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Reaction Products from *N*-Methyl-*N*-nitrosourea and Deoxyribonucleic Acid Containing Thymidine Residues

SYNTHESIS AND IDENTIFICATION OF A NEW METHYLATION PRODUCT, O^4-METHYLTHYMIDINE

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1. DNA was treated with *N*-methyl-*N*-nitrosourea at pH 7–8, 37°C, degraded to yield 3- and 7-methylpurines and deoxyribonucleosides and the reaction products were separated by chromatography on ion-exchange resins. The following methods for identification and determination of products were used: with unlabelled *N*-methyl-*N*-nitrosourea, u.v. absorption; use of *methyl*-^{14}C-labelled *N*-methyl-*N*-nitrosourea and use of [^{14}C]thymine-labelled DNA. 2. The synthesis of O^4-methylthymidine and its identification by u.v. and mass spectroscopy are reported. 3. 3-Methylthymidine and O^4-methylthymidine were found as methylation products from *N*-methyl-*N*-nitrosourea with thymidine and with DNA, in relatively small yields. Unidentified products containing thymine were found in enzymic digests of *N*-methyl-*N*-nitrosourea-treated DNA, which may be phosphotriesters. 4. The possible role of formation of methylthymines in mutagenesis by *N*-methyl-*N*-nitrosourea is discussed.

Methylating agents are known to react at the following sites in base residues of nucleic acids: N-1, N-3 and N-7 of adenine; N-3 of cytosine; N-3, N-7 and O-6 of guanine (reviewed by Lawley, 1972*a*,*b*). The remaining principal bases, uracil in RNA, or thymine in DNA, have received less attention because of their evidently low reactivities towards alkylation. Recently, methods for separation and measurement of methylation products in nucleic acids, involving degradation of methylated nucleic acids enzymically to nucleosides and the chromatographic separation of the latter, have been described and applied to both types of nucleic acid (Lawley & Shah, 1972*a*,*b*; Lawley, 1972*b*). It was therefore decided to apply these methods to investigate the possible methylation of the thymidine residue in DNA. The mutagen and carcinogen *N*-methyl-*N*-nitrosourea, which has been quite extensively studied previously (reviewed by Lawley, 1972*b*) and is known to react at the O-6 atom of guanine, was selected as methylating agent.

This study was expected to be relevant to the general question whether specific DNA-methylation reactions might lead to induction of potentially miscoding bases in the scheme of mutagenesis by anomalous base-pairing as proposed by Watson & Crick (1953). It may be noted in this regard that Tessman *et al.* (1964) had provided evidence, from studies of ethylation mutagenesis with bacteriophage containing single-stranded DNA, that thymine was a mutable base.

At that time alkylation of thymine residues in DNA had not been detected, although subsequently Loveless & Hampton (1969) found that 3-methylthymine was a product from methylation of DNA by *N*-methyl-*N*-nitrosourea. On the other hand, Craddock (1971–72) investigated the possible occurrence of this product in DNA methylated *in vivo* by dimethylnitrosamine but did not detect it.

With regard to other possible sites of thymine methylation, Wong & Fuchs (1971) reported that diazomethane can methylate 1-methyluracil at the extranuclear O-4 atom, but not at O-2. It therefore seemed likely that thymidine would be methylated at O-4, and this possibility has now been established to occur by Farmer *et al.* (1973). In the present work, the methylation of thymidine and of thymidine residues in DNA by *N*-methyl-*N*-nitrosourea has been investigated. This necessitated the synthesis of the hitherto unreported O^4-methylthymidine.

The occurrence of this product in DNA methylated by the mutagen *N*-methyl-*N*-nitrosourea would be of particular interest with regard to the possible molecular mechanism of mutagenesis. The structure of the corresponding methylated base residue in DNA is

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analogous to that of the previously proposed miscoding base O^6-methylguanine (Loveless, 1969). In both cases O-alkylation fixes the tautomeric structures that are anomalous in the Watson–Crick sense, in that they permit guanine–thymine pairing without distortion of the overall dimensions of base-pairs, which had previously been noted for ionized forms of 7-alkyldeoxyguanosine residues or of the thymidine analogue, 5-bromodeoxyuridine (Lawley & Brookes, 1962).

Since the amounts of thymine-derived products in methylated DNA were expected to be small, it was clear that methods of separation of these derivatives from major methylation products would require attention. It was decided to avoid acid hydrolysis, since apart from the expected instability of the O-methylated derivative, this method had not enabled the detection of 3-methylthymine in the work of Craddock (1971–72). This raised the difficulty that the major methylated nucleoside, 7-methyldeoxyguanosine, would under the conditions used for enzymic degradation of DNA be converted into a number of pyrimidine derivatives [as found by Lawley & Shah (1972*a*) in parallel analyses of methylated RNA].

However, it was expected that this problem could be obviated by removal of certain alkylpurines from methylated DNA by hydrolysis at neutral pH values; those methylpurines that are released by hydrolysis on heating a neutral solution of methylated DNA include the 3- and 7-methylated derivatives of both adenine and guanine. The partially depurinated DNA could then be subjected to enzymic degradation. With regard to methods for separation of products, the chromatographic system using Dowex 50 (NH_4^+ form), which proved useful for methylated RNA (Lawley & Shah, 1972*a*), had already been applied to methylated DNA (Lawley & Shah, 1972*b*; Lawley, 1972*b*). In addition it seemed desirable to identify the new products on other systems, and Dowex 1 (formate form) was therefore used.

