodium nitrocyanamide m.p. 195–197°C. For the potassium derivative, the residue after evaporation was crystallized from ethanol as prisms, m.p. 133–135°C [m.p. reported by McKay, Ott, Taylor, Buchanan & Crooker (1950), 135–136°C] (Found: C, 9.8; H, <0.03; N, 33.7%; CKN_3O_2 requires C, 9.6; N, 33.6%).

Reaction between MNNG and cysteine. Cysteine hydrochloride (6mmol) was neutralized with M-NaOH; 24ml of sodium phosphate buffer, pH7.0 (35mM-Na_2HPO_4–30mM-NaH_2PO_4) was added, and then to the mixture at 37°C MNNG (6mmol) in *NN*-dimethylformamide (3ml) was rapidly added. The solution turned a pale red, gas was evolved and a clear colourless solution was obtained after about 3min. Then a white precipitate began to form and the solution slowly turned yellow. Portions (10μl) of the mixture were applied to paper and chromatographed with solvents (2) or (4). The mixture was left overnight at room temperature, the precipitate was filtered off and the clear solution was applied to a column (30cm×4cm) of Dowex50 (H^+ form), eluted with water (160×10ml fractions). Two major u.v.-absorbing peaks were obtained, in fractions 50–75 and in fractions 115–155; further elution with 0.1M–HCl (100 fractions) gave no further significant absorption. These peaks contained 54 and 35% of the total E_{260} units and 83 and 15% of the E_{280} units respectively. The first peak was not homogeneous, since the 'tail' from fractions 66–75 contained material with E_{280}/E_{260} ratio less than that observed for the main peak (3.1). The second peak was homogeneous, E_{280}/E_{260} ratio 0.76. The 'tail' of the first peak was rejected and the fractions containing the two principal products were evaporated. The major product, 2-nitramino-2-thiazoline-4-carboxylic acid, was obtained as pale green-blue prisms, recrystallized from water; the yield was 280mg, m.p. 190°C (decomp.) (Found: C, 25.1; H, 2.4; N, 21.9; S, 17.0. $C_4H_5N_3O_4S$ requires C, 25.1; H, 2.6; N, 22.0; S, 16.8%). The ionization equilibria of this product were examined by spectrophotometric titration and by paper electrophoresis, showing it to be a weak acid with two pK'_a values (I 0.0025, 23°C) of 2.5 and 9.0.

The principal minor product was crystallized from propan-2-ol–ether, and its absorption spectra in acid, neutral and alkaline solutions were shown to be identical with those of *N*-methyl-*N'*-nitroguanidine.

The residual precipitate from the reaction mixture was sparingly soluble in water but soluble in M-HCl; on examination by paper chromatography with solvents (2) and (4) no u.v.-absorbing spots were detected and the sole ninhydrin-positive spot had R_F values identical with those of cystine. Examination of the precipitate by i.r. spectroscopy in Nujol mull confirmed its identity with that of authentic cystine.

Reactions of methylating agents with DNA: effect of thiols. Stock solutions contained salmon sperm DNA (10mM-DNA P) in 10mM-sodium acetate or in sodium EDTA buffer, pH7.1 (3.4mM-disodium EDTA neutralized with 3.2mM-NaOH). The ^{14}C-labelled methylating agents (MNNG, methyl methanesulphonate or dimethyl sulphate) were added in ether solution to give concentrations of up to 1mM. Portions of the mixtures were then immediately added to appropriate volumes of buffer at 37°C containing various concentrations of thiols (*N*-acetylcysteine or GSH). After various times portions of the reaction mixtures (generally 0.5ml, containing about 1mg of DNA) were added to cold 2-ethoxyethanol containing 0.1 vol. of 2.5M-sodium acetate (2ml). The precipitate of fibrous DNA was redissolved in the EDTA buffer and reprecipitated, washed with ethanol and then ether, dried and assayed for radioactivity; in some cases samples of DNA were hydrolysed and analysed chromatographically. To check the efficacy of the isolation procedure for DNA, a solution containing [^{14}C]MNNG and GSH at the highest concentrations used was incubated at 37°C for 40min; then DNA was added. The resultant precipitate contained no radioactivity.

Neutral and acid hydrolysis of DNA. A sample of [^{14}C] methylated DNA (1–5mg.) in mM-sodium phosphate buffer, pH7.0 (0.5–1ml), was heated at 100°C for 20min. To the cooled hydrolysate was added M-HCl (0.1ml, containing 7-methylguanine and 3-methyladenine, 50μg). The precipitate of polynucleotide material was obtained by centrifugation and washed with 0.1M-HCl (0.5ml). The combined supernatants were evaporated to 50μl and applied to paper for chromatography. The residual polynucleotides were dissolved in M-HCl (50μl) at 100°C and applied separately to paper, with the marker bases. Solvents (1), (3) or (6) were used. The dried and neutralized papers were then examined under u.v. light (254nm), cut into segments and assayed for radioactivity, at a counting efficiency of 44%.

For hydrolysis of DNA in dilute acid, to a solution (up to 3mg) in water (0.2ml) was added M-HCl (0.02ml) and the mixture was incubated at 37°C for 16h or heated at 70°C for 10min. For hydrolysis of nucleic acids with aq. 72% (w/v) $HClO_4$, 15μl/mg of nucleic acid was used. After 1h at 100°C the hydrolysate was diluted threefold with water and clarified by centrifugation.

Chromatographic methods. The R_F values of the added marker bases 7-methylguanine, 1-methyladenine, 3-methyladenine and 3-methylcytosine were observed to be as recorded by Lawley & Brookes (1963), and a two-dimensional paper chromatogram, with solvent (1) then solvent (3), achieved their separation from the normal DNA bases; some overlap of guanine with 7-methylguanine was sometimes found. 2-Amino-6-methoxypurine was identified as a strongly fluorescent spot running ahead of 3-methyladenine in solvents (3) and (6). R_F values are given in Table 4.

For column chromatography, in a typical experiment, an $HClO_4$ hydrolysate of DNA (6.5mg, 6.4mmol of [^{14}C]methyl groups/mol of DNA P), obtained by reaction with [^{14}C]MNNG in the presence of GSH, was applied to a column (13cm × 1cm) of Dowex 50 (H^+ form) and eluted with a linear 0.75–2M-HCl gradient (600ml) in 10ml fractions at 0.5ml/min. The E_{254} of the effluent was monitored and radioactivity in each fraction was measured after evaporation of 1ml portions in scintillation vials. With ^{14}C-labelled DNA or RNA obtained from reaction with whole cells the whole fractions were evaporated.

Bases in the u.v.-absorbing fractions were identified and estimated from their absorption spectra. The fractions containing the normal bases of DNA, and their molar proportions, were as follows: thymine (3–4, 0.29); cytosine (8–11, 0.22); guanine (13–18, 0.22); adenine (39–50, 0.27). For cellular RNA, the molar proportions of bases were: uracil, 0.23; cytosine, 0.29; guanine, 0.31; adenine, 0.18. When marker methylated bases were added they appeared at the following positions: 3-methylcytosine, together with the later part of the cytosine peak and immediately after; 3-methyladenine and 7-methylguanine, together in a single peak between guanine and adenine (fractions 20–30) with 3-methyladenine richer in the earlier fractions: these bases could be separated by paper chromatography of the residue from the evaporated fractions; 1-methyladenine, together with adenine, more in the earlier fractions of the adenine peak.

