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Alkylation of Deoxyribonucleic Acid by Carcinogens Dimethyl Sulphate, Ethyl Methanesulphonate, *N*-Ethyl-*N*-nitrosourea and *N*-Methyl-*N*-nitrosourea

RELATIVE REACTIVITY OF THE PHOSPHODIESTER SITE THYMIDYLYL(3′-5′)THYMIDINE

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1. The ethyl phosphotriester of thymidylyl(3′-5′)thymidine, dTp(Et)dT, was identified as a product from reaction of DNA with *N*-ethyl-*N*-nitrosourea, by procedures parallel to those reported previously for the methyl homologue produced by *N*-methyl-*N*-nitrosourea. 2. Enzymic degradation to yield alkyl phosphotriesters from DNA alkylated by these carcinogens and by dimethyl sulphate and ethyl methanesulphonate was studied quantitatively, and the relative yields of the triesters dTp(Alk)dT were determined. The relative reactivity of the phosphodiester group dTpdT to each of the four carcinogens was thus obtained, and compared with that of DNA overall, or with that of the N-7 atom of guanine in DNA. Relative reactivity of the phosphodiester group was lowest towards dimethyl sulphate, the least electrophilic of the reagents used, and was highest towards *N*-ethyl-*N*-nitrosourea, the most electrophilic reagent. 3. The nature of the alkyl group transferred also influenced reactivity of the phosphodiester site, since this site was relatively more reactive towards ethylation than would be predicted simply from the known Swain–Scott *s* values of the alkylating agents. It was therefore suggested that the steric accessibility of the weakly nucleophilic phosphodiester group on the outside of the DNA macromolecule favours its reaction with ethylating, as opposed to methylating, reagents. 4. Taking a value of the Swain–Scott nucleophilicity (*n*) of 2.5 for an average DNA nucleotide unit [Walles & Ehrenberg (1969) *Acta Chem. Scand.* **23**, 1080–1084], a value of *n* of about 1 for the phosphodiester group was deduced, and this value was found to be 2–3 units less than that for the N-7 atom of guanine in DNA. 5. The reactivity of DNA overall was markedly high towards the alkylnitrosoureas, despite their relatively low *s* values. This was ascribed to an electrostatic factor that favoured reaction of the negatively charged polymer with alkyldiazonium cation intermediates.

A previous report from this laboratory (Swenson *et al.*, 1976) described the isolation and positive identification, with a synthetic standard, of the phosphotriester dTp(Me)dT from DNA methylated by *N*-methyl-*N*-nitrosourea. We now report the extension of this work to analytical procedures for determination of this triester and its homologues in DNA alkylated by a series of alkylating carcinogens of graded chemical reactivity. These reagents, in increasing order of electrophilic reactivity [or of decreasing Swain–Scott (1953) *s* factor], are dimethyl sulphate, ethyl methanesulphonate, *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea (Osterman-Golkar *et al.*, 1970; Veleminsky *et al.*, 1970).

Having devised such methods, we proceeded to use them to compare the overall reactivities of these carcinogens with DNA under standard reaction conditions, and further, to compare the reactivity to these carcinogens of the phosphodiester group (typified mainly by the dTpdT group in DNA) with that of a characteristic nucleophilic centre, the N-7 atom of guanine in DNA.

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Abbreviations used: dTp(Alk)dA, dTp(Alk)dC, dTp(Alk)dT, alkyl phosphotriesters of thymidylyl-(3′-5′)-deoxyadenosine, -deoxycytidine or -thymidine, where Alk is Me, methyl or Et, ethyl; dTpdA, thymidylyl-(3′-5′)deoxyadenosine; dTpdC, thymidylyl(3′-5′)-deoxycytidine; dTpdT, thymidylyl(3′-5′)thymidine; dTpEt, thymidine 3′-ethyl phosphate; dTpMe, thymidine 3′-methyl phosphate; EtpdT, thymidine 5-′ethyl phosphate; MedpT, thymidine 5′-methylphosphate; R_T, retention time.

Experimental

Reagents

Di[^{14}C]methyl sulphate (31 mCi/mmol), di[^{3}H]-methyl sulphate (16.7 mCi/mmol), [^{14}C]ethyl methanesulphonate (8.7 mCi/mmol), *N*-[^{14}C]methyl-

N-nitrosourea (11 mCi/mmol) and [^{3}H]ethylamine hydrochloride (360 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *N*-[^{3}H]Ethyl-*N*-nitrosourea (13.6 mCi/mmol) was synthesized from [^{3}H]ethylamine hydrochloride after dilution with unlabelled ethylamine hydrochloride as described for the synthesis of *N*-methyl-*N*-nitrosourea (Lawley & Shah, 1972).

DNA from salmon sperm, dT, dA, dG, dC, dTpdT, dTpdA, dTpdC, *Escherichia coli* alkaline phosphatase (Type III) (EC 3.1.3.1), venom phosphodiesterase (Type II, from *Crotalus adamanteus*) (EC 3.1.4.1), and deoxyribonuclease I from bovine pancreas (EC 3.1.4.5) were products of Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Venom phosphodiesterase was dissolved in 0.1 M-sodium borate buffer, pH 8.5, at 0.4 unit/ml, and deoxyribonuclease was dissolved at 1000 units/ml in 0.1 M-sodium borate buffer (pH 8.5)/0.01 M-$MgSO_4$. (In each case, 1 unit of enzyme activity is defined as 1 μmol of substrate transformed/min.) The enzymes were stored at –20°C.

5′-*O*-(*p*-Monomethoxytrityl)thymidine (Schaller *et al.*, 1963), dTp(Me)dT (Swenson *et al.*, 1976), *N*,3′-*O*-diacetyldeoxycytidine 5′-phosphate, 3′-*O*-acetylthymidine 5′-phosphate (Khorana & Vizsolyi, 1961) and *N*,3′-*O*-diacetyldeoxyadenosine 5′-phosphate (Schaller & Khorana, 1963) were prepared as described in the literature. dTpMe, MepdT, EtpdT and dTpEt were prepared by standard procedures (Khorana, 1959; De Boer *et al.*, 1973); *N*,3′-*O*-Diacetyldeoxycytidine 5′-phosphate (20 μmol) was similarly methylated, and the acetyl-blocking groups were removed in conc. NH_3 (sp.gr. 0.880) in 1 h at 20°C to yield deoxycytidine 5′-methylphosphate (16 μmol). The following chromatographic standards were obtained as described in previous studies: O^6-methyl- or O^6-ethyl-deoxyguanosine, O^6-methyl- or O^6-ethyl-guanine, 7-methyl- or 7-ethyl-guanine, and 3-methyl- or 3-ethyl-adenine (Farmer *et al.*, 1973; Lawley *et al.*, 1973; Lawley & Shah, 1972; Denayer, 1962).

Chromatography

MN Cel 300 UV_{254} sheets of cellulose on plastic (Macherey–Nagel, Düren, Germany) were purchased from Camlab, Cambridge, U.K., and 0.5 or 1.0 mm thick layers of silica (Kieselgel HF_{254}; BDH Chemicals, Poole, Dorset, U.K.) were prepared on glass plates. Thin-layer chromatograms were developed in the following solvent systems: A, chloroform/methanol (17:3, v/v); B, 2-methylpropan-2-ol/butan-2-one / conc. NH_3 (sp.gr. 0.880) / water (4:3:2:1, by vol.); C, chloroform/methanol/ethanol/conc. NH_3 (sp.gr. 0.880) (34:10:5:1, by vol.); D, chloroform/methanol (3:1, v/v); E, butan-1-ol/water/acetic acid (50:25:11, by vol.); F, chloroform/methanol/formic acid (85:15:1, by vol.).

High-pressure liquid chromatography was carried out on a Waters ALC/GPC 204 liquid chromatograph equipped with a model U6K injector, a model 660 programmer and a model 440 dual-wavelength u.v. detector set at 254 and 280 nm (Waters Associates, Northwich, Cheshire, U.K.). Cation-exchange chromatography was on a silica-based Partisil 10-SCX Magnum 9 column (9.2 mm × 250 mm) preceded by a Partisil 10-SCX precolumn (4.6 mm × 100 mm) (Whatman, Bromley, Kent, U.K.); reversed-phase chromatography was with a Waters μBondapak C_{18} column (4.6 mm × 250 mm) coupled to a precolumn (4.6 mm × 100 mm) of Partisil 10-ODS (Whatman); and chromatography on silica was with a column of Partisil 10 (4.6 mm × 250 mm).