It was also decided to identify the products by showing that they contained both the methyl group derived from *N*-methyl-*N*-nitrosourea radioisotopically labelled in the methyl group, and the thymine residue; for the latter purpose DNA prelabelled with [^{14}C]thymine was obtained from a thymine-requiring strain of *Escherichia coli*.

Materials and Methods

Materials

DNA from salmon sperm [Sigma (London) Chemical Co., London SW6, U.K.] was always used. [^{14}C]Thymine-labelled DNA was obtained by the methods described by Lawley & Brookes (1968), from a culture of *E. coli* B/r T$^-$ (500ml; 8×10^8 cells/ml) grown in M9 medium containing [^{14}C]thymine (10μg, 0.15μCi/ml; sp. radioactivity 2μCi/mg) (yield 10mg).

Radioisotopically labelled *N*-methyl-*N*-nitrosourea was obtained as described by Lawley & Shah (1972*a*) at a sp. radioactivity of 0.74mCi/mmol (*methyl*-^{14}C-labelled). Unlabelled *N*-methyl-*N*-nitrosourea was prepared similarly on a larger scale.

3-Methylthymidine was obtained from Dr. D. M. Brown (University Chemical Laboratory, Cambridge, U.K.). O^4-Methylthymidine was synthesized as described below. O^6-Methyldeoxyguanosine was obtained from deoxyguanosine by the method of Farmer *et al.* (1973). Other methylation products required as marker compounds were obtained as described by Lawley & Shah (1972*a*), except the deoxyribonucleosides rather than ribonucleosides were used. In chromatographic eluents, 'ammonium formate' denotes appropriate mixtures from diluted A.R. conc. aq. NH_3 (sp.gr. 0.88) and A.R. formic acid; the molarities stated are approximate, based on the assumption that conc. aq. NH_3 was 15M.

Methylation of DNA

Reactions were generally carried out at pH7–8, 37°C.

When unlabelled *N*-methyl-*N*-nitrosourea was used, in a typical experiment, the reaction mixture (25ml) contained DNA (approx. 10mM, 4.5mg/ml) in Tris–HCl buffer (0.5M), pH8; *N*-methyl-*N*-nitrosourea was added, with stirring, to give a concentration of 0.4M. Evolution of N_2 ceased after about 30min, when the cooled solution was made 0.25M in sodium acetate and DNA was precipitated in fibrous form with ethanol (2vol.). The DNA was washed with ethanol and dried in a desiccator.

When [^{14}C]*N*-methyl-*N*-nitrosourea was used, the reaction mixture (10ml) contained DNA (7mM-P), 280mM-sodium acetate, pH7.1, and [^{14}C]*N*-methyl-*N*-nitrosourea (10mM; 7.4μCi/ml). After 4h at 37°C, the DNA was precipitated and washed twice; the extent of reaction was 6mmol of methyl groups/mol of DNA P.

When ^{14}C-labelled DNA was used, the reaction mixture (5ml) contained DNA (1mM-P), Tris–HCl buffer, pH8 (0.4M), and *N*-methyl-*N*-nitrosourea (0.2M).

Degradation of methylated DNA

For enzymic degradation, DNA (about 10mg/ml) was dissolved in water, and deoxyribonuclease (from bovine pancreas; Sigma) (130 Kunitz units/ml, in 10mM-magnesium acetate, pH about 6.5) was added; after 30min at 37°C, the non-viscous solution was adjusted to about pH8 by adding 1M-Tris–HCl buffer (0.1vol.), and venom phosphodiesterase (about 0.03EC unit/ml) was added. Generally digestion was continued for 16h.

In some cases, methylated DNA was prehydrolysed at pH7, 100°C, for 30min; when [*methyl*-^{14}C]*N*-methyl-*N*-nitrosourea was used, the residual polynucleotides were then precipitated by adding, in the cold, 1M-HCl (0.1vol.). The precipitate after washing with cold 0.1M-HCl was redissolved and degraded enzymically, otherwise the whole solution was treated with the enzymes. The supernatant after removal of polynucleotide material contained 3- and 7-methyladenine and 3- and 7-methyl guanine, as shown by chromatography (next section).

Chromatography

The general procedures have been described by Lawley & Shah (1972*a*) for products from methylated RNA. In general, fractions of 6.4ml were collected, and the positions of elution of marker compounds were determined from their u.v. absorption.

Additional procedures used here were first, that in separation of pyrimidine nucleosides from enzymic digests on a column of Dowex 50 (NH_4^+ form), it was found advantageous to use a more dilute eluent (0.1M-ammonium formate) at somewhat lower pH (8.0) than the previously used pH8.9. With a column of dimensions 60cm × 1.5cm, the order of elution was: thymidine, peak fraction 25; 3-methylthymidine, 31; deoxycytidine, 34; O^4-methylthymidine, 42; deoxyguanosine, 73; deoxyadenosine, 84; O^6-methyldeoxyguanosine, 128; after fraction 140, elution was continued with 1M-ammonium formate, pH8.9, which eluted 3-methyldeoxycytidine at fraction 220 and 1-methyldeoxyadenosine at fraction 237. If methylpurines were present in the mixture, they were eluted as follows: 3-methylguanine, fraction 49; 3-methyladenine, 58; 7-methylpurines were eluted together with the purine deoxyribonucleosides.