Identification of the extranuclear 6-oxygen atom of guanine in DNA as a site of methylation by MNNG. With non-labelled MNNG, to a mixture of DNA (from salmon sperm, 0.1mmol of DNA P in 50mM-sodium acetate, 5ml) and *N*-acetylcysteine (10mmol in 1.5M-tris–HCl buffer, pH7.4) was added at 37°C MNNG (10mmol in *NN*-dimethylformamide, 5ml). After 20min 2-ethoxyethanol was added to the reaction mixture (1.5vol.) and the resulting fibrous precipitate of DNA was redissolved in 50mM-sodium acetate; 0.2vol. of 2.5M-sodium acetate was added, then 1.5vol. of 2-ethoxyethanol. After this dissolution and reprecipitation had been repeated, the DNA was washed with ethanol and then ether and dried. Portions (10mg) of DNA were hydrolysed by dissolving in water (0.5ml) and then adding M-HCl (0.05ml) and maintaining the suspension at 37°C for 16h, after which the clear hydrolysate was chromatographed on Whatman 3MM paper with solvent (6). The blue fluorescent spot of R_F0.62, identical with that of authentic 2-amino-6-methoxypurine, was eluted into 0.1M-HCl (5ml) and the absorption spectrum of the eluate read against an appropriate blank. The eluate was then neutralized with 2M-tris and its spectrum redetermined; it was then adjusted to pH12 with NaOH. The peak wavelengths of the spectra were identical with those of authentic 2-amino-6-methoxypurine at the three pH values (at pH1, 286nm; pH7, 281nm; pH12, 284nm). From the observed extinctions at pH1 the yield of this product was estimated to be 3mmol/mol of DNA P.

With [^{14}C]methyl-labelled MNNG (0.06mmol) with DNA (0.25mmol) in the presence of GSH (0.1mmol) in 0.1M-tris–HCl buffer, pH7.4 (20ml), the extent of methylation of DNA isolated as described above was 11mmol/mol of DNA P. Portions (3mg) of [^{14}C]methylated DNA were hydrolysed in 0.1M-HCl (0.2ml) at 37°C for 16h or at 100°C for 10min and the hydrolysates were chromatographed with unlabelled marker bases 7-methylguanine, 3-methyladenine and 2-amino-6-methoxypurine. These products were separated by solvents (3) or (6). The proportions of the products were estimated by cutting the papers into segments and assaying them for radioactivity. Some streaking back to the origin was observed with 7[^{14}C]-methylguanine and the proportion of this base was better estimated by using the acid solvent (1); but this

solvent did not separate the other two methylated purines completely.

Alternatively, DNA was hydrolysed at 100°C at pH 7 for 20min, liberating ^{14}C-labelled 7-methylguanine and 3-methyladenine, which were separated by paper chromatography with solvents (3) or (6). The acid-insoluble residual polynucleotide was hydrolysed with 0.1M-HCl and was found to contain the major part of the 2-amino-6-[^{14}C]-methoxypurine.

In other experiments [^{14}C]methylated DNA (25mg), obtained as described from [^{14}C]MNNG, was degraded enzymically by the method of Hall (1967). After preliminary digestion with deoxyribonuclease, snake venom phosphodiesterase (1mg/ml) and alkaline phosphatase (0.05mg/ml) were added, after adjustment of the pH to 8.6 with tris, and the mixture was incubated for 3 or 16h at 37°C (total vol. 4ml). Portions of the freeze-dried digest (1ml) were chromatographed on paper with solvents (3), (4) or (6), with an added marker of O^6-methyldeoxyguanosine. The added marker was well separated ahead of other products, with R_F 0.62 in solvent (3), 0.84 in solvent (4) and 0.76 in solvent (6). The ^{14}C-labelled O^6-methyldeoxyguanosine, amounting to about 7% of total ^{14}C, was apparently liberated after 3h digestion, when most of the ^{14}C remained near the origin of the chromatograms. After 16h digestion the main peaks of radioactivity were at R_F 0.42 and 0.56 in solvent (4) and at 0.33 and 0.50 in solvent (6), but the far-running peaks of O^6[^{14}C]-methyldeoxyguanosine were still clearly defined.

As a further check on the identity of the O^6-methylation product from guanine of DNA, the [^{14}C]methylated DNA was hydrolysed with 72% (w/v) $HClO_4$ for 1h at 100°C. Paper chromatograms showed the presence of 7-methylguanine and 3-methyladenine, but the peaks of 2-amino-6-methoxypurine were absent. None of the procedures used showed any appreciable amount of 2-amino-6-methoxypurine in DNA methylated by di[^{14}C]methyl sulphate.

Methods for cell culture. For hamster embryo cells the modification of the method of Sheinin (1962) as described in detail by Thatcher & Walker (1969) was used. 'Active' cells in secondary cultures were obtained by resuspending confluent secondary cultures with trypsin and plating at a density of 10^6 cells/16oz glass medicine bottle. Such cultures were termed 'active' during a period 24–48h after plating. 'Confluent' cultures were obtained after growth for 12–14 days. The medium used was CRML1066 (Grand Island Biological Co., New York, N.Y., U.S.A.) supplemented with 10% (w/v) of foetal calf serum and 1mg of RNA nucleosides/ml.

With mouse L-cells the methods described in detail by Walker & Helleiner (1963) were used; growth medium was CMRL1066 with 10% (w/v) of bovine serum, minus thymidine and cytidine, and cells were grown in suspension in spinner flasks. In agreement with the work of Sparkes & Walker (1966) the concentration of acid-soluble thiol in these cells reached a maximum when the population density was between 1×10^5 and 2×10^5 cells/ml, then decreased as the density increased.

Determination of cellular acid-soluble thiol. Acid-soluble thiol was determined by a modification of Ellman's (1959) method. About 10^7–10^8 cells (a known number determined with a Coulter counter) were suspended in cold 5% trichloroacetic acid, and a portion (0.5ml) of the clear supernatant after centrifugation was added to 3.5ml of a solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (200μg/ml in 0.2M-sodium phosphate buffer, pH 7.6). The absorption spectrum of the resultant yellow solution was measured immediately and the concentration of thiol was determined from the value $\epsilon_{max.}$ at 410nm 1.36×10^4.

Uptake of MNNG by cultured mammalian cells. In a typical experiment, to a suspension of cells (confluent hamster embryo, 10ml, 2×10^7 cells/ml in growth medium at 37°C) was added [^{14}C]MNNG (in 0.1ml of ethanol to give 0.19mM concentration in the medium, as measured by assay of radioactivity in 0.1ml). After 0.5, 1, 2, 5, 10, 15, 20, 40 and 80 min, portions (1ml) of the suspension were removed and the cells were immediately collected by centrifugation in the cold. The clear supernatant was removed and added to cold 50% (w/v) trichloroacetic acid (0.1ml); the pellet was resuspended in cold phosphate-buffered saline (Grand Island Biological Co.) (5ml). The re-harvested washed cells were then suspended in cold 5% trichloroacetic acid (0.5ml). The acidified samples were then clarified by centrifugation and the acid-insoluble residue from the cells was further washed with cold 5% trichloroacetic acid, ethanol and ether, and dried. The radioactivity in 0.1ml portions of clarified medium and of cellular supernatant was assayed.

The acid-insoluble fraction of the cells was dissolved in 10% (w/v) tetraethylammonium hydroxide (0.4ml) and a portion (0.2ml) was assayed for radioactivity. The residual acid-soluble supernatants from the cells were evaporated after addition of *N*-methyl-*N'*-nitroguanidine (the product of acid hydrolysis of MNNG; 100μg) and chromatographed on paper with solvent (3). Portions (0.05ml) of the residual supernatants from the acidified and heated media were chromatographed similarly.