Synthesis of dTp(Et)dT

dTp(Et)dT was prepared along the general lines of the synthetic procedures of Miller *et al.* (1974), which were modified for the synthesis of dTp(Me)dT (Swenson *et al.*, 1976).

Thus 5′-*O*-(*p*-monomethoxytrityl)thymidine was condensed with 3′-*O*-acetylthymidine 5′-phosphate to yield 5′-*O*-(*p*-monomethoxytrityl)thymidylyl(3′-5′)-thymidine 3′-*O*-acetate, and this protected phosphodiester was converted into the protected ethyl triester in 77% yield by dry ethanol and toluene-*p*-sulphonyl chloride in the solvent *NN*-dimethylformamide/2,6-lutidine (Miller *et al.*, 1974). The acetyl and monomethoxytrityl protecting groups were removed (Swenson *et al.*, 1976) and the dTp(Et)dT was purified by chromatography on 0.5 mm-thick layers of Kieselgel HF_{254} in solvent system A [R_F, 0.41; R_F for Tp(Me)T, 0.32; 53% yield]. The $\lambda_{max.}$ (266 nm) and $\lambda_{min.}$ (234 nm) in the u.v. spectrum were identical with those found for dTp(Me)dT (Swenson *et al.*, 1976).

The identity of the synthesized material as dTp-(Et)dT was confirmed by its products of alkaline hydrolysis. dTp(Et)dT (4.0 μmol) was dissolved in 0.1 M-NaOH (2.0 ml). At various times, samples (0.2 ml) were added to 0.2 M-acetic acid (0.2 ml) and chromatographed on 0.5 mm-thick layers of Kieselgel HF_{254} developed in system B [product, R_F: dT, 0.68; dTp(Et)dT, 0.63; dTpEt, 0.49; EtpdT, 0.46; dTpdT, 0.43]. In some cases, samples were chromatographed on 0.5 mm-thick layers of Kieselgel HF_{254} in system C [product, R_F: dT, 0.88; dTp(Me)dT, 0.91; dTpEt, 0.48; EtpdT, 0.39; dTpdT, 0.14]. dTp(Et)dT was converted under the alkaline conditions with a half-life $t_{\frac{1}{2}}$ of 6.5 ± 0.3 h into the four expected u.v.-absorbing products, and the amount of dT produced was equal to the total amounts of the ethyl esters of 3′- and 5′-TMP (see Table 2). Product yields were determined by using the molar absorption coefficients of 1.84×10^4 litre·mol^{-1}·cm^{-1} for dTpdT, 1.80×10^4 litre·mol^{-1}·cm^{-1} for dTp(Et)dT, and 9.6×10^3 for

dT (Miller *et al.*, 1971); the absorption coefficients of EtpdT and dTpEt were taken to be 9.6×10^3 litre·mol^{-1}·cm^{-1}.

Synthesis of dTp(Me)dC

dTp(Me)dC was prepared by coupling 5′-*O*-(*p*-monomethoxytrityl)thymidine and *N*,3′-*O*-diacetyl-deoxycytidine 5′-phosphate in the presence of toluene-*p*-sulphonyl chloride, followed by methylation of the phosphate group by methanol and toluene-*p*-sulphonyl chloride in *NN*-dimethylformamide and 2,6-lutidine (54% yield) as described previously for dTp(Me)dT (Swenson *et al.*, 1976). The *O*- and *N*-acetyl groups were removed from this protected phosphotriester, in an aqueous methanol/pyridine/NaOH system (Swenson *et al.*, 1976) at 27°C for 10 min, and the 5′-*O*-*p*-monomethoxytrityl group was removed in 80% (v/v) acetic acid kept at 28°C for 5h. The product, dTp(Me)dC, was purified by chromatography on 0.5mm-thick layers of Kieselgel HF_{254} in solvent system D (R_F 0.18; 67% yield). The u.v. spectrum of the sample was indistinguishable from that of dTpdC at pH7.0 ($\lambda_{max.}$ 268nm; $\lambda_{min.}$ 236nm) and at pH1.0 ($\lambda_{max.}$ 272nm; $\lambda_{min.}$ 235nm).

The identity of the product as dTp(Me)dC was confirmed when it was hydrolysed in 0.1M-NaOH at 37°C with a half-life ($t_{\frac{1}{2}}$ 1.7h) similar to that of dTp(Me)dT under these conditions ($t_{\frac{1}{2}}$ 2.3h) (Swenson *et al.*, 1976). Portions of the hydrolysed sample were chromatographed on 0.5mm-thick layers of Kieselgel HF_{254} in system C [product, R_F: dT, 0.82; dTp(Me)dC, 0.59; dC, 0.31; dTpMe, 0.20; MepdC, 0.06; dTpdC, 0.06], and the fractions containing dTpMe, MepdC and dTpdC were rechromatographed on MN-cellulose sheets in system B (product, R_F: MepdC, 0.41; dTpMe, 0.34; MepdT, 0.29; dTpdC, 0.19). Under these conditions, only the five expected u.v.-absorbing products, dT, dC, dTpMe, MepdC and dTpdC, were observed.

Synthesis of dTp(Me)dA

dTp(Me)dA was prepared similarly, starting with *N*,3′-*O*-diacetyldeoxyadenosine 5′-phosphate. The fully blocked dTp(Me)dA isolated was deacetylated by incubation in conc. NH_3 (sp.gr. 0.088) in pyridine (3:2, v/v) at 27°C for 2h (71% yield). Parallel studies on the lability of the methyl phosphotriester group to NH_3 showed that under these conditions dTp(Me)dT was degraded with a half-life of about 4.5h but dTp(Et)dT appeared to be stable. The monomethoxytrityl group of the deacetylated triester was removed in 80% acetic acid at 28°C for 4h, and the product was purified by chromatography on silica in system B to give dTp(Me)dA (R_F 0.58) in 60–70% yield. The u.v. spectra of the product at pH7.0 ($\lambda_{max.}$ 260nm; $\lambda_{min.}$ 233nm) and at pH1.0 ($\lambda_{max.}$ 259nm; $\lambda_{min.}$ 233nm) were similar to those of dTpdA.

Reaction of alkylating agents with DNA

DNA was dissolved in 0.1M-Tris/HCl buffer, pH8.0, at 37°C at a concentration of 15μmol of DNA P/ml of reaction mixture (measurement based on $\varepsilon_p = 6500$) and was caused to react with ^{14}C-labelled *N*-methyl-*N*-nitrosourea or ethyl methanesulphonate or ^3H-labelled dimethyl sulphate or *N*-ethyl-*N*-nitrosourea (20μmol/ml of reaction mixture) at 37°C. After 2.5h, 2.5M-sodium acetate (0.1 vol.) and ethanol (2 vol.) were added to the solution. The DNA was removed, washed in ethanol and then ether, and redissolved in water. The cycle of precipitation and washing was repeated twice to free the DNA from unbound radioactivity.

Samples of DNA for which different extents of overall alkylation were required were obtained by adjusting the concentration of alkylating agent in the reaction mixture.

Enzymic degradation of DNA to yield phosphotriesters

In early experiments [e.g. Swenson *et al.* (1976) and results shown in Tables 1 and 2], DNA was enzymically digested in the presence of Tris/HCl buffers. However, it was found that dTp(Me)dT was slowly degraded by the Tris under the conditions for enzymic digestion. Thus in 42h at 37°C about 30% of dTp(Me)dT (1.3mM) in 0.2M-Tris/HCl, pH8.0, was converted into polar products that could be digested by venom phosphodiesterase to yield dT. This Tris-dependent degradation is presumably attack of the amine by the S_N2 mechanism on the alkyl group of the phosphotriester (Cox & Ramsay, 1964). Ethyl triesters would therefore be expected to be more resistant to this type of reaction, in agreement with their observed stability towards NH_3. Subsequent enzyme degradations were therefore carried out in 0.1M-sodium borate, pH8.5, and under these conditions no degradation of dTp(Me)dT or dTp(Me)dC could be detected by t.l.c. after 40h of incubation, in the presence or absence of enzymes.