The 3- and 7-methylpurine liberated by neutral hydrolysis were separated on Dowex 50 (NH_4^+ form), eluted with 0.3M-ammonium formate, pH4.3. With a column of dimensions 22cm × 1.5cm, the peak fractions were: 7-methylguanine, 50; 7-methyladenine, 65; 3-methylguanine, 74; 3-methyladenine, 127.

As an alternative to cation-exchange chromatography, anion exchange was used to separate deoxyribonucleosides. With a column (80cm × 1.5cm) of Dowex AG-1 (formate form) it was found necessary to use graded concentrations of ammonium formate, pH8.9: 0.01M for 50 fractions; gradient of 0.01–1M, a further 70 fractions; then 1M. The order of elution was: thymidine, fraction no. 10; 3-methyldeoxycytidine, 25; O^4-methylthymidine, 37; 3-methylthymidine, 52; O^6-methyldeoxyguanosine, 170.

When neutral hydrolysis of methylated DNA was used, removal of 7-methylguanine from the residual polynucleotide was not complete, presumably because of adsorption of a small proportion of this base to the polymer. However, the positions of the elution of this base were well removed from the region of elution of pyrimidine nucleosides; when Dowex 1 was used, 7-methylguanine was eluted just before O^6-methyldeoxyguanosine.

Determinations of proportions of radioactive products were made by measurement of radioactivity in fractions by liquid-scintillation counting in a phosphor containing the detergent Triton X-100 (Lawley & Shah, 1972*a*); the positions of elution of products were determined by u.v. monitoring of appropriate added marker compounds. In some cases, portions (1ml) of methylthymidine-containing fractions were counted for radioactivity, and then the residual fractions were rechromatographed. This was particularly necessary for 3-methylthymidine, which was eluted together with the 'tail' of the thymidine peak. The O^4-methylthymidine peak showed only a single peak on rechromatography.

Determinations of amounts of non-radioactive products were made by u.v.-absorption spectroscopy with a Unicam SP.8000 spectrophotometer; with a 40mm cell, the limit of detection of absorbance was about 0.002, corresponding to about 0.001 μmol of products. It was necessary to rechromatograph the fractions in the region of elution of O^4-methylthymidine, since these contained the 'tail' of the deoxycytidine peak.

Syntheses

Synthesis of O^4-methylthymidine (*α-anomer*). 3′5′-Di-*O*-*p*-toluoyl-O^4-methylthymidine (α-anomer) was obtained from 3,5-di-*O*-*p*-toluoyl-2′-deoxyribofuranosyl chloride and 2,4-dimethoxy-5-methylpyrimidine, as described by Prystas *et al.* (1963); to a solution of this derivative (0.5g) in methanol (40ml) was added sodium metal (50mg). After 16h at 5°C, the solution was neutralized by the addition of Amberlite MB 1 resin, and then filtered and concentrated. An aqueous solution of the residue was extracted with ether. Concentration of the aqueous phase gave an oil, which was purified by chromatography on a column (10cm × 2cm) of silicic acid (Merck Kieselgel G; Anderman and Co., London S.E.1, U.K.), with methanol–chloroform (2:3, v/v) as eluent. The product (0.174g, m.p. 165–168°C) was recrystallized from ethyl acetate.

O^4-Methylthymidine (*α-anomer*) gave prismatic needles (0.135g, 52%); m.p. 166–168°C; $[\alpha]_D^{25} = -57.4°$ (*c*1.0 in water) (Found: C, 51.8; H, 6.5; N, 10.7; $C_{11}H_{16}N_2O_5$ requires C, 51.55; H, 6.3; N, 10.9%). The u.v. spectra of the product were as follows: in 0.1M-HCl, $\lambda_{max.}$ 279nm ($10^{-3}\epsilon_{max.}$ 6.0), $\lambda_{m.in.}$ 245nm; in water, $\lambda_{max.}$ 279nm ($10^{-3}\epsilon_{max.}$ 6.1), $\lambda_{min.}$ 241nm; in 0.1M-NaOH, $\lambda_{max.}$ 279nm ($10^{-3}\epsilon_{max.}$ 6.1), $\lambda_{min.}$ 243nm. The mass spectrum is shown in Fig. 1(*b*).

Synthesis of O^4-methylthymidine (*β-anomer*). This

compound was similarly prepared (see above) from 3',5'-di-*O*-*p*-toluoyl-O^4-methylthymidine (β-anomer). The product, after washing with ether, gave colourless needles (0.197g), m.p. 164–168°C. Recrystallization from ethyl acetate gave O^4-methylthymidine β-anomer as prismatic needles (0.164g, 64%); m.p. 171.5–173.5°C; $[\alpha]_D^{25} = +60.9°$ (*c* 1.0 in water) (Found: C, 51.85; H, 6.5; N, 10.8%). The u.v. spectra were as follows: in 0.1M-HCl, $\lambda_{max.}$ 279nm ($10^{-3}\epsilon_{max.}$ 6.1), $\lambda_{min.}$ 241nm; in water, $\lambda_{max.}$ 280nm ($10^{-3}\epsilon_{max.}$ 6.3), $\lambda_{min.}$ 241nm; in 0.1M-NaOH, $\lambda_{max.}$ 280nm ($10^{-3}\epsilon_{max.}$ 6.1), $\lambda_{min.}$ 243nm. The mass spectrum is shown in Fig. 1(*a*).