Methylation of constituents of cells by MNNG. In a typical experiment with cultured mammalian cells, to a suspension of mouse L-cells (grown to 5×10^5 cells/ml, resuspended at 37°C in growth medium to a density of 2.8×10^7/ml; 20ml) [^{14}C]MNNG was added in ethanol (0.1ml) to give a concentration of 0.33mM. Nucleic acids were then isolated by a modification of the method described by Kirby (1957), as follows. After 40min the suspension was added to the cold phosphate-buffered saline (200ml); the cells were harvested by centrifugation and resuspended in aq. 6% (w/v) sodium 4-aminosalicylate (40ml). The suspension was lysed by addition of 10% (w/v) of sodium dodecyl sulphate (0.1 vol.) and the viscous lysate was extracted with 1 vol. of phenol reagent (containing 500g of phenol, 62ml of *m*-cresol, 62ml of water and 0.62g of 8-hydroxyquinoline). The mixture was stirred or shaken for about 5 min and then centrifuged to clarify the layers. The upper aqueous layer was removed by pipetting and fibrous DNA was obtained by adding 2-ethoxyethanol (1.5 vol.). To the residue, ethanol (2 vol.) was added to precipitate RNA. The DNA was dissolved in 40mM-sodium acetate (8ml) and 2.5M-sodium acetate (1ml) and 4M-NaCl (1ml) were added. The solution was then incubated with ribonuclease (50μg/ml) for 20min at 37°C, re-extracted with phenol reagent, and from the aqueous layer DNA was precipitated with 2-ethoxyethanol (0.5 vol.) and redissolved in 0.25M-sodium acetate containing 0.3M-NaCl (5ml). RNA was washed with aq. 70% (w/v) ethanol containing sodium acetate (0.25M) and redissolved as described for DNA. The solutions were

then centrifuged at 35000rev./min in the SW50 rotor of a Beckman L65 ultracentrifuge for 30min and the nucleic acids were reprecipitated from the clear supernatants, washed with ethanol and ether, dried and assayed for radioactivity. In some cases nucleic acids were hydrolysed and chromatographed. Protein was precipitated from the first phenol layer with methanol (10vol.) and washed with methanol and ether, dried and assayed for radioactivity.

RESULTS AND DISCUSSION

Decomposition of MNNG in aqueous solutions of various pH *values, and reaction with cysteine*

The rate of disappearance of the absorption spectrum of MNNG in the visible region reflects the loss of the nitroso group. The reaction was catalysed by either acid or alkali, and the alkali-catalysed mechanism predominated at pH values above about 4.5.

Fig. 1 shows that the decomposition exhibited unimolecular kinetics, and from such data the half-lives ($t_{\frac{1}{2}}$) of MNNG in various media were calculated (Table 1). It was noted that in tris–HCl buffers over the range pH 7–8 approximately a tenfold decrease in $t_{\frac{1}{2}}$ resulted from the increase in pH of 1 unit. Paper chromatography of the final reaction mixture, after decolorization was complete, with solvent (5) showed that the only u.v.-absorbing products were nitrocyanamide salts at pH6–9. Phosphate catalysed the decomposition and gave a relatively lower yield of nitrocyanamide with some non-u.v.-absorbing product. Thiosulphate markedly catalysed the decomposition of MNNG and the predominant product was *N*-methyl-*N'*-nitroguanidine.

In 5% trichloroacetic acid, pH1.4, the acid-catalysed decomposition of MNNG gave *N*-methyl-*N'*-nitroguanidine as sole u.v.-absorbing product. During the reaction the transient appearance of spectral bands due to nitrous acid was noted (Table 2).

All the thiols tested proved to be powerful activators of the decomposition of MNNG. Other amino acids apart from cysteine had a much weaker effect (Figs. 2 and 3). The reaction between cysteine and MNNG at neutral pH gave initially a mixture of products, some being ninhydrin-positive. But chromatography of the reaction mixture on a column of Dowex 50 (H^+ form) eluted with water gave only two main products. These were identified as a carboxylic acid, to which the structure 2-nitraminothiazoline-4-carboxylic acid was assigned, and *N*-methyl-*N'*-nitroguanidine. Paper chromatography with solvent (5) separated these products, the R_F values being 0.03 and 0.4 respectively. Elution of the u.v.-absorbing spots from the paper showed that the molar ratio was 0.57 at pH6.05,

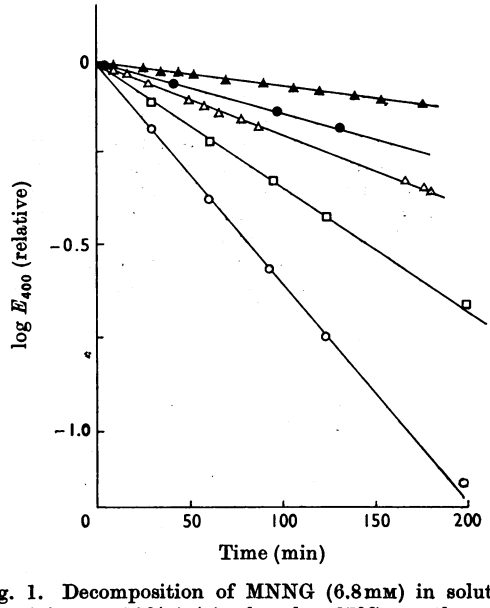


Fig. 1. Decomposition of MNNG (6.8mM) in solution containing aq. 10% (v/v) ethanol at 37°C, together with the following: ▲, 180mM-tris–HCl buffer, pH7.15; ●, 180mM-sodium phosphate buffer, pH6.95; △, 180mM-tris–HCl buffer, pH7.15, plus $Na_2S_2O_3$ (6.8mM); □, 220mM-sodium phosphate buffer, pH7.4; ○, 440mM-sodium phosphate buffer, pH7.25. The changes with time of log E_{400} are shown.

Table 1. *Decomposition of MNNG* (6.8mM) *in solutions containing aq.* 10% (*v/v*) *ethanol at* 37°C, *together with various buffers or reagents as shown*

The half-life of MNNG was estimated from plots of the first-order reactions, as shown in Fig. 1, or from the second-order reactions with cysteine.

Buffer	pH	$t_{\frac{1}{2}}$ (min)
200mM-Sodium acetate	5.55	3300
200mM-Sodium acetate 6.8mM-cysteine	5.55	22
180mM-Sodium phosphate	6.15	990
160mM-Sodium phosphate 6.8mM-cysteine	6.07	10
180mM-Sodium phosphate	6.95	220
160mM-Sodium phosphate 6.8mM-cysteine	6.97	1
180mM-Sodium phosphate	7.85	48
160mM-Sodium phosphate 6.8mM-cysteine	7.79	0.2
200mM-tris–HCl	7.04	584
200mM-tris–HCl	7.78	99
200mM-tris–HCl	8.12	43
160mM-tris–HCl 6.8mM-$Na_2S_2O_3$	7.15	138
180mM-tris–HCl 10mM-lysine	7.1	88
180mM-Sodium carbonate	9.95	0.4

Table 2. *Absorption spectra of MNNG and related compounds*

	Solvent or pH	$\lambda_{max.}$ (nm)	$\epsilon_{max.}$	$\epsilon_{280}/\epsilon_{260}$
MNNG	7	402	163	
		278	17600	1.6
	95% ethanol	419	142	—
		402	179	
'Nitrous acid'	5% tri-	385	31	—
	chloroacetic	371	53	
	acid	356	51	
		346	37	
N-Methyl-*N'*-nitroguanidine	2–10	267	14500	0.74
	0.2M-NaOH	252	8000	0.55
	10M-HCl	225	8900	0.13
MNNG: acid hydrolysis product	2–10	267	—	0.73
MNNG: neutral hydrolysis product	7	263	—	0.63
MNNG: alkaline hydrolysis product (nitrocyanamide salt)	0–12	263	19200	0.63
MNNG: cysteine, purified product (2-nitraminothiazoline-	0	280	16900	2.6
4-carboxylic acid)	6	282	17200	3.2
	12	269	11300	0.95

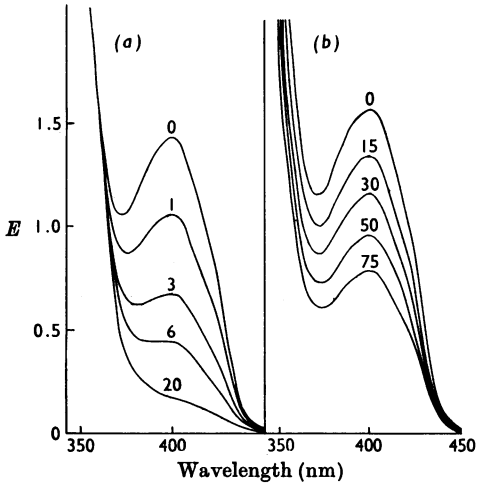


Fig. 2. Spectra showing decomposition of MNNG in the presence of cysteine (*a*) and histidine (*b*) at 37°C. MNNG (100mM in ethanol, 0.3ml) was added to solutions containing 0.2M-sodium phosphate buffer, pH7.0 (2.4ml) and 100mM amino acids (0.3ml, in buffer) in a thermostatically controlled cell in the Unicam SP800 spectrophotometer. Spectra were scanned at intervals. The numbers on the curves show the time (in min).