DNA samples were dissolved in water at concentrations of 2–4μmol of DNA P/ml and adjusted to pH8.5 by addition of 1M-sodium borate buffer (0.1vol.) The samples were digested at 37°C for 30min with 100 units (0.1ml) of deoxyribonuclease/ml of DNA, and then with 0.04 unit (0.1ml) of venom phosphodiesterase and 2.5 units of *E. coli* alkaline phosphatase/ml of DNA. At 24h, the addition of phosphodiesterase and alkaline phosphatase was repeated and the hydrolyses were terminated at 46–52h by addition of 1M-formic acid (0.04ml/ml of hydrolysate).

Time course of enzymic hydrolysis

DNA alkylated by N-[^{14}C]methyl-N-nitrosourea at an overall content of 1.3 mmol of methyl groups/mol of DNA P was dissolved in water at 4.0 μmol of DNA P/ml and digested as described above, but with a third addition, at 48 h, of venom phosphodiesterase and alkaline phosphatase. At various times, samples (0.5 ml) were added to 0.02 ml of 1 M-formic acid to stop the digestion.

A fraction of the alkylated DNA was dissolved in 1 mM-sodium cacodylate/formic acid buffer, pH 6.5, and heated at 100°C for 30 min to liberate 3- and 7-methylpurines. The solution was cooled, adjusted to pH 8.5 with 1 M-sodium borate buffer (0.1 vol.) and digested as described above. At various times, portions (0.5 ml) were added to 1 M-formic acid (0.02 ml) and dTp(Me)dT was determined by high-pressure liquid chromatography (see below).

Separation of dTp(Et)dT by chromatography on Dowex 50 (NH_4^+ form)

DNA that had been treated with N-[^{3}H]ethyl-N-nitrosourea at the ratio 4.3 mmol of ethyl groups/mol of DNA P (1.4 μCi total) was heated at 100°C for 30 min at pH 7 to liberate 3- and 7-ethyl purines. The partially apurinic residue was precipitated with 1 M-HCl (0.1 vol.) and was digested in Tris buffer as described for N-methyl-N-nitrosourea-treated DNA (Swenson *et al.*, 1976). The sample (1.0 μCi) was applied, with unlabelled dTp(Et)dT, to a column (1.5 cm × 50 cm) of Dowex AG 50W (X4; NH_4^+ form), which was eluted as described by Lawley (1973). A peak of radioactivity (0.044 μCi) which chromatographed with dTp(Et)dT and dA was collected, evaporated *in vacuo*, and rechromatographed on 0.5 mm-thick layers of Kieselgel HF_{254} in system A. The dTp(Et)dT band was eluted with methanol, the specific radioactivity was measured (Swenson *et al.*, 1976), and the dTp(Et)dT was rechromatographed on a silica t.l.c. in system B. The specific radioactivity was again measured, and the cycle was repeated with solvent E (see Table 1).

Separation by t.l.c. of dTp(Alk)dT from digests of alkylated DNA

Alkylated DNA samples were directly digested by enzymes for 46 h in 0.1 M-sodium borate buffer, pH 8.5, as described above, but in the presence of dTp(Alk)dT (3.5–4.5 μmol/ml). The unlabelled triesters served as internal standards by which corrections for losses in the chromatography of hydroly-

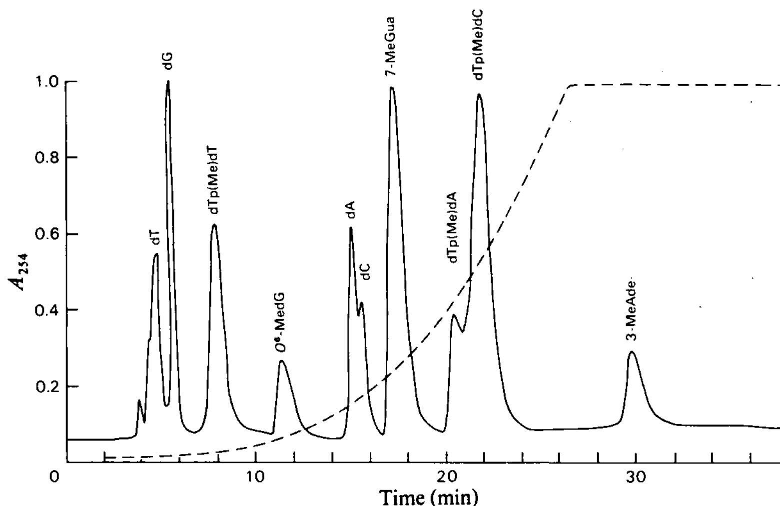

Fig. 1. *Typical u.v.-absorption profile of an enzymic digest of methylated salmon sperm DNA, with added markers, subjected to high-pressure liquid chromatography on Partisil 10-SCX cation exchanger eluted with a gradient of ammonium formate, pH 3.7, in dilute aqueous methanol*

Details of methods for methylation of DNA and enzymic digestion are given in the text. The sample containing 0.5–1.5 ml of enzymic digest was adjusted to approx. pH 4 by addition of 1 M-formic acid (0.04 ml/ml of digest) and was injected on to the column together with unlabelled marker compounds (total volume less than 1.9 ml). ——, A_{254}. ----, Relative proportion of the initial solvent (0.01 M-ammonium formate, pH 3.7, in 0.1% methanol) to the final solvent (0.5 M-ammonium formate, pH 3.7, in aqueous 5% methanol). The gradient, according to a preset programme (curve 8), was started 2 min after injection and was run for 25 min, then the final concentration of solvent was maintained for a further 10 min. The rate of elution was 4 ml/min throughout.

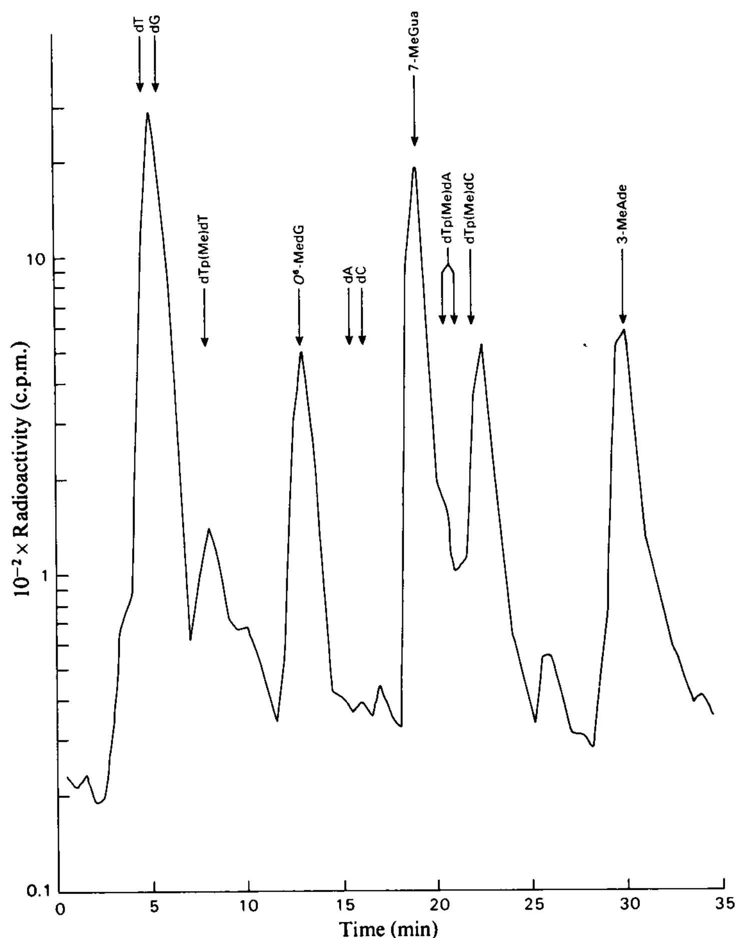

Fig. 2. *Typical profile of radioactivity of an enzymic digest of DNA methylated by N-[^{14}C]methyl-N-nitrosourea, subjected to high-pressure liquid chromatography on Partisil* 10-*SCX cation exchanger eluted with a gradient of ammonium formate, pH*3.7, *in dilute aqueous methanol*

The procedures were as given for Fig. 1, with collection of fractions (2ml) at intervals of 0.5min for scintillation counting.

sates could be made. The hydrolysates were applied to 1.0mm-thick layers of Kieselgel HF_{254} which were developed in system E. The dTp(Alk)dT band that was recovered was rechromatographed in system B and then in system F. From the recovery of the unlabelled phosphotriester [40–45% for dTp(Me)dT and 63–67% for dTp(Et)dT] and from the amount of radioactivity that chromatographed with the triester, the amount of dTp(Alk)dT released from the DNA by the enzymes was determined.