Reaction of thymidine with N-methyl-N-nitrosourea

Thymidine (1mmol) was dissolved in deionized water (10ml) in a pH-stat (Radiometer, Copenhagen, Denmark) set at pH7.2, 37°C; after addition of *N*-methyl-*N*-nitrosourea (4mmol) with stirring, the pH was maintained by automated addition of Tris base (2M). Reaction mixtures obtained in this way, after addition of base had ceased, were chromatographed separately on columns (80cm×1.5cm) of Dowex 50 (NH_4^+ form), eluted with 0.3M-ammonium formate, pH8.9 or pH6.65. Three main u.v.-absorbing peaks were found in each case; the first to be eluted was identified by u.v. spectra as unchanged thymidine; the second was 3-methylthymidine, $\lambda_{max.}$ 267nm, E_{280}/E_{260} 0.71, $\epsilon_{max.}$ 9100; the third product had $\lambda_{max.}$ 279.5nm, E_{280}/E_{260} 2.0, $\epsilon_{max.}$ 6300, at both pH7 and pH13. This last product was shown to be converted into thymidine ($\lambda_{max.}$ 267nm, E_{280}/E_{260} 0.71) by the change in u.v. spectra of solutions maintained at pH1, 70°C, virtually complete in 20min, or at pH12.7, 35°C, with a half-life of about 3h (product, $\lambda_{max.}$ 266.5nm, E_{280}/E_{260} 0.67, i.e. identical with thymidine, isosbestic point at 275nm). It was identified as O^4-methylthymidine by comparison with the authentic synthetic product, as follows.

The product (from fractions 37–42) was freeze-dried. The resulting mixture of the suspected O^4-methylthymidine and ammonium formate was dissolved in methanol and the solution was applied to a plate (20cm×5cm) coated with silicic acid (Merck Kieselgel GF_{254}), which was then developed in

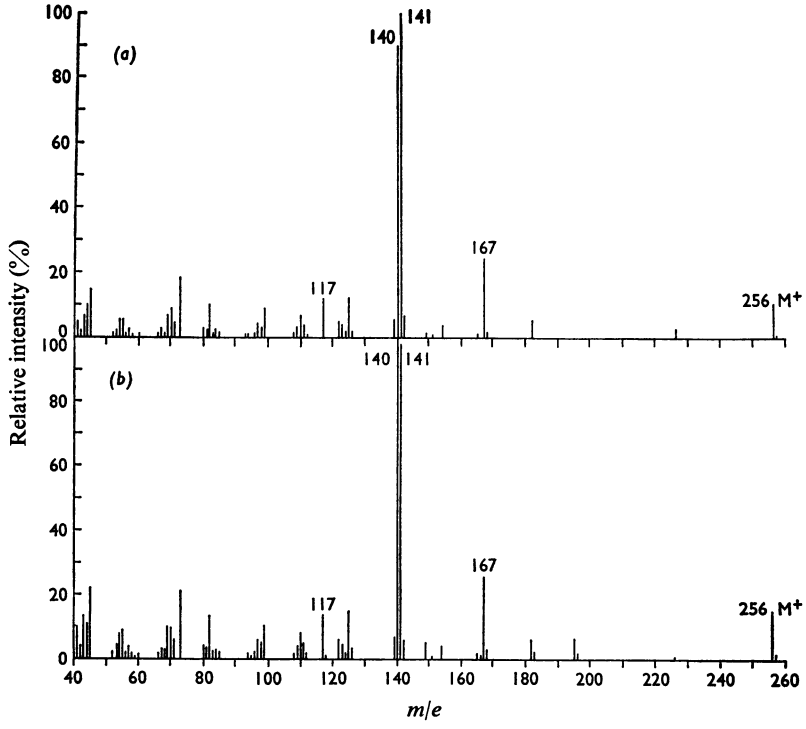

Fig. 1. *Mass spectra of O^4-methylthymidine*

(*a*) β-Anomer; (*b*) α-anomer. Spectra were obtained with an AEI MS-12 instrument by the direct insertion technique at an ionizing potential of 70eV and source temperature of 135–150°C.

methanol–chloroform (1:9, v/v). The mobile u.v.-detectable component was eluted with methanol. Ammonium formate was detected on the plate as a brown band only after exposure to the vapour of iodine. It was scarcely mobile in this system. The eluted component, after t.l.c. again as described above, gave a strong u.v.-detectable band at an R_F value (0.28) identical with that of a sample, identically developed, of synthetic O^4-methylthymidine (β-anomer). The mass spectrum of the product derived from *N*-methyl-*N*-nitrosourea obtained after elution by the method of Rix *et al.* (1969) contained all the features present in the spectrum (Fig. 1*b*) of the authentic sample.