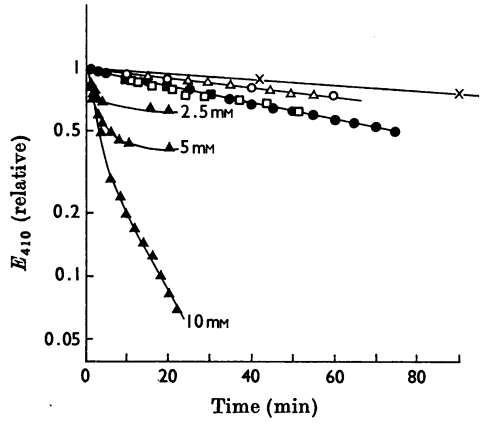


Fig. 3. Rate of decomposition of MNNG in the presence of amino acids at pH7.0 at 37°C. The method described for Fig. 2 was used, except that where indicated the concentration of cysteine was 2.5 or 5mM; otherwise MNNG and amino acids were used at a concentration of 10mM. x, Buffer only (180mM-sodium phosphate buffer, pH7.0); △, glutamic acid; ○, *S*-methylcysteine; ●, histidine; □, lysine; ■, glycine; ▲, cysteine (upper curve, 2.5mM; middle curve, 5mM; lower curve, 10mM).

0.67 at pH6.95 and 0.80 at pH7.79. The reactions between MNNG and equimolar thiols were of second-order kinetics.

Since completing this work we learned that Schulz & McCalla (1969) had obtained analogous results, which have now been published.

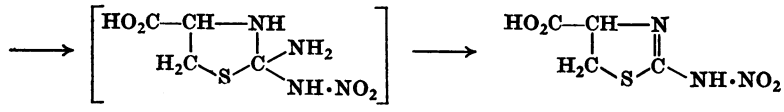
The various mechanisms for decomposition of MNNG are summarized in the formulae on the next page.

The alkali-catalysed decomposition (*a*) could alternatively be represented as the breakdown of the anionic form of MNNG. Both mechanisms have been discussed for the analogous decomposition of *N*-methyl-*N*-nitrosourea (Garrett, Goto &

(*a*) $CH_3 \cdot N(NO) \cdot C(NH) \cdot NH \cdot NO_2 + OH^- \longrightarrow [CH_3 \cdot N : N \cdot OH] + NC \cdot N \cdot NO_2^- + H_2O$

(*b*,1) $CH_3 \cdot N(NO) \cdot C(NH) \cdot NH \cdot NO_2 + HS \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H \longrightarrow$

$[CH_3 \cdot N : N \cdot OH] + [HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot C(NH) \cdot NH \cdot NO_2]$



(*b*,2) $CH_3 \cdot N(NO) \cdot C(NH) \cdot NH \cdot NO_2 + HS \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H \longrightarrow$

$CH_3 \cdot N : C(NH_2) \cdot NH \cdot NO_2 + [HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot NO] \longrightarrow$

$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$

Stubbins, 1965). According to these authors the latter route, i.e. decomposition of the anion, would be susceptible to generalized base catalysis by buffer anions. With MNNG, in agreement with the report of McCalla *et al.* (1968), phosphate ions were found to catalyse the decomposition (Table 1), but it was notable that the yield of nitrocyanamide was less for the phosphate-catalysed reaction than for the reaction at the same pH in the presence of tris–HCl buffer. The nature of the other product(s), which did not absorb in the u.v. above 220nm, was not investigated. Ionization of MNNG in tris–HCl buffer in the range pH 6–8 was indicated by a progressive shift in the absorption maximum of MNNG from 401nm at pH 7.0 to 386nm at pH 8.1, again supporting the concept of the participation of the alternative mode of decomposition.

In agreement with the work of Schulz & McCalla (1969), the reaction of MNNG with cysteine involves principally mechanism (*b*1), i.e. attack of ionized thiol on the electron-deficient carbon atom of the nitroguanidine moiety of MNNG, followed by cyclization of the unstable open-chain intermediate, then elimination of ammonia from the cyclic product to yield the thiazoline derivative isolated. This reaction is analogous to the cyclization of *S*-2-aminoethylisothiourea studied by Doherty, Shapira & Burnett (1957) and Khym, Shapira & Doherty (1957).

Again in agreement with the work of Schulz & McCalla (1969), the second main process involves attack by the thiol on the nitroso group of MNNG (mechanism *b*2), liberating free radicals and ultimately leading to formation of cystine. The ratio of extents of reaction by these two routes, determined as the ratio of the yield of 2-nitraminothiazoline-4-carboxylic acid to that of *N*-methyl-*N'*-nitroguanidine, showed increasing predominance of route (*b*1) as the pH increased from 6 to 8. However, according to Schulz & McCalla (1969), who used 2mol of cysteine/mol of MNNG, the yield of cystine was greater than that of *N*-methyl-*N'*-nitroguanidine.

Several thiols in addition to cysteine were examined for their ability to catalyse the decomposition of MNNG. With 10mM-MNNG and equimolar concentrations of 2-mercaptoethanol, *N*-acetylcysteine or GSH in 180mM-sodium phosphate buffer, pH 6.9, the half-life of MNNG was found to be about 2min at 37°C in all cases.

Surprisingly, the powerfully nucleophilic anion thiosulphate was also found to yield *N*-methyl-*N'*-nitroguanidine as principal product from reaction with MNNG at pH 7, and therefore this anion attacks mainly the nitroso group of MNNG, not the electron-deficient carbon atom.

Methylation of DNA in vitro *by MNNG, dimethyl sulphate or methyl methanesulphonate*

Effect of thiols. The rates of methylation of DNA by [^{14}C]methyl-labelled MNNG in the presence of *N*-acetylcysteine or GSH were determined by precipitation of DNA at various times; no ^{14}C label entered DNA if the mixture of [^{14}C]MNNG and thiol was allowed to react for 40min before addition of DNA. The concentrations of thiol used were of the same order as those encountered in mammalian cells; e.g. Sparkes & Walker (1966) found that the average concentration of acid-soluble thiol (shown to be mainly GSH) ranged from about 1 to 7mM in mouse L-cells. These concentrations of thiol sufficed to decompose MNNG rapidly and also to catalyse the relatively rapid methylation of DNA (see Fig. 4). The extent of methylation of DNA achieved within 40min in the presence of 8mM-*N*-acetylcysteine was as high as that achieved in 21h in the absence of the thiol at the same pH.

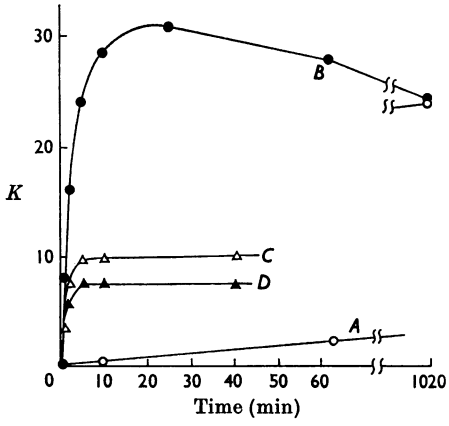


Fig. 4. Effect of thiols on methylation of DNA by [^{14}C]MNNG at 37°C and pH7.0. The reaction mixture contained: (A) ○, 0.25mM-[^{14}C]MNNG, salmon sperm DNA, (11.4mM-DNA P), 15mM-sodium phosphate buffer, pH7.0; (*B*) ●, as (*A*), but with 8mM-*N*-acetylcysteine; (*C*) △, as (*A*), but with 8mM-GSH; (*D*) ▲, as (*A*), but with 16mM-GSH. Methods for isolation and assay of DNA are described in detail in the text. *K*=extent of methylation, μmol of [^{14}C]methyl/g of DNA divided by concentration of MNNG (μmol/ml).