Separation of dTp(Alk)dT by high-pressure liquid chromatography from DNA hydrolysates

Enzymic hydrolysates of alkylated DNA were injected on the Partisil 10-SCX column (0.5–1.5ml samples) together with the appropriate unlabelled synthetic dTp(Alk)dT, 7-alkylguanine, 3-alkyladenine, O^6-alkyldeoxyguanosine, and in some cases, dTp(Me)dC and dTp(Me)dA standards. The sample was eluted at 4ml/min with a buffer consisting of 0.01M-ammonium formate, pH3.7, in 0.1% (v/v) methanol for 2min. This buffer was then varied in a concave fashion (see Fig. 1) (programme curve 8 of the Waters instrument) over 25min to a final concentration of 0.5M-ammonium formate, pH3.7, in 5% (v/v) methanol; expressions of buffer concentration are based on the NH_4^+ ion. Fractions containing dTp(Alk)dT [R_T dTp(Me)dT, 7.5min; R_T dTp(Et)dT, 9.6min] were collected manually, or 0.5min (2.0ml) fractions were collected directly into scintillation vials on an Isco model 128 fraction

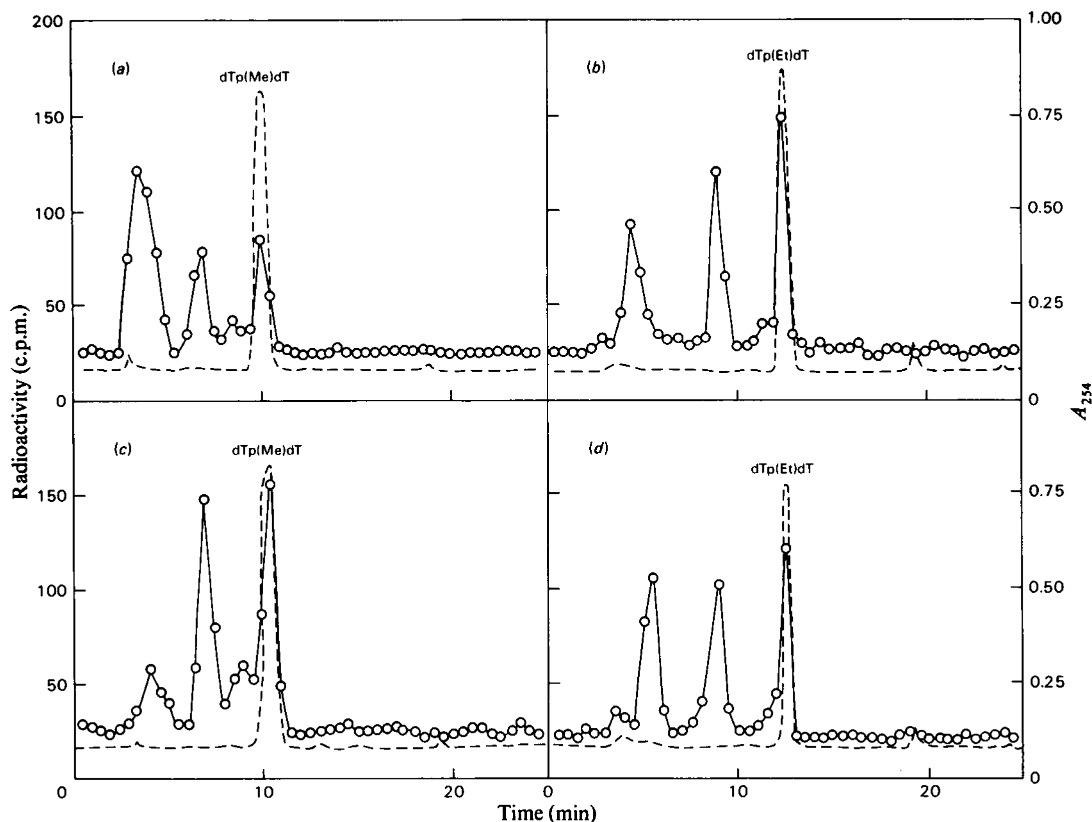

Fig. 3. *Typical chromatograms showing the isolation of methyl or ethyl phosphotriesters of thymidylyl(3′-5′)thymidine by rechromatography of fractions containing these products obtained as in Figs.* 1 *and* 2

The appropriate fractions associated with u.v. absorption of added marker triesters were evaporated and the residues redissolved in aq. 30% (v/v) methanol (2ml), and a portion (1.8ml) was injected on to the ODS-μBondapak column and eluted for 2min with the same solvent. Elution was then continued with a gradient (programme curve 7) of aq. 30–90% (v/v) methanol over 20min at 2ml/min. Fractions (1 ml) were collected and their radioactivity was measured by scintillation counting. ——, Radioactivity (c.p.m.); ----, A_{254}. (*a*) dTp(Me)dT from di[^{14}C]methyl sulphate-treated DNA; (*b*) dTp(Et)dT from [^{14}C]ethyl methanesulphonate-treated DNA; (*c*) dTp(Me)dT from *N*-[^{14}C]methyl-*N*-nitrosourea-treated DNA; (*d*) dTp(Et)dT from *N*-[^{3}H]ethyl-*N*-nitrosourea-treated DNA.

collector (MSE Scientific Instruments, Crawley, Sussex, U.K.). The radioactivity was determined by liquid-scintillation counting (Lawley & Shah, 1972).

An example of a separation on this system with methylated markers is given in Fig. 1, and a profile of radioactivity that was obtained from a digest of DNA alkylated by *N*-[^{14}C]methyl-*N*-nitrosourea is given in Fig. 2. Recovery of radioactivity was quantitative.

The dT(Alk)dT-containing fractions were evaporated on a rotary evaporator at 45°C, dissolved in aq. 30% (v/v) methanol (2.0ml), and portions (1.8ml) were injected on the ODS-μBondapak C_{18} column. The sample was eluted at 2.0ml/min with 30% methanol for 2min followed by a concave gradient (programme curve 7) that raised the methanol concentration from 30% to 90% (v/v) in 20min [R_T dTp(Me)dT, 9.6min; R_T dTp(Et)dT, 12.4min]. Either the dTp(Me)dT and dTp(Et)dT fractions were collected manually or 0.5min (1.0ml) fractions were collected directly into scintillation vials (Fig. 3).