Results and Discussion

Synthesis of the anomeric O^4-methylthymidines

Although O^4-methyluridine (β-anomer) has been synthesized (Robins & Naik, 1971) the anomeric O^4-methylthymidines have not hitherto been reported. Formerly, structural modification at the 4-position of the presently utilized intermediates, the anomeric 3′,5′-di-*O*-*p*-toluoyl-O^4-methylthymidines (Prystas *et al.*, 1963), had preceded the removal of the toluoyl groups. The O^4-methylthymidine (β-anomer) here described, and which has been prepared from a rigorously characterized intermediate, was identical with a minor product formed (see Farmer *et al.*, 1973) from the reaction between thymidine and diazomethane. This identity served to characterize the product from the last-mentioned, less-rigorous procedure, which, being a one-stage procedure, might more conveniently be used for the synthesis of other O^4-alkylthymidines by using homologous diazoalkanes.

Mass spectrometry of the O^4-methylthymidines

Mass spectrometry was used as an additional aid to the characterization of O^4-methylthymidine among the products of the alkylation of thymidine with *N*-methyl-*N*-nitrosourea. For the confirmation, by mass spectrometry, of the sites of alkylation on the purine bases, the bases were preferred to the deoxyribosides (see Farmer *et al.*, 1973). However, the pyrimidine–deoxyribose linkage is less labile to acid. In the specific case of O^4-alkylation of thymine the lability to acidic hydrolysis of the methoxy groups dictates that O^4-alkylation be detected at the level of the deoxyriboside.

The mass spectra of the anomeric O^4-methylthymidines (Fig. 1) appear little influenced by configurational differences at the glycosidic linkage. A few major fragments in the spectra can be assigned a similar origin to analogous fragments in the mass spectrum of 2′-deoxyuridine (Biemann & McCloskey, 1962). Thus the signals at *m/e* 112 and 113 in the spectrum of 2′-deoxyuridine correspond to those at *m/e* 140 $[(B+H)^+]$ where B denotes the O^4-methylthymidinyl radical, and 141 $[(B+2H)^+]$ in the present spectra. Similarly there are present (Fig. 1) peaks at *m/e* 117 (deoxyribose$^+$) and *m/e* 167 ($B.CH_2CH_2^+$), the latter corresponding to the (*m*-89) peak in the spectrum of the deoxyuridine. A detailed, unambiguous determination of the fragmentations of the base moiety would require deuterium-labelled analogues (cf. 2-amino-6-methoxypurine; Farmer *et al.*, 1973).

Table 1. *Products from salmon sperm DNA and [methyl-^{14}C]N-methyl-N-nitrosourea*

The methylated DNA was prehydrolysed at pH7, 100°C, and the liberated 3- and 7-methylpurine were chromatographed separately. The residual polynucleotide was degraded enzymically and the resultant deoxyribonucleosides were then chromatographed. Details of methods are given in the text. Relative yields of products refer to the percentages of radioactivity in the total of products identified; it should be noted that about 18% of radioactivity was in unidentified material (possibly derived from phosphotriesters; see the text). The overall extent of methylation was 6mmol of methyl groups/mol of DNA P.

Method for chromatography of deoxyribonucleosides ...	Relative yield of product (%)	
	Dowex 1 (formate form)	Dowex 50 (NH_4^+ form)
1-Methyldeoxyadenosine	0.4	2.3
3-Methyladenine	8.0	7.9
7-Methyladenine	1.5	2.3
3-Methyldeoxycytidine	0.8	1.0
3-Methylguanine	0.8	0.8
O^6-Methyldeoxyguanosine	7.8	6.9
7-Methylguanine	79	78
3-Methylthymidine	0.3	0.3
O^4-Methylthymidine	0.6	0.2

However, a comparison with other alkylthymidines is in this case unnecessary, since the *O*-alkylated thymidine is distinguished additionally by the lability of the alkyl substituent to acidic hydrolysis.

Reaction of N-methyl-N-nitrosourea with thymidine

Thymidine (1 mmol), on reaction with *N*-methyl-*N*-nitrosourea (4 mmol) at pH 7, 37°C, gave small amounts of two products identified as 3-methylthymidine (yield, estimated from u.v. spectra, about 20 μmol) and O^4-methylthymidine (yield, about 6 μmol). The identification of the latter product, not previously reported from methylation of thymidine, was based on its identity with the synthetic product by u.v. and mass spectra. It was also shown to be converted into thymidine on mild acid (pH 1, 70°C) or alkali (pH 12.7, 35°C) treatments, but was stable under the conditions used for isolation of deoxynucleoside products from enzymically degraded DNA.

Reaction of N-methyl-N-nitrosourea with DNA; identification of products from thymidine residues

DNA was methylated with *methyl*-^{14}C-labelled *N*-methyl-*N*-nitrosourea (to an extent of 0.6 mmol of methyl groups/mol of DNA P), 3- and 7-methylpurines were removed by hydrolysis from the methylated DNA at neutral pH values and the residual polynucleotide was degraded to nucleosides. Small amounts of labelled products were eluted coincident with the u.v.-absorbing markers of 3-methylthymidine and O^4-methylthymidine, in two chromatographic systems, one using a cation-exchange resin, Dowex 50 (NH_4^+ form) and the other an anion-exchange resin, Dowex 1 (formate form) (see Table 1 and Fig. 2). It was checked that no other known methylated nucleoside ran at the position of elution of O^4-methylthymidine. With regard to the other possible methylation product from thymidine, O^2-methylthymidine, it should be pointed out that at present this compound is not known. Furthermore, in the methylation of thymidine with diazomethane, no evidence for such a product was found (Farmer *et al.*, 1973) and Wong & Fuchs (1971) failed to find methylation at O-2 with the analogue 1-methyluracil. However, in the absence of an authentic marker, the presence of O^2-methylthymidine in the present work with *methyl*-^{14}C-labelled DNA cannot entirely be eliminated. It was also difficult to rule out the possibility that a derivative of 7-methyldeoxyguanosine could have been eluted near to the position of 3-methylthymidine. It should be noted, however, that the prior hydrolysis at neutral pH, 100°C, was expected to convert 7-methyldeoxyguanosine into 7-methylguanine completely.