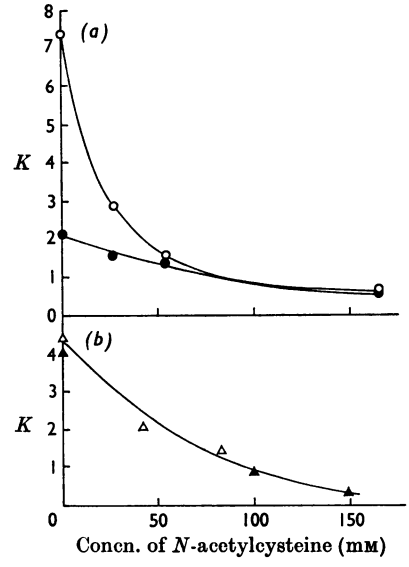


Fig. 5. Effect of *N*-acetylcysteine on methylation of DNA by [^{14}C]methyl methanesulphonate (*b*) and di[^{14}C]methyl sulphate (*a*) at 37°C. For the lower graph (*b*; △ and ▲), reaction mixtures contained salmon sperm DNA (6.6mM-DNA P), disodium EDTA buffer, pH7.6 (50mM), and 1.4mM-di[^{14}C]methyl sulphate; after reaction for 2h in the presence of the concentrations of *N*-acetylcysteine shown, DNA was isolated as described in the text. For the upper graph (*a*), the reaction mixture contained salmon sperm DNA (9.7mM-DNA P), 15mM-sodium phosphate buffer, pH7.0, and 0.09mM-[^{14}C]methyl methanesulphonate; DNA was isolated after 2.8h (●) or 17h (○).

The supernatants from the reaction mixtures after removal of methylated DNA were examined by paper chromatography with solvents (1) or (3) and showed the presence of *N*[^{14}C]-methyl-*N'*-nitroguanidine, accounting for 33% of the total radioactivity in the reaction mixture [R_F values 0.76 in solvent (1) and 0.45 in solvent (3)]. Small amounts of another isotopically labelled product from GSH [R_F values 0.92 in solvent (1) and 0.03 in solvent (3)] were found but were not identified. It appears therefore that the reaction of the type (*b*1) accounts for the liberation of the methylating species from MNNG. Whether the principal side reaction of the type (*b*2) affects DNA has not been investigated.

The effects of thiols on the methylation of DNA by dimethyl sulphate or by methyl methanesulphonate were in the opposite sense to those on methylation by MNNG. The extent of methylation was decreased as the thiol concentration increased (Fig. 5), but much higher concentrations were required than sufficed to catalyse the MNNG methylations. It seemed unlikely that the concentrations of thiol encountered in cells could cause any appreciable effects on methylations by these alkanesulphonates. The kinetics of these methylations were similar to those of the corresponding hydrolyses of the methylating agents (with methyl methanesulphonate a time of half-reaction of about 5h and with dimethyl sulphate about 16min).

Reaction of MNNG with mammalian cells

Effects of thiol concentrations in cells: comparisons with dimethyl sulphate and methyl methanesulphonate. The kinetics of methylation of acid-insoluble material in cultured mammalian cells suspended in growth medium were determined. With [^{14}C]MNNG a rapid uptake of the compound into the soluble fraction of the cells was detected over a period of a few minutes after its addition, then the concentration declined. The maximal uptake was about 10% of the total present. The methylation of the acid-insoluble cellular constituents was also rapid but reached a maximum somewhat later, after about 10min. Both processes were much more rapid than the decomposition of MNNG in growth medium, which had a half-life of about 80min. The reasons for the apparent exclusion of the compound from the cells after a short time of exposure are not obvious, although some effect on the cellular membrane might account for this effect.

The relatively rapid methylation of cellular

Table 3. *Extent of methylation of cellular constituents of cultured mammalian cells by* [^{14}C]*MNNG or by di*[^{14}C]*methyl sulphate*

Cells were grown as described in detail in the text and resuspended in growth medium at about 10^7 cells/ml at 37°C. After treatment the cells were collected by centrifugation and washed and cellular constituents were isolated and assayed for radioactivity. Abbreviations: L, mouse L-cells, culture grown to high density, approx. 5×10^5/ml; AL, mouse L-cells, low density, 10^5/ml, in active growth; SL, mouse L-cells, synchronized by 0.1 M-5-fluorodeoxyuridine, 90% S-phase cells; CHE, hamster embryo, secondary culture grown to 'confluence'; AHE, hamster embryo, 'sparse' secondary culture, in active growth; AME, mouse embryo, 'sparse' secondary culture, in active growth; SH, acid-soluble thiol groups; K, (μmol of [^{14}C]methyl groups/g of cellular constituent)/(concn. of ^{14}C-labelled reagent, mM).

Concn. of MNNG ...	0.23 mM	0.09 mM	0.45 mM	0.45 mM	0.29 mM	0.46 mM	0.51 mM	0.30 mM	0.47 mM	0.27 mM	0.27 mM
Time of treatment at 37°C ...	20 min	40 min	2 min	40 min	40 min	40 min	40 min	40 min	40 min	20 min	40 min
Type of cell	CHE	CHE	CHE	CHE	CHE	CHE	CHE	AHE	AHE	AME	AME
SH (μmol/10^{10} cells)	—	26	35	35	226	31	45	90	37	89	89
K_{DNA}	10.1	10.0	2.4	8.3	8.2	9.9	8.7	22.6	19.0	17.4	20.2
K_{RNA}	18.2	18.6	9.6	23.7	19.7	20.4	17.4	39.5	33.0	27.6	31.2
$K_{protein}$	2.4	2.7	1.3	2.3	3.1	3.3	2.1	5.7	4.3	4.9	—
Concn. of MNNG ...	0.27 mM	0.27 mM	0.35 mM	0.49 mM	0.33 mM	0.65 mM	0.20 mM				
Time of treatment at 37°C ...	40 min	80 min	40 min	40 min	40 min	40 min	40 min				
Type of cell	L	L	L	L	AL	AL	SL				
SH (μmol/10^{10} cells)	41	41	34	—	243	95	250				
K_{DNA}	2.0	1.7	2.0	2.1	7.7	7.0	8.7				
K_{RNA}	5.6	5.3	5.4	8.0	12.2	12.5	11.0				
$K_{protein}$	0.7	0.6	0.3	0.4	0.6	0.6	0.8				
Concn. of dimethyl sulphate ...	0.076 mM	0.038 mM	0.124 mM	0.134 mM	0.09 mM						
Time of treatment at 37°C ...	40 min	40 min	40 min	40 min	40 min						
Type of cell	AL	AL	L	SL	CHE						
SH (μmol/10^{10} cells)	181	—	35	—	—						
K_{DNA}	5.0	5.8	4.8	4.8	8.8						
K_{RNA}	4.6	5.2	4.8	6.4	5.8						
$K_{protein}$	9.2	10.4	9.2	8.4	12.2						

constituents was confirmed by studies of the extents of methylation of isolated cellular DNA, RNA and protein. These were about one-half of the maximal extent (for RNA and protein) and about one-third of this (for DNA) when cells were disrupted 2min after addition of MNNG, but between 20 and 80min after this addition no increase in methylation was found (Table 3).