In some cases samples of ^{14}C-labelled dTp(Me)dT from *N*-[^{14}C]methyl-*N*-nitrosourea-treated DNA or ^{3}H-labelled dTp(Et)dT from *N*-[^{3}H]ethyl-*N*-nitrosourea-treated DNA which were obtained by these methods were further chromatographed on the ODS-μBondapak column at 2.0ml/min with aq. 15% (v/v) acetonitrile [R_T dTp(Me)dT, 7.2min; R_T dTp(Et)dT, 11.4min] and on the silica column with diethyl ether/ethanol (3:1, v/v); [R_T dTp(Me)dT,

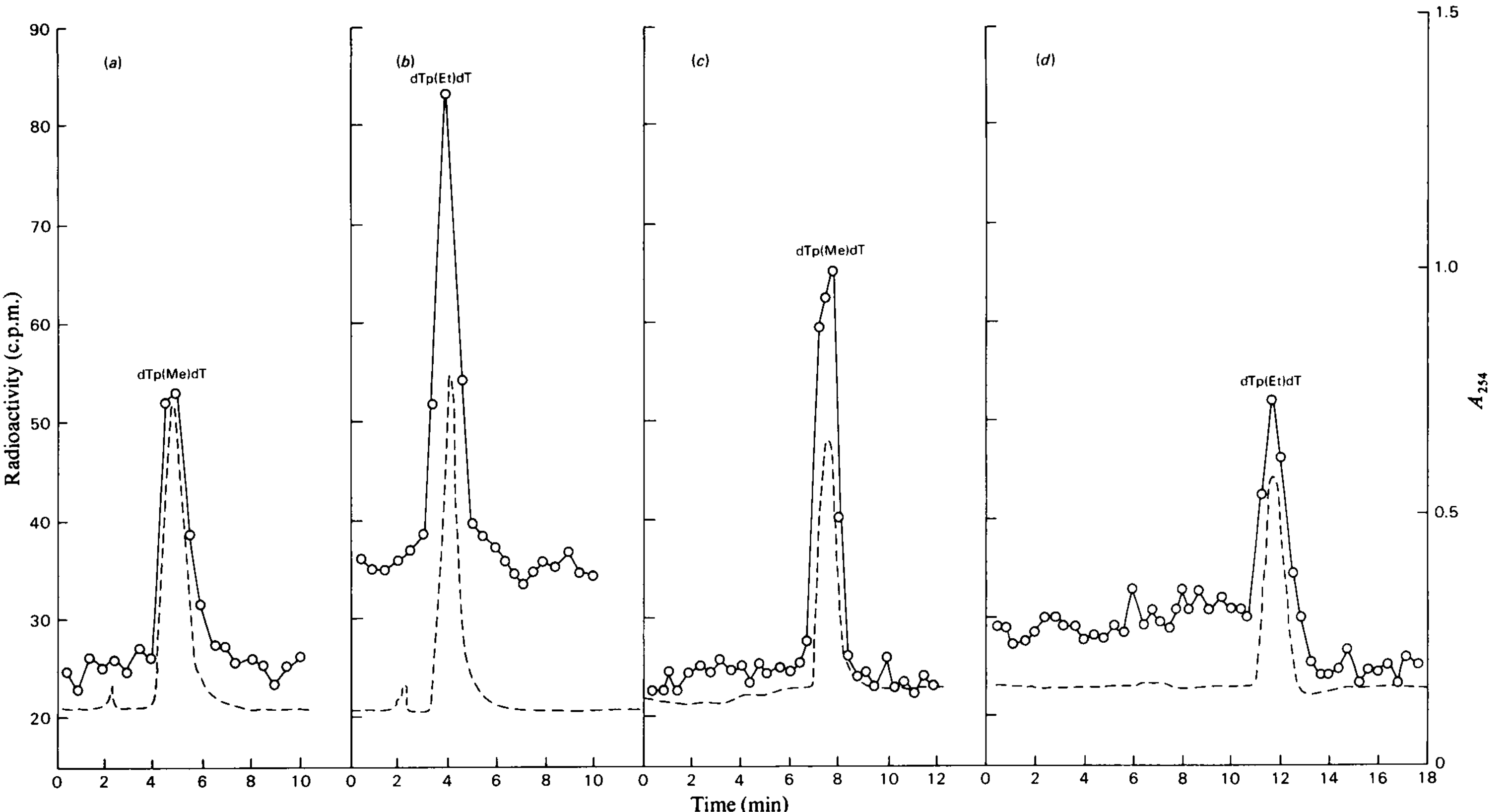

Fig. 4. *Rechromatography of methyl or ethyl phosphotriesters of thymidylyl(3′-5′)thymidine isolated from DNA treated with N-[^{14}C]methyl-N-nitrosourea or N-[^{3}H]ethyl-N-nitrosourea as shown in Figs.* 2 *and* 3

Fractions associated with u.v. absorption (Fig. 3) of added triester markers were evaporated. For rechromatography on silica, the sample was dissolved in methanol (0.1 ml) and a portion (0.07 ml) was applied to the column, followed by isocratic elution with ether/ethanol (3:1, v/v). For rechromatography on the ODS μBondapak column, the sample was dissolved in 15% (v/v) acetonitrile (1 ml) and a portion (0.9 ml) was applied to the column, followed by isocratic elution with the same solvent at 2 ml/min. (*a*) dTp(Me)dT, rechromatographed on silica eluted with ether/ethanol (3:1, v/v); (*b*) dTp(Et)dT, rechromatographed on silica eluted with ether/ethanol (3:1, v/v); (*c*) dTp(Me)dT, rechromatographed on ODS-μBondapak eluted with aq. 15% acetonitrile; (*d*) dTp(Et)dT, rechromatographed on ODS-μBondapak eluted with aq. 15% acetonitrile. ——, Radioactivity; ----, A_{254}.

4.4min; R_T dTp(Et)dT, 3.6min]. In all cases, a single peak of radioactivity coincided with the unlabelled triester marker (Fig. 4) and dTp(Alk)dT could be quantitatively recovered.

Determination of 7-alkylguanine

Samples of alkylated DNA were depurinated by heating in 0.1M-HCl at 70°C for 20min, and the purines liberated were chromatographed with markers of 7-alkylguanine, O^6-alkylguanine and 3-alkyladenine on the SCX column as described for the phosphotriesters. Fractions (0.5min, 2.0ml) were collected directly into scintillation vials, and the amounts of 7-methylguanine or 7-ethylguanine (R_T 17.4 and 20.9min respectively) were determined from the radioactivity that was co-eluted with the appropriate marker (Table 4). The two other major purine-alkylation products, O^6-methyl- or O^6-ethyl-guanine (R_T 22.2 and 27.4min respectively) and 3-methyl- or 3-ethyl-adenine (R_T 28.4 and 31.6min respectively) were eluted later and did not interfere with the determination of 7-alkylguanine.

Results

Characterization of dTp(Et)dT as a product from ethylated DNA

By using techniques previously described for the characterization of dTp(Me)dT as a product released by enzymes from N-[^{14}C]methyl-N-nitrosourea-treated DNA (Swenson *et al.*, 1976), it was shown that dTp(Et)dT was a product that could be obtained from DNA ethylated by N-[^{3}H]ethyl-N-nitrosourea. Ethylated DNA (4.3mmol of ethyl groups/mol of DNA P, 24μmol of DNA P; 1.4μCi) was heated at 100°C, pH7, digested by deoxyribonuclease, venom phosphodiesterase and alkaline phosphatase, and chromatographed on a column of Dowex 50 (NH_4^+ form) eluted at pH8.0 with a marker of synthetic dTp(Et)dT as described in the Experimental section.

The pattern of elution of radioactivity from the column was broadly similar to that previously obtained with hydrolysates of methylated DNA, except that the ethylated products were generally eluted at greater retention volumes than those for the corresponding methylated derivatives, so that dTp-(Et)dT was co-eluted with dA. A peak of radioactivity containing 0.044μCi was eluted with the synthetic dTp(Et)dT marker in this system. The identity of this radioactive product with dTp(Et)dT was shown by its further chromatography with the unlabelled marker on silica gel in three solvent systems at constant specific radioactivity (Table 1).

This identity was confirmed by hydrolysis of the ^{3}H-labelled dTp(Et)dT in 0.1M-NaOH at 37°C. The labelled material was hydrolysed at the same rate ($t_{\frac{1}{2}}$ 6.3h) as that of unlabelled dTp(Et)dT ($t_{\frac{1}{2}}$ 6.5± 0.3h). After 48h in alkali, a sample corresponding to 0.2μmol (1400d.p.m.) of dTp(Et)dT was applied to a sheet of cellulose and developed in system B. The specific radioactivities of the dTpEt (R_F 0.54) and EtpdT (R_F 0.48) obtained were closely similar to that of the dTp(Et)dT (Table 2); the amount of radioactivity (3c.p.m.) associated with dTpdT (R_F 0.25) was not significant. These data also confirm that the [^{3}H]ethyl group is attached to the phosphate group and not to the thymine-base residue of dTpdT. In parallel studies with unlabelled dTp(Et)dT, it was determined from the u.v. absorption of the eluted

Table 1. *T.l.c. of ^{3}H-labelled dTp(Et)dT from DNA alkylated by N-[^{3}H]ethyl-N-nitrosourea, enzymically degraded and chromatographed on Dowex 50 (NH_4^+ form)*

Ethylated DNA (24μmol of DNA P; 1.4μCi) was heated at pH6.5, 100°C, and the residual polynucleotide (1μCi) was enzymically digested (see the text for details) and chromatographed with unlabelled dTp(Et)dT marker (3.0μmol) on Dowex 50 (NH_4^+ form) eluted with 0.01M-ammonium formate, pH8.0. The fractions that contained marker dTp(Et)dT and 0.044μCi of ^{3}H were condensed *in vacuo* and rechromatographed on silica in the three solvent systems given below.