Further experiments were therefore carried out to support the identification of methylated thymidines.

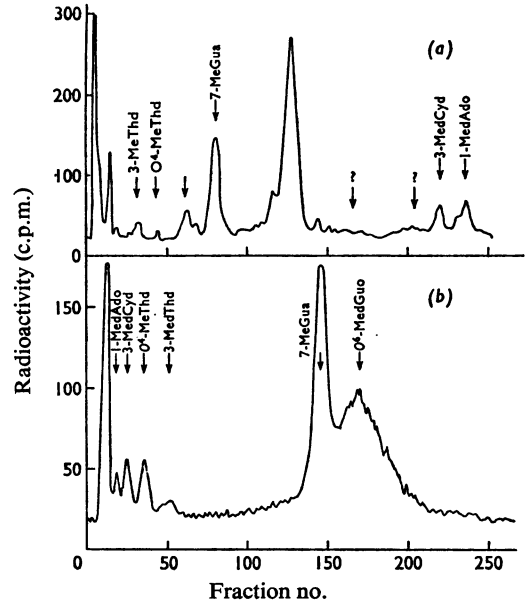

Fig. 2. *Chromatography of products from salmon sperm DNA treated with [methyl-*^{14}*C]N-methyl-N-nitrosourea*

The ^{14}C-methylated DNA was prehydrolysed at pH 7, 100°C, to remove 3- and 7-methylpurines (which were chromatographed separately, as described in the text). The residual polynucleotide was degraded enzymically with deoxyribonuclease, venom phosphodiesterase and alkaline phosphatase and chromatographed as a mixture of nucleosides and (probably, see the text) of phosphotriesters or degradation products therefrom. (*a*) Chromatogram obtained by using Dowex 50 (NH_4^+ form), eluted with 0.1 M-ammonium formate, pH 8.0, up to fraction 140, and then with 1 M-ammonium formate, pH 8.9. (*b*) Chromatogram obtained by using Dowex 1 (formate form) eluted as described in detail in the Materials and Methods section. The positions of elution of identified nucleosides are shown by arrows: 1-MedAdo, 1-methyldeoxyadenosine; 3-MedCyd, 3-methyldeoxycytidine; O^6-MedGuo, O^6-methyldeoxyguanosine; 3-MeThd, 3-methylthymidine; O^4-MeThd, O^4-methylthymidine. Some 7-methylguanine (denoted 7-MeGua) had evidently remained adsorbed to the polynucleotide. Unidentified products from Dowex 50 (NH_4^+ form) are denoted by question marks (for discussion of their probable nature as phosphotriesters, see the text). Radioactivity eluted in early fractions may contain methanol from partial alkaline hydrolysis of phosphotriesters.

First, DNA (250 μmol of P) was treated with *N*-methyl-*N*-nitrosourea (40 mol/mol of DNA P), degraded enzymically and chromatographed on Dowex

50 (NH_4^+ form). The u.v. absorption of fractions known to coincide with the positions of elution of the methylthymidines was measured. 3-Methylthymidine could not be identified positively by this procedure, since the large peak of thymidine extended into its region of elution. At the position of elution of O^4-methylthymidine, u.v. absorption with $\lambda_{max.}$ about 280nm and E_{280}/E_{260} greater than unity was detected, and this could be rechromatographed and re-detected (Fig. 3). The amount was small, and near the limits of detection of the methods used. The yield of O^4-methylthymidine calculated from the u.v. absorption of the appropriate fractions was compared with that of the principal methylated pyrimidine, 3-methyldeoxycytidine, and other products that could be measured by this method (Table 2). The relative yields obtained in this way were in reasonable agreement with those obtained from radioisotopically labelled DNA. Evidently the thymidine products were the least abundant of those detected. Discrepancies in their estimated proportions may be ascribed either to some decomposition during the procedures used, or to incomplete liberation by enzymic hydrolysis.

Secondly, DNA (5μmol of P) prelabelled with [^{14}C]thymine was treated with excess of *N*-methyl-*N*-nitrosourea (200mol/mol of DNA P) and then degraded and chromatographed as described above. In the region of the pyrimidine nucleosides, assay of samples of fractions for radioactivity showed labelled peaks at the positions of elution of thymidine, 3-methylthymidine, deoxycytidine and O^4-methylthymidine (Fig. 4). The methylthymidine peaks were then rechromatographed to separate any overlapping material; as expected, this was necessary to separate 3-methylthymidine from the 'tail' of the thymidine peak; the O^4-methylthymidine was quantitatively recovered as a single peak. The molar proportions relative to thymidine were 0.04% for 3-methylthymidine and 0.03% for O^4-methylthymidine (Table 3).