The results in Table 3 are presented in terms of the factor K, extent of methylation (μmol of [^{14}C] methyl groups/g of cellular constituent) divided by concentration of the methylating agent (in μmol/ml of suspension medium). This factor is constant, independent of MNNG concentration for a given type of cell at a given stage of growth. The influence of the stage of growth was also found to be consistent, in that 'active' cells, i.e. mouse L-cells grown in suspension to low densities, or 'sparse' cultures of rodent embryo cells, gave higher values of K than did 'inactive' L-cells grown to high densities, or confluent cultures of embryo cells. Synchronized L-cells (mostly in the DNA-synthetic phase) behaved in similar fashion to 'active' cells. However, embryo cells showed consistently higher values of K than did mouse L-cells. Thus, apart from this latter difference, the extents of methylation were consistently higher for cells with higher contents of acid-soluble thiol.

Taken together with the observed relative rapidity of the methylations of cellular constituents, these observations suggest that the methylations are thiol-catalysed. This conclusion is supported by the finding that the extents of methylation of protein are much less than those of nucleic acid, possibly indicating that the thiol groups of proteins, known to be the most readily alkylated groups, are blocked by reaction with the nitroguanidine or nitroso moieties of MNNG according to the reactions described for cysteine. However, the possibility that metabolism of MNNG could liberate a methylating species cannot be eliminated. Such metabolism might occur to a greater extent in cells containing higher concentrations of acid-soluble thiol.

Comparison of the cellular methylations by dimethyl sulphate and methyl methanesulphonate with those by MNNG showed that the half-times of reaction of the methyl alkanesulphonates with acid-insoluble cellular constituents were approximately the same as those of their respective hydrolyses, i.e. about 12min for dimethyl sulphate and about 4h for methyl methanesulphonates. The preferential methylation of nucleic acids rather than protein was not found, and the state of 'activity' of the cells did not influence the extent of methylation (Table 3).

Direct evidence for an effect of MNNG on cellular thiols was obtained by using a modification of Ellman's (1959) method for determination of acid-soluble thiol in mouse L-cells treated with MNNG.

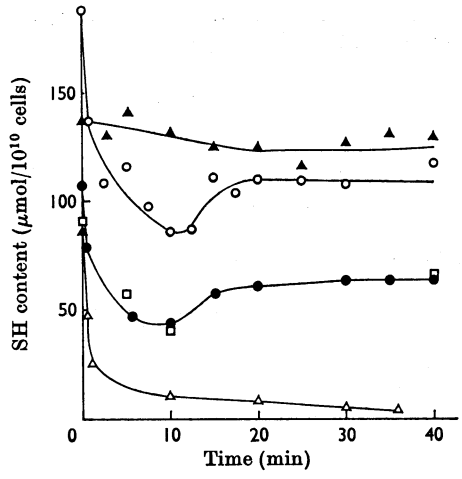


Fig. 6. Effect of MNNG on acid-soluble cellular thiol. To L-cells suspended in growth medium at 37°C MNNG was added to give the concentration stated. After various times the acid-soluble thiol content of washed cells was determined by Ellman's (1959) method, as described in detail in the text. Concentrations of MNNG: ○, 0.69mM; ●, 0.62mM; □, 0.94 mM; △, 3.23mM. For comparison the comparative lack of effect of dimethyl sulphate (0.67mM) is also shown (▲).

At relatively low doses (below 2mM-MNNG) the cellular thiol concentration fell rapidly, but after about 10min a partial recovery was observed (Fig. 6). Higher doses (greater than 2mM) caused a permanent decrease in the thiol concentration.

The coincidence of the onset of this recovery and of the cessation of cellular methylation may be noted, but the results do not permit any detailed interpretation of the cause of either effect. It is not clear whether the loss of thiol is due to simple chemical reaction with MNNG, or whether metabolism of the compound occurs, with concomitant effect on the thiol concentrations. It should be emphasized that the concentrations of MNNG used to demonstrate the effect are high in comparison with the concentrations that prevent cell division. For example, colony formation by active L-cells was decreased to 10^{-3} of that of controls after 40min treatment with 0.02mM-MNNG. After unlimited exposure to the same dose of this compound further inactivation, to 4×10^{-5} of the control value, was found. The high cytotoxicity of MNNG to cultured mammalian cells was also shown by a comparison with the cytotoxic action of dimethyl sulphate. It would be desirable to compare the extents of methylation of cellular constituents by these agents at doses permitting appreciable survival, but the specific radioactivity of MNNG in the present work was inadequate for this purpose. Whether a specific effect of

MNNG on cellular thiols contributes to its cytotoxic action remains to be investigated.

Sites of methylation of DNA by MNNG and by dimethyl sulphate

It was known that the principal product from methylation of DNA by MNNG *in vitro* is 7-methylguanine (McCalla, 1968; Craddock, 1968; Lawley, 1968), and that the main minor product is 3-methyladenine (Lawley, 1968; Craddock, 1969). Since Loveless (1969) had shown that *N*-methyl-*N*-nitrosourea reacts with deoxyguanosine to yield, in addition to 7-methyldeoxyguanosine, O^6-methyldeoxyguanosine, the corresponding O^6-methylated base was sought in hydrolysates of DNA treated with MNNG, which is thought to methylate through the same intermediates as the nitrosourea. In addition, the presence of the expected minor methylation products 3-methylcytosine and 1-methyladenine was investigated. The search for these various products necessitated the use of several methods for hydrolysis of methylated DNA.

In the first place, since O^6-methyldeoxyguanosine was known to be demethylated by vigorous acid hydrolysis (Friedman *et al.* 1965), its hydrolysis by dilute acid was investigated. It was found that this deoxyribonucleoside is stable at pH 2 at 23°C, but spectroscopic examination showed that it was rapidly hydrolysed at pH 1 with a half-life of a few minutes. The free base 2-amino-6-methoxypurine, obtained by the method of Balsiger & Montgomery (1960), was shown to be stable at pH 1 at 37°C for at least 50h and at 70°C for at least 1h, but it decomposed at a measurable rate at pH 1 at 100°C (half-life about 1.4h) to yield guanine. Accordingly methylated DNA was hydrolysed at pH 1 at 37°C for 16h or at 70°C for 30min to liberate methylated purines without appreciable destruction of this acid-labile product.

As described in detail in the Materials and Methods section the following evidence was obtained in support of the identification of the 6-oxygen atom of guanine in DNA as a site methylated by MNNG. Reaction with excess of MNNG and mild acid hydrolysis of methylated DNA gave a blue fluorescent spot of R_F identical with that of 2-amino-6-methoxypurine and with u.v.-absorption spectra at three pH values identical with those of the authentic base. When [^{14}C]MNNG was used a peak of radioactivity at the R_F of this base was consistently observed for hydrolysates of [^{14}C]methylated DNA produced under conditions of mild acid hydrolysis (Fig. 7). But more vigorous conditions, with perchloric acid at 100°C, which are known to demethylate the base, also destroyed the 2-amino-6[^{14}C]-methoxypurine residues in [^{14}C]methylated DNA. When enzymic degradation of this DNA was used,

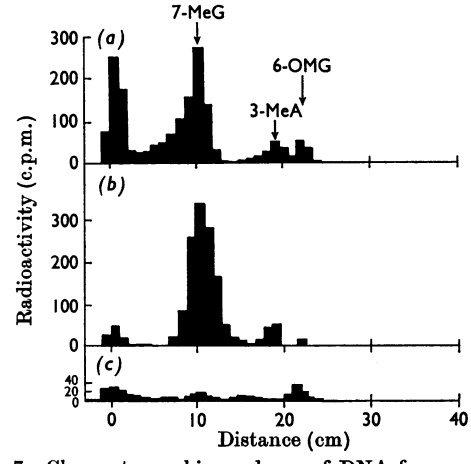


Fig. 7. Chromatographic analyses of DNA from mouse L-cells isolated after treatment of cells with [^{14}C]MNNG (0.49mM) for 40min at 37°C; the extent of methylation of DNA was 0.35mmol/mol of DNA P. Hydrolysed DNA (6mg) was chromatographed on Whatman 3MM paper with appropriate marker bases with solvent (6). Methods for hydrolysis: (*a*) 0.1M-HCl for 16h at 37°C; (*b*) mM-sodium phosphate buffer, pH 7.0, for 20min at 100°C; the [^{14}C]methylated bases liberated were chromatographed; (*c*) residual polynucleotide from (*b*) hydrolysed as for (*a*). The radioactivity on segments of paper (1cm wide) was counted in toluene scintillation fluid as described in the text. The abscissae denote distance (cm) from the origin of the chromatogram. A background of 25c.p.m. was subtracted; efficiency of counting was 44%. Abbreviations: 7-MeG, 7-methylguanine; 3-MeA, 3-methyladenine; OMG, 2-amino-6-methoxypurine.