Solvent system	R_F	Specific radioactivity [d.p.m./μmol of dTp(Et)dT]
A	0.33	6640
B	0.60	6360
E	0.49	6900

Table 2. *T.l.c. of alkaline-hydrolysis products of dTp(Et)dT*

dTp(Et)dT (4μmol) was dissolved in 0.1M-NaOH (2ml) at 37°C. At 48h, samples (0.2ml) were neutralized with 0.2M-acetic acid (0.2ml) and chromatographed on silica in solvent system C (see the text). The proportions of products, which were based on u.v. absorption, are given as μmol of product/μmol dTp(Et)dT (±S.D for five determinations). Likewise, a sample of [*ethyl*-^{3}H]dTp(Et)dT (0.2μmol, 1400 d.p.m.) was chromatographed on cellulose in solvent system B after 48h in alkali, and the specific radioactivities of dTpEt, EtpdT and dTpdT were determined; dT was not recovered. The ^{3}H-labelled product was hydrolysed at the same rate ($t_{\frac{1}{2}}$, 6.3h, based on ^{3}H) as that of the unlabelled synthetic dTp(Et)dT ($t_{\frac{1}{2}}$, 6·5±0.3h).

Product	Product [μmol/μmol of dTp(Et)dT]	Specific radioactivity of product (d.p.m./μmol)
dTp(Et)dT	—	6900
dT	0.78±0.02	Not determined
dTpEt	0.30±0.01	6100
EtpdT	0.47±0.02	6700
dTpdT	0.22±0.01	300

Table 3. *Comparative reactivities towards alkylating agents of DNA and of a phosphodiester site in DNA [thymidylyl(3′-5′)-thymidine, dTpdT]*

Salmon sperm DNA (15mM-DNA P) in 100mM Tris/HCl buffer, pH8 (3ml), was treated with labelled alkylating agent (20mM) at 37°C for 2.5h and isolated by precipitation. The alkylated DNA was enzymically digested and analysed by t.l.c. as described in detail in the text. Relative reactivities are expressed as

$$\frac{k_{DNA}}{k_{dTpdT}} = \frac{[\text{Alk.DNA}]}{[\text{DNA P}]} \cdot \frac{[\text{dTpdT}]}{[\text{dTp(Alk)dT}]}$$

on the basis that the proportion of dTpdT sequences in DNA (equal to [dTpdT]/[DNA P]) is the square of the molar proportion of T, i.e. 0.073, $(0.27)^2$; [Alk.DNA]/[dTp(Alk)dT] is from the proportion of products as dTp(Alk)dT (see column 4). The differences in apparent nucleophilicities of DNA overall (per average nucleotide) and of dTpdT are derived from $\log (k_{DNA}/k_{dTpdT}) = s\,(n_{DNA} - n_{dTpdT})$. From the half-lives of hydrolysis of dimethyl sulphate, *N*-methyl- and *N*-ethyl-*N*-nitrosourea (approx. 0.25h) the reactions had gone essentially to completion, whereas for ethyl methanesulphonate ($t_{\frac{1}{2}}$ approx. 11h) the extent of reaction was about 15% of maximal. The relative reactivities of DNA are therefore comparable as extents of reaction, except for ethyl methanesulphonate, for which the estimated comparable value is 0.6/0.15 = 4mmol of ethyl groups/mol of DNA P (shown in parentheses).

Reagent	Swain–Scott factor *s*	Extent of reaction (mmol of alkyl group/mol of DNA P)	Percentage of products as dTp(Alk)dT	k_{DNA}/k_{dTpdT}	Difference in nucleophilicity ($n_{DNA} - n_{dTpdT}$)
Dimethyl sulphate	0.86	5.3	0.09	8.1	2.2
Ethyl methanesulphonate	0.64	0.6 (4.0)	0.93	7.8	1.4
N-Methyl-*N*-nitrosourea	0.42	55.0	1.2	6.1	1.9
N-Ethyl-*N*-nitrosourea	0.26	4.6	4.3	1.7	0.9

products that 47% of the degradation occurred between the 3′-bond and the phosphate group, 30% at the 5′-site, and 22% between the ethyl group and the phosphate (Table 2); these proportions are similar to those observed for the alkaline hydrolysis of ^{14}C-labelled dTp(Me)dT which was isolated from [^{14}C]methylated DNA (Swenson *et al.*, 1976).

Reactions of alkylating agents with DNA

When DNA was treated with each of the four alkylating agents under standard conditions at pH8.5, the relative order of reactivity of the agents was *N*-methyl-*N*-nitrosourea > dimethyl sulphate > *N*-ethyl-*N*-nitrosourea > ethyl methanesulphonate (Table 3). Since the half-lives of hydrolysis of dimethyl sulphate, *N*-ethyl-*N*-nitrosourea and *N*-methyl-*N*-nitrosourea determined under similar conditions were approx. 15min, the reactions with these alkylating agents had gone essentially to completion when they were terminated at 2.5h. However, as noted in the legend to Table 3, the reaction with ethyl methanesulphonate ($t_{\frac{1}{2}}$ approx. 11h) had proceeded only to about 15% of maximal by 2.5h.

Time course of enzymic release of dTp(Me)dT from [^{14}C]methylated DNA

The time course of enzymic hydrolysis of *N*-[^{14}C]methyl-*N*-nitrosourea-treated DNA (1.3mmol of methyl groups/mol of DNA P) showed that the

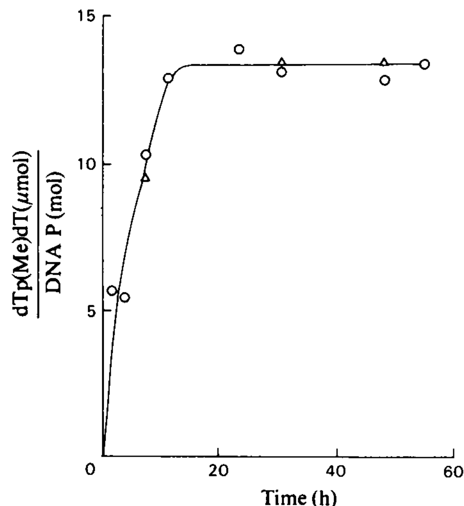

Fig. 5. *Time course of liberation of the methyl phosphotriester of thymidylyl(3′-5′)thymidine from DNA methylated by N-[^{14}C]methyl-N-nitrosourea by enzymic digestion with deoxyribonuclease venom phosphodiesterase and bacterial alkaline phosphatase*

The triester was isolated and determined after various times of incubation at 37°C, as described in detail in the text, by using the chromatographic procedures shown in Figs. 1, 2 and 3. ○, Methylated DNA digested directly; △, methylated DNA heated at pH6.5, 100°C for 30min, to remove 3- and 7-methylpurines before enzymic digestion.

release of ^{14}C-labelled dTp(Me)dT [13.3±0.2 μmol of dTp(Me)dT/mol of DNA P] was complete by 24 h (Fig. 5); longer incubations or addition of more enzymes at 24 and 48 h did not increase the amount of [*methyl*-^{14}C]dTp(Me)dT in the hydrolysate. In addition, as observed with synthetic dTp(Me)dT and dTp(Me)dC, the ^{14}C-labelled dTp(Me)dT from DNA appeared to be stable to the enzymic hydrolysis system, since its concentration did not decrease with prolonged incubation (Fig. 5).