A somewhat surprising outcome of this last experiment was the elution of considerable amounts of radioactive products in the region of the purine nucleosides and beyond. These products were not

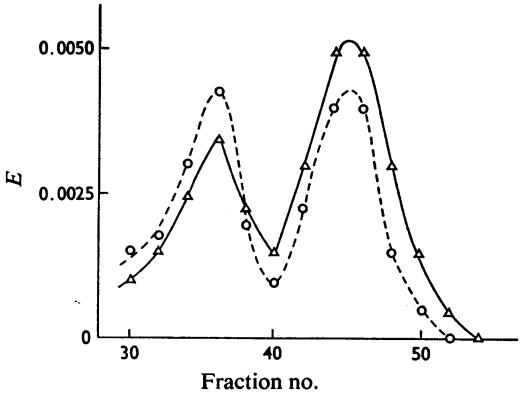

Fig. 3. *Rechromatography of fractions from an enzymic digest of N-methyl-N-nitrosourea-treated salmon sperm DNA containing O^4-methylthymidine, monitored by u.v. absorption*

A chromatogram of a digest obtained on Dowex 50 (NH_4^+ form) as for Fig. 2(*a*), but by using unlabelled *N*-methyl-*N*-nitrosourea, contained u.v.-absorbing material in fractions 42–43, at the known position of elution of O^4-methylthymidine. These fractions were rechromatographed in the same system and the u.v. absorption was measured in a 40mm path-length cuvette on a Unicam SP.8000 spectrophotometer, at a maximum absorbance setting corresponding to $E_{1cm} = 0.05$, i.e. 0.0005 per chart division; ○, E_{260}; △, E_{280}. The first peak is the 'tail' absorption due to deoxycytidine; the second with $\lambda_{max.}$ 280nm has the spectral characteristics of O^4-methylthymidine.

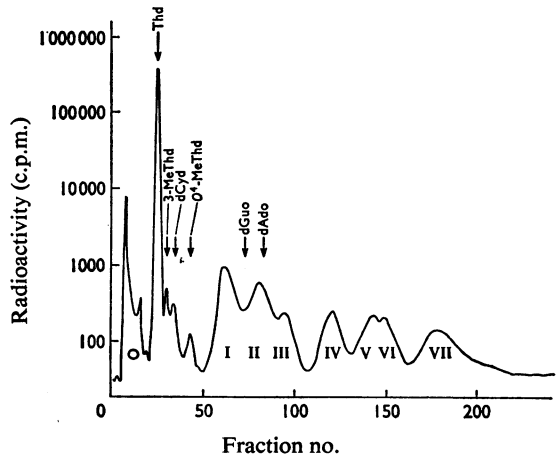

Fig. 4. *Chromatogram of an enzymic digest of N-methyl-N-nitrosourea-treated [^{14}C]thymine-labelled DNA from E. coli*

The chromatogram, on Dowex 50 (NH_4^+ form), was obtained as for Fig. 2(*a*). Arrows show the positions of u.v.-absorption peaks due to the normal deoxyribonucleosides: Thd, thymidine; dCyd, deoxycytidine; dGuo, deoxyguanosine; dAdo, deoxyadenosine; and of added markers, 3- and O^4-methylthymidine (3-MeThd, O^4-MeThd). The ordinate scale of c.p.m. is logarithmic; background was 30c.p.m. The products eluted after O^4-methylthymidine are probably phosphotriesters (see the text).

deoxyguanosine or deoxyadenosine, and in untreated control DNA no labelled products apart from thymidine and a trace of deoxycytidine were found. The relative amount of O^4-methylthymidine to the total of these products was about 1:100.

Table 2. *Products from salmon sperm DNA and N-methyl-N-nitrosourea, measured from u.v. absorption of chromatographic fractions*

The reaction mixture contained 40mol of *N*-methyl-*N*-nitrosourea/mol of DNA P. *N*-Methyl-*N*-nitrosourea-treated DNA was prehydrolysed at pH7, 100°C, liberating 3- and 7-methylpurines; the residue was degraded enzymically and the whole of the products were chromatographed on Dowex 50 (NH_4^+ form), eluted with 0.1M-ammonium formate, pH8.0, to fraction 150, and then with 1M-ammonium formate, pH8.9. The products shown were obtained from appropriate u.v.-absorbing regions; other products were not separated by this method. The relative molar yields were estimated by summation of the u.v. absorption of the fractions. The region containing O^4-methylthymidine was rechromatographed (see Fig. 3).

Product	Fractions	Recovery (μmol)
Thymidine	20–29	72
Deoxycytidine	30–39	49
O^4-Methylthymidine	42–44	0.013
3-Methylguanine	48–52	0.09
O^6-Methyldeoxyguanosine	120–140	0.68
3-Methyldeoxycytidine	230–240	0.08
1-Methyldeoxyadenosine	250–260	0.08

The region of elution of these products was also that in which some radioactivity from [*methyl*-^{14}C]-*N*-methyl-*N*-nitrosourea-treated DNA was eluted and in which some u.v. absorption was also noted when unlabelled *N*-methyl-*N*-nitrosourea was used as reagent. Further, labelled products from *N*-methyl-*N*-nitrosourea-treated ^{32}P-labelled DNA are found in this region (P. D. Lawley, unpublished work).