O^6[^{14}C]-methyldeoxyguanosine was identified in the hydrolysate by co-chromatography with the authentic deoxynucleoside in three solvent systems.

When DNA was methylated *in vitro* or in L-cells with di[^{14}C]methyl sulphate, paper chromatography of hydrolysates obtained with either 0.1M-HCl or with perchloric acid showed the presence of ^{14}C-labelled 7-methylguanine and 3-methyladenine as products but 2-amino-6-methoxypurine was not detected.

The proportion of 3-methyladenine in DNA methylated by dimethyl sulphate was consistently higher than that in DNA methylated by MNNG (Table 4).

Other differences in proportions of methylation products were noted for these two agents. In the Dowex 50 chromatograms of perchloric acid-hydrolysed DNA (Fig. 8) more unidentified minor peaks were found with [^{14}C]MNNG. One was eluted near to thymine; one near to cytosine, not identical with the known product 3-methylcytosine; another near to guanine, separated from the single main peak of 7-methylguanine and 3-methyladenine, which are eluted together in this system; and another

Table 4. *Products from methylation of DNA by* [^{14}C]*MNNG or di*[^{14}C]*methyl sulphate, either* in vitro *at neutral* pH *(with 5mM-GSH added for the reaction with MNNG), or in L-cells treated in suspension in growth medium for 40min at 37°C*

DNA, after isolation as described in the text, was hydrolysed either with 72% (w/v) $HClO_4$ for 1h at 100°C, or with 0.1M-HCl at 37°C for 16h (denoted as pH1), or by treatment at pH7 at 100°C for 20min followed by precipitation of the polynucleotide residue with cold 0.1M-HCl. Methods for chromatography of [^{14}C]methylated bases were; (*a*) with a column of Dowex 50 (H^+ form) eluted with a gradient of 0.75–2M-HCl (7-methylguanine and 3-methyladenine were not separated); or (*b*) chromatography on Whatman 3MM paper with solvents (1), (3) or (6), as described in the Materials and Methods section. Proportions of bases in the various hydrolysates are given as percentages.

DNA ...	Salmon sperm						L-cells					Salmon sperm			L-cells			R_F		
Reagent ...	MNNG						MNNG					Dimethyl sulphate			Dimethyl sulphate			Solvent		
Extent of methylation (mmol/mol of DNA P) ...	6						0.3					3			0.4			(1)	(3)	(6)
Method for hydrolysis	$HClO_4$	$HClO_4$	pH1	pH1	pH1	pH7	$HClO_4$	$HClO_4$	pH1	pH1	pH7	$HClO_4$	pH1	pH7	$HClO_4$	pH1	pH1			
Method for chromatography	*a*	3	3	6	6	1	*a*	6	6	6	6	*a*	3	1	6	3	6			
7-Methylguanine					67	76				70	71			76	84	80	71	0.31	0.14	0.35
3-Methyladenine		3	11	9	12	11		7	7	7	7		19	15	15	15	11	0.50	0.31	0.58
7-Methylguanine+ 3-methyladenine	83						83					94						—	—	—
2-Amino-6-methoxy-purine		<0.2	6	7	7			<0.6	6	6			<0.2		<0.5	<1	<1	0.50	0.55	0.68
3-Methylcytosine	2						2					2						0.57		
1-Methyladenine	1						1					3						0.35	0.23	—

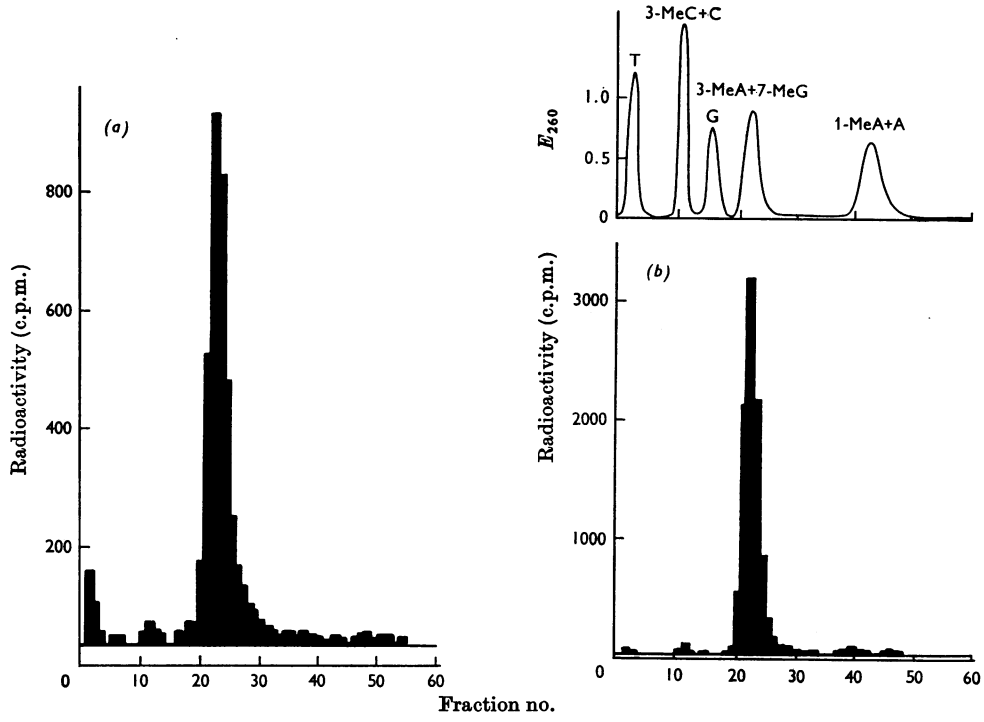


Fig. 8. Analyses of salmon sperm DNA methylated by: (*a*), [^{14}C]MNNG in the presence of GSH (extent of methylation, 6mmol/mol of DNA P); (*b*), di[^{14}C]methyl sulphate (3mmol/mol of DNA P). DNA (6mg for *a*; 4mg for *b*) was hydrolysed with 72% (w/v) $HClO_4$ for 1h at 100°C and the hydrolysate was applied to a column of Dowex 50 (H^+ form) and eluted with a gradient of 0.75–2M-HCl, as described in detail in the text. The positions of bases eluted are indicated: T, thymine; C, cytosine; G, guanine; A, adenine; and the positions of added marker bases; 3-MeC, 3-methylcytosine; 7-MeG, 7-methylguanine; 3-MeA, 3-methyladenine; 1-MeA, 1-methyladenine. These ^{14}C-labelled bases were further identified by evaporation of appropriate fractions and by paper chromatography of the residues. [^{14}C]Methylated DNA from cells treated with the respective agents gave identical chromatographic profiles.

eluted after adenine, distinct from 1-methyladenine, which precedes adenine.

In the corresponding chromatograms of [^{14}C]-methylated RNA, it was notable that dimethyl sulphate yielded more of the minor products 3-methylcytosine and 1-methyladenine than did MNNG. Mild acid hydrolysis of RNA methylated with [^{14}C]MNNG did yield some 2-amino-6[^{14}C]-methoxypurine, but a method for quantitative determination has yet to be devised.