Preheating the alkylated DNA in pH 6.5 buffer at 100°C for 30 min, to liberate 3- and 7-methylpurines, appeared to have no effect on the enzymic hydrolysis of the alkylated DNA (triangles, Fig. 5). This step is therefore shown to be useful in studies where accurate measurements of 3- and 7-alkylpurines, in addition to dTp(Alk)dT, are needed.

Determination of dTp(Alk)dT from alkylated DNA

The quantitative determination of dTp(Alk)dT from DNA treated with dimethyl sulphate, ethyl methanesulphonate, *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*-nitrosourea was approached by two independent methods. The first method was to hydrolyse the alkylated DNA enzymically in the presence of unlabelled dTp(Alk)dT and separate the hydrolysate by t.l.c. on silica in solvent systems E, B and F as described in the Experimental section. The unlabelled dTp(Alk)dT served as an internal standard from which corrections could be made for recoveries of the DNA-derived triesters. Thus di[^{3}H]methyl sulphate-treated DNA (17.5 μmol of DNA P; 0.77 μCi) was enzymically degraded in the presence of 10.2 μmol of dTp(Me)dT. After chromatography, 4.6 μmol of carrier dTp(Me)dT was recovered, containing 680 d.p.m. From this it could be calculated that 0.09% of the alkyl groups bound to DNA after reaction with di[^{3}H]methyl sulphate were released as [*methyl*-^{3}H]dTp(Me)dT (Table 3).

Corresponding determinations were made of the proportion of products obtained as dTp(Alk)T from DNA alkylated by [^{14}C]ethyl methanesulphonate, *N*-[^{14}C]methyl-*N*-nitrosourea and *N*-[^{3}H]ethyl-*N*-nitrosourea. From these data the values of reactivities of the phosphodiester group relative to DNA overall and the corresponding differences in nucleophilicity were calculated as shown in the legend to Table 3.

The second method for the quantitative determination of dTp(Alk)dT in enzymic hydrolysates of alkylated DNA used two high-pressure liquid-chromatography systems to separate and purify the products of interest. This method is preferred because the chromatographic methods are non-destructive to the triesters, therefore eliminating the need to correct for losses of product, and the method is faster than that by t.l.c. Samples of labelled alkylated DNA were digested and chromatographed on the SCX column (Figs. 1 and 2). The radioactive material that coincided with dTp(Alk)dT was condensed and a sample was rechromatographed on the ODS-μBondapak column (Fig. 3). The amounts of dTp-(Alk)dT recovered (155–1250 d.p.m.) are expressed as a percentage of total DNA-bound alkyl groups in Table 4. These values are in reasonable agreement with those determined by the t.l.c. method (Table 3).

The identities of the radioactive products obtained by this method with dTp(Me)dT or dTp(Et)dT were confirmed when the samples were rechromatographed by high-pressure liquid chromatography on silica eluted with ether/ethanol (3:1, v/v) or on the ODS-μBondapak column with aq. 15% (v/v) acetonitrile. In all cases, a single peak of radioactivity coincided with the synthetic marker (Fig. 4).

Determination of dTp(Me)dA and dTp(Me)dC

In addition to the dTp(Me)dT that was recovered from *N*-[^{14}C]methyl-*N*-nitrosourea-treated DNA (above), the radioactivity that co-chromatographed with synthetic dTp(Me)dA and dTp(Me)dC on the SCX column [R_T dTp(Me)dA, 20.4 and 20.9 min incompletely resolved diastereoisomers; R_T dTp,

Table 4. *Comparative reactivities towards alkylating agents of the phosphodiester group dTpdT and of the N-7 atom of guanine residues in DNA*

Alkylation of DNA was as in Table 3, except that the amounts of the reagents were adjusted to give extents of reaction close to 1 mmol of alkyl group/mol of DNA P, and analyses of DNA were by high-pressure liquid chromatography as described in the text. The relative molar proportions of dTpdT and dG in DNA were taken as 0.073 and 0.23 respectively per mol of DNA P.

Reagent	Swain–Scott factor s	Overall alkylation (mmol/mol of DNA P)	Percentage of products as: 7-Alkylguanine	dTp(Alk)dT	$\frac{k_{N-7}}{k_{dTpdT}}$	Difference in nucleophilicity ($n_{N-7}-n_{dTpdT}$)
Dimethyl sulphate	0.86	2.9	79	0.07	358	3.0
Ethyl methanesulphonate	0.64	1.0	59	1.0	19	2.0
N-Methyl-*N*-nitrosourea	0.42	1.5	67	1.0	21	3.1
N-Ethyl-*N*-nitrosourea	0.26	0.9	14	3.9	1.2	0.3

(Me)dC, 21.6min (Fig. 2)] was collected into a single fraction and was rechromatographed on the ODS-μBondapak column as described for dTp(Me)dT. The dTp(Me)dA was incompletely resolved into its two diastereoisomers under these conditions (R_T 10.0 and 10.6min), and dTp(Me)dC was eluted as a single peak (R_T 7.8min); the complete resolution of the diastereoisomers of the ethyl phosphotriester of dTpdA by high-pressure liquid chromatography in an anion-exchange system has been reported (Jensen & Reed, 1977). A broad peak of radioactivity accounting for 3.5% of the total DNA-bound alkyl groups was eluted with the dTp(Me)dA and dTp-(Me)dC. The radioactivity was not clearly eluted as peaks coincident with the synthetic markers; this is most likely due to the high probability that the methyl phosphotriesters of deoxycytidylyl(3′-5′)thymidine and deoxyadenylyl(3′-5′)thymidine, and their diastereoisomers, chromatographed at or near the positions of dTp(Me)dC and dTp(Me)dA in these systems. This probability is supported by the finding that the amount of radioactivity eluted with these markers was approximately the amount that would be expected (3.8%) for these four phosphotriesters, assuming molar proportions of 0.23 for dC, 0.27 for dT and 0.27 for dA in salmon sperm DNA (Felix *et al.*, 1956), and taking the amount of dTp(Me)dT as 1% of the DNA-bound alkyl groups (Table 4).

With di[^{14}C]methyl sulphate-treated DNA the radioactivity recovered with dTp(Me)dC and dTp-(Me)dA amounted to 0.8% of that initially bound to DNA; this is slightly more than that expected (0.3%) on the basis of assumptions and taking the amount of dTp(Me)dT in this DNA as 0.07% of the total bound alkyl groups (Table 4). It seems that although the high-pressure liquid-chromatographic systems used could readily separate dTp(Me)dT from other alkylation products, they do not appear to deal as efficiently with the separations of the other triesters. Further studies using all the appropriate synthetic markers and using other chromatographic systems may resolve this problem.

Discussion

To determine the extents of alkylation of the phosphodiester sites of DNA by the four carcinogens chosen, it was first necessary to isolate and establish the identity of the ethyl homologue of the previously identified methylation product dTp(Me)dT. Accordingly, dTp(Me)dT was synthesized and characterized as a product of ethylation of DNA by procedures analogous to those described for dTp(Me)dT (Swenson *et al.*, 1976). In addition to the Dowex 50 and t.l.c. systems used previously, methods involving high-pressure liquid chromatography were devised. The following systems were found useful: firstly, a silica-based cation exchanger (Partisil 10-SCX) eluted with a gradient of ammonium formate buffer, pH 3.7, in dilute aqueous methanol; secondly, a reversed-phase column (ODS-μBondapak C_{18}) eluted with either a gradient of aq. 30–90% (v/v) methanol, or with isocratic 15% aq. (v/v) acetonitrile; thirdly, a silica column eluted with ether/ethanol (3:1, v/v).

The SCX system proved valuable for preliminary separations of dTp(Me)dT and certain other alkylation products found in enzymic digests of alkylated DNA (Figs. 1 and 2). To purify and determine the phosphotriesters, it was necessary to rechromatograph, on the ODS-μBondapak/methanol system (Fig. 3), those fractions from the SCX column containing the u.v. absorption associated with the marker phosphotriesters. The purities of the resulting products were then checked by using the second ODS-μBondapak system and the silica system (Fig. 4); these indicated that the separations were adequate.