The [^{14}C]thymine-labelled products appeared as seven peaks, not well separated; the other procedures did not generally resolve peaks in this region; an increased background of either u.v. absorption or *methyl*-^{14}C was found, although the earliest unidentified product on Dowex 50 (NH_4^+ form) was quite well resolved (cf. Fig. 2*a*). A possible explanation of the available results is that these products are phosphotriesters of the type Xp(Me)Y, where X and Y represent deoxyribonucleoside residues (there are seven possible products of this type out of 16 possible triesters of the four non-methylated deoxyribonucleosides). Bannon & Verly (1972) have found evidence for formation of phosphotriesters in DNA alkylated by methyl methanesulphonate and ethyl methanesulphonate, much less for the former reagent (about 1% of the methylation products and about 20% of ethylation products). These phosphotriesters were stable at neutral pH values and were not degraded by phosphodiesterases.

It may be recalled that Lawley & Shah (1972*a*) found that rather more than 10% of radioactivity in digests from RNA methylated by [*methyl*-^{14}C]-*N*-methyl-*N*-nitrosourea could not be accounted for in base-methylation products, and was eluted early from Dowex 50 columns. The possibility was considered that some of these products were derived from

Table 3. [^{14}C]*Thymine-labelled products from* [^{14}C]*thymine-labelled E. coli DNA and N-methyl-N-nitrosourea*

The reaction mixture contained 200mol of *N*-methyl-*N*-nitrosourea/mol of DNA P. *N*-Methyl-*N*-nitrosourea-treated DNA (4.5×10^6c.p.m.) was degraded enzymically to nucleosides and chromatographed on Dowex 50 (NH_4^+ form), eluted with 0.1M-ammonium formate, pH8.0, to fraction 140, and then with 1M-ammonium formate, pH8.9. Control DNA (6.2×10^5c.p.m.) was treated as for *N*-methyl-*N*-nitrosourea-treated DNA, but with omission of the *N*-methyl-*N*-nitrosourea reaction. Some radioactive peaks, denoted O, I . . . VII (see Fig. 4) contained unidentified products, possibly derived from phosphotriesters (see the text).

		% of radioactivity	
Product	Fractions	*N*-Methyl-*N*-nitrosourea-treated DNA	Control DNA
O	1–20	1.5	0.02
Thymidine	21–29	94.7	99.92
3-Methylthymidine	30–32	0.15	Nil
3-Methylthymidine (re-run)	28–32	0.04	Nil
Deoxycytidine	33–40	0.1	0.06
O^4-Methylthymidine	41–44	0.03	Nil
O^4-Methylthymidine (re-run)	41–44	0.03	Nil
I-VII	50–220	2.9	Nil

hydrolysis of phosphotriester groups in the methylated RNA, which were expected to be unstable at neutral pH values, unlike those from methylated DNA. The present work on methylation of DNA suggests that it may be possible to isolate and identify DNA phosphotriester products by procedures analogous to those used here.

In summary, an investigation of products derived from methylation of thymidine residues in DNA by *N*-methyl-*N*-nitrosourea at pH8 has shown that 3-methylthymidine and O^4-methylthymidine are formed, but in relatively small proportions of total products, less than 1%. These products were also obtained by methylation of thymidine at neutral pH values, also in small yield.

Methylation of thymine residues in DNA at N-3, if it occurred *in vivo*, would presumably block the formation of hydrogen bonds with its partner base adenine according to the Watson–Crick model, and would thus parallel the methylation of cytosine at N-3 and of adenine at N-1. Methylation at O-4 of the thymine might, however, be expected to permit anomalous base-pairing with guanine, since the proton attached to N-3 would presumably be lost to give the neutral O^4-methylthymidine residue, and would no longer be available to participate in hydrogen-bond formation.

There is thus an analogy between O^4-methylthymidine and O^6-methyldeoxyguanosine in that both are 'promutagenic' bases, in the sense described. If mutations could be induced only by formation of these bases at random in DNA, and they were equally likely to yield such an effect, the ratio of their extents of formation would equal the ratio of the probability of induction of AT→GC transitions to that of GC→AT transitions. According to the present work, this ratio would therefore be very approximately about 1:50.

However, the accuracy of estimations of the two products is very different. The proportion of O^6-methyldeoxyguanosine in the methylation products from *N*-methyl-*N*-nitrosourea-treated DNA has been estimated consistently as between 6 and 7%, and moreover the same results are obtained if this product is determined as the base (by acid hydrolysis of DNA) or as the deoxyribonucleoside (by enzymic hydrolysis). O^4-Methylthymidine cannot be estimated by chemical hydrolysis of DNA, since it is too unstable. The present methods have detected this product, but evidently improved methods will be necessary to obtain a good quantitative estimate of its proportion relative to other products.

The general conclusions derived from investigations of induced mutagenesis by alkylating agents, that GC→AT transitions generally predominate markedly over AT→GC transitions (see Krieg, 1963; Drake, 1970), could thus accord with the present hypothesis that these result from mispairing of O^6-alkylguanines and O^4-alkylthymines, since it may be expected that generally the relative predominance of reactivity at the extranuclear O atom of the purine will be maintained for other alkylating mutagens. However, further studies will clearly be needed to support this hypothesis.

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