The results, in summary, do therefore provide some support for the view that the differences in activation of MNNG and dimethyl sulphate with respect to methylation of DNA *in vitro* and in cells are reflected in the ability of MNNG to methylate a wider spectrum of sites than can dimethyl sulphate. This difference may well be due to the significant contribution of the methylcarbonium ion to the mode of reaction of MNNG. However, the possibility that the methyldiazonium ion could react with DNA in a manner different from the presumed bimolecular mechanism operating with dimethyl sulphate cannot be ruled out. Sites of *O*-methylation by MNNG other than the extranuclear 6-oxygen atom of guanine established by the present work may well exist. The possibility of phosphotriester formation in DNA has already been stressed by Olson & Baird (1969). Whether this leads to the rapid chain fission of DNA in neutral media was not, however, proved by these authors, since the observed degradation of DNA followed treatment in alkali. Nevertheless phosphotriester formation in RNA would be expected to cause chain fission at neutral pH values (cf. Brown & Todd, 1955).

It seems likely on theoretical grounds that the alkylnitrosamidines and alkylnitrosamides in general would alkylate through alkyldiazonium intermediates. Therefore, if *O*-methylation in nucleic acids were characteristic of such compounds, in contrast with alkylating agents such as dimethyl sulphate, which appear to be confined to *N*-methylation, such *O*-methylation might be held responsible for certain biological effects of alkylnitrosamides. Loveless & Hampton (1969) have already established

that *N*-methyl-*N*-nitrosourea can cause mutations in bacteriophage T2 by extracellular treatment, but methyl methanesulphonate was not mutagenic in this system. Swann & Magee (1968) found that methylation of rat kidney DNA could not of itself account for carcinogenesis by methylating agents, since *N*-methyl-*N*-nitrosourea, a potent carcinogen for this organ, gave less methylation than did the non-carcinogen methyl methanesulphonate. As pointed out by Loveless (1969), the ability of the alkylnitrosamide to methylate the 6-oxygen atom of guanine in DNA could readily account for the causation of transition mutations in bacteriophage, and this process could therefore be important for alkylation carcinogenesis if the hypothetical cancer-initiating mutations were of this type. Alternatively the formation of unstable phosphotriester groups in cellular RNA might be invoked as a process specific to the *O*-alkylating carcinogens (Mizrahi & Emmelot, 1964).

The specific mutagenic effects of ethylating, as opposed to methylating, agents, first noted by Kølmark (1956) and further emphasized by the work of Loveless (1959), may also be attributed to the ability of the former to ethylate the 6-oxygen of guanine in DNA, since this has been shown to occur with deoxyguanosine (Loveless, 1969). However, the alternative possibility has been suggested that formation of phosphotriester groups in DNA may also be a factor in ethylation mutagenesis, since ethylation by diethyl sulphate, but not methylation by dimethyl sulphate, leads to degradation of a polyribonucleotide at neutral pH, a reaction thought to be diagnostic for phosphotriester formation (Ludlum, 1969). Here again, *O*-alkylation may be due to the greater tendency of the ethylating agent to react through the S_N1 mechanism. Some differences between MNNG and its ethyl analogue are also likely, since on theoretical grounds the ethyldiazonium intermediate is expected to be less stable than the methyldiazonium ion, and a comparison between these analogues now seems desirable.

Some of the preliminary chemical studies were performed by P. D. L. at the Chester Beatty Research Institute, London S.W.3, U.K. with the skilled technical assistance of Mrs J. H. Lethbridge. We also thank Mrs G. Milne and Mrs I. Beauchamp for skilled technical assistance, Mr S. Bryniak for performing some of the experiments on decomposition of MNNG, and Dr R. Lumley Jones for determination of i.r. spectra. We acknowledge the benefit of discussions with Dr A. Loveless, Chester Beatty Research Institute, and thank him for allowing us to see the manuscript of a paper before publication. This work was supported by a grant from the National Cancer Institute of Canada.

REFERENCES

Balsiger, R. W. & Montgomery, J. A. (1960). *J. org. Chem.* **25**, 1573.
Brookes, P. & Lawley, P. D. (1960). *J. chem. Soc.* p. 539.
Brookes, P. & Lawley, P. D. (1962). *J. chem. Soc.* p. 1348.
Brown, D. M. & Todd, A. R. (1955). In *The Nucleic Acids*, Vol. 1, p. 455. Ed. by Chargaff E. & Davidson, J. N. New York: Academic Press Inc.
Craddock, V. M. (1968). *Biochem. J.* **106**, 921.
Craddock, V. M. (1969). *Biochem. J.* **111**, 615.
Doherty, D. G., Shapira, R. & Burnett, W. T. (1957). *J. Am. chem. Soc.* **79**, 5667.
Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
Freese, E. (1959). *Brookhaven Symp. Biol.* **12**, 63.
Friedman, D. M., Mahapatra, G. N., Dash, B. & Stevenson, R. (1965). *Biochim. biophys. Acta*, **103**, 286.
Garrett, E. R., Goto, S. & Stubbins, J. F. (1965). *J. pharm. Sci.* **54**, 119.
Hall, R. H. (1967). In *Methods in Enzymology*, vol. 12, p. 305. Ed. by Grossman, L. & Moldave, K. New York: Academic Press Inc.
Harris, S. R. (1958). *J. Am. chem. Soc.* **80**, 2303.
Ingold, C. K. (1953). *Structure and Mechanism in Organic Chemistry*, Chapter 7. Ithaca, N.Y.: Cornell University Press.
Jones, J. W. & Robins, R. K. (1963). *J. Am. chem. Soc.* **85**, 193.
Khym, J. X., Shapira, R. & Doherty, D. G. (1957). *J. Am. chem. Soc.* **79**, 5663.
Kirby, K. S. (1957). *Biochem. J.* **66**, 495.
Kølmark, G. (1956). *C. r. Trav. Lab. Carlsberg*, **26**, 205.
Lawley, P. D. (1966). *Prog. nucleic Acid Res. molec. Biol.* **5**, 89.
Lawley, P. D. (1968). *Nature, Lond.*, **218**, 580.
Lawley, P. D. & Brookes, P. (1961). *Nature, Lond.*, **192**, 1081.
Lawley, P. D. & Brookes, P. (1963). *Biochem. J.* **89**, 127.
Loveless, A. (1959). *Proc. R. Soc.* B, **150**, 497.
Loveless, A. (1969). *Nature, Lond.*, **223**, 206.
Loveless, A. & Hampton, C. L. (1969). *Mutation Res.* **7**, 1.
Ludlum, D. B. (1969). *Biochim. biophys. Acta*, **174**, 773.
McCalla, D. R. (1968). *Biochim. biophys. Acta*, **155**, 114.
McCalla, D. R., Reuvers, A. & Kitai, R. (1968). *Can. J. Biochem.* **46**, 807.
McKay, A. F. (1949). *J. Am. chem. Soc.* **71**, 1968.
McKay, A. F., Ott, W. L., Taylor, G. W., Buchanan, M. N. & Crooker, J. F. (1950). *Can. J. Res.* B **28**, 683.
McKay, A. F. & Wright, G. F. (1947). *J. Am. chem. Soc.* **69**, 3028.
Mizrahi, J. J. & Emmelot, P. (1964). *Biochim. biophys. Acta*, **91**, 362.
Nagata, C., Imamura, A., Saito, H. & Fukui, K. (1963). *Gann*, **54**, 109.
Olson, A. O. & Baird, K. M. (1969). *Biochim. biophys. Acta*, **179**, 513.
Pal, B. C. (1962). *Biochemistry, Easton*, **1**, 558.
Schoental, R. (1965). *Nature, Lond.*, **209**, 148.
Schulz, U. & McCalla, D. R. (1969). *Can. J. Chem.* **47**, 2021.
Sheinin, R. (1962). *Virology*, **17**, 426.
Sparkes, B. G. & Walker, I. G. (1966). *Can. J. Biochem.* **44**, 1159.
Swann, P. F. & Magee, P. N. (1968). *Biochem. J.* **110**, 39.
Thatcher, C. J. & Walker, I. G. (1969). *J. natn. Cancer Inst.* **42**, 363.
Walker, I. G. & Helleiner, C. W. (1963). *Cancer Res.* **23**, 734.