It was further necessary to check that the enzymic degradation of alkylated DNA resulted in complete liberation of the phosphotriesters. A suitable procedure was to incubate alkylated DNA in a borate-buffered phosphodiesterase/alkaline phosphatase system; use of Tris-based buffers was contra-indicated by the observed appreciable loss of methyl phosphotriesters in such buffer, presumably because of reaction of the amine at the methyl group of the triester (Cox & Ramsay, 1964). The concentration of enzymes used (0.04 unit of venom phosphodiesterase and 2.5 units of alkaline phosphatase/ml) was sufficient to give complete degradation of alkylated DNA in 24h at 37°C as judged from the time course of liberation of dTp(Alk)dT, and by the fact that further additions of enzyme did not release more of this product (Fig. 5). Prior removal of 3- and 7-alkylpurines from alkylated DNA by heating at 100°C, neutral pH, did not appear to interfere with this enzymic degradation.

These procedures, which rely on direct measurement of a radioactively labelled phosphotriester after co-isolation with a synthetic marker, have been used for determination of phosphotriesters in DNA alkylated *in vivo* (J. V. Frei, P. D. Lawley, D. H. Swenson & W. Warren, unpublished work). With less direct methods of measurement, which rely on the isolation of labelled alcohols or alkyl phosphates after chemical degradation procedures (Bannon & Verly, 1972; Sun & Singer, 1975; O'Connor *et al.*, 1975), it may be expected that certain labelled impurities in DNA might interfere with such analyses. It may be noted that a physicochemical method for analysis of phosphotriesters in DNA *in vitro* and *in vivo* has been developed by Shooter & Merrifield (1976), which appears to overcome this potential problem.

To express relative reactivities of the phosphodiester and N-7 of guanine sites towards the four carcinogens in more absolute terms, it was necessary to assume that the alkylation of phosphodiester sites

occurred at random and that the base sequence in DNA was random. Some support for the validity of these assumptions was obtained from comparison of the observed relative extents of formation of dTp-(Me)dT, dTp(Me)dA and dTp(Me)dC, in the *N*-[^{14}C]methyl-*N*-nitrosourea-treated DNA. Therefore, in Tables 3 and 4, it has been assumed that the proportion of dTpdT to total phosphodiester groups equalled the square of the molar proportion of dT, i.e., $(0.27)^2 = 0.073$.

To express the relative reactivities of DNA, per average nucleotide unit, and the relative reactivities of the specific sites, N-7 of guanine and a phosphodiester group in DNA, recourse was made to the concept of relative nucleophilicities of these groups, derived from the work of Swain & Scott (1953) as discussed previously (Osterman-Golkar *et al.*, 1970; Lawley *et al.*, 1975). To convert the relative extents of alkylation into apparent nucleophilicities (*n* of Tables 3 and 4), values of the Swain–Scott substrate constant *s* for the four alkylating agents were taken as those given by Osterman-Golkar *et al.* (1970) and Veleminsky *et al.* (1970).

As expected, the nucleophilic reactivity of the phosphodiester group was least towards dimethyl sulphate, the alkylating agent of highest *s* value, and this reactivity increased with decreasing *s* of the alkylating agent (Tables 3 and 4).

As discussed previously (Lawley *et al.*, 1975), data for the comparative reactivities (*k*) enable values for differences in nucleophilicity (Δn) between various sites in DNA to be derived, according to the expression $\Delta \log k = s \cdot \Delta n$. No absolute value of *n* for any specific site in DNA appears to have been obtained yet, but a value $n = 2.5$ has been derived by Walles & Ehrenberg (1969) for an average DNA nucleotide unit with respect to alkylation by alkyl methanesulphonates. Since the main site of alkylation is the N-7 atom of guanine (Lawley *et al.*, 1975), it follows that the value of *n* for this specific site will be rather greater than this overall value for DNA, i.e. probably about 3.5.

The values of Δn for the phosphodiester group in DNA in comparison with the overall average nucleotide unit (Table 3), or for the specific site N-7 of guanine (Table 4), show consistent variations with the nature of the alkylating agents, and this is discussed below. However, taking an average value of Δn in each case, i.e. $n_{\mathrm{DNA}} - n_{\mathrm{dTpdT}} = 1.6$ from Table 3, and $n_{\mathrm{N\text{-}7}} - n_{\mathrm{dTpdT}} = 2.6$ from Table 4, a value of $n =$ about 1 seems reasonable for the phosphodiester group.

Clearly the concept of nucleophilicity is not itself adequate to explain the relative reactivities of the phosphodiester group (or of DNA overall) towards the alkylating agents. This same conclusion was also reached for relative reactivities of purine sites in DNA (Lawley *et al.*, 1975).

In the first place, the relatively high reactivities towards DNA of *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea, compared with those of dimethyl sulphate and ethyl methanesulphonate, do not accord with the *s* values of the reagents. A possible explanation is that the relative reactivity of the multiply negatively charged DNA polymer is greater towards the nitrosamides, because they react through positively charged alkyldiazonium intermediates, whereas the alkanesulphonates react as neutral molecules mainly by the S_N2 mechanism.

It can be envisaged further that the alkyldiazonium ions are S_N1 agents, liberating highly reactive carbonium ions, with the ethyldiazonium ion being more reactive than the methyldiazonium (see Lawley & Thatcher, 1970). According to this concept, the alkyldiazonium ions are specifically concentrated in the vicinity of the negatively charged DNA polymer, but will attack various sites in the polymer to some extent according to their relative nucleophilicities, and to some extent according to their steric accessibilities.

It can be deduced from the present data that the steric factor is greater for the ethylating agents, in the sense that their reaction with the relatively accessible phosphodiester groups on the exterior of the DNA double helix is greater than that for the methylating agents. Evidently this steric effect can overcome the factor of relative nucleophilicity, since the ratio of reactivities of phosphodiester sites to those of the other sites in DNA is about the same for ethyl methanesulphonate as for *N*-methyl-*N*-nitrosourea, despite the higher *s* value of the former.

In the absence of direct measurement of all the 16 possible phosphotriesters that could be formed by alkylation of the DNA, it was possible to estimate this value from the yields of dTp(Alk)dT, and from the known molar ratio of dT in salmon sperm DNA (Felix *et al.*, 1956). By assuming a random distribution of dT in this DNA, such that dTpdT would account for 7.3% of the possible dideoxynucleoside monophosphates, and assuming a random attack on any of the diester bonds, the total extent of phosphotriester formation by the alkylating agents (expressed as percentage of total DNA-bound alkyl groups) is thereby estimated from the t.l.c. methods as 1% for dimethyl sulphate, 13% for ethyl methanesulphonate, 16% for *N*-methyl-*N*-nitrosourea and 59% for *N*-ethyl-*N*-nitrosourea; from high-pressure liquid chromatography, the estimates are 1, 14, 14 and 52% respectively.

These values may be compared with those in the literature, which were deduced from data on liberation of alcohols or alkyl phosphates from DNA alkylated by the following alkylating carcinogens: methyl methanesulphonate (an agent with *s* value similar to that of dimethyl sulphate; Osterman-Golkar *et al.*, 1970), 1% (Bannon & Verly, 1972);

ethyl methanesulphonate, 13% (Sun & Singer, 1975) or 15% (Bannon & Verly, 1972); *N*-methyl-*N*-nitrosourea, about 18% (Lawley, 1973) or 25% (Sun & Singer, 1975); *N*-ethyl-*N*-nitrosourea, 70% (Sun & Singer, 1975). Evidently, the values obtained by us on the stated assumptions are in some cases lower than the previous estimates. Further work will be required to reconcile the various data, for example, by a rigorous summation of the amounts of all known products in various alkylated DNA molecules.

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References

Bannon, P. & Verly, W. G. (1972) *Eur. J. Biochem.* **31**, 103–111

Cox, J. R. & Ramsay, O.B. (1964) *Chem. Rev.* **64**, 317–352

De Boer, G., Miller, P. S. & Ts'o, P. O. P. (1973) *Biochemistry* **12**, 720–726

Denayer, R. (1962) *Bull. Soc. Chim. Fr.* 1358–1364

Farmer, P. B., Foster, A. B., Jarman, M. & Tisdale, M. J. (1973) *Biochem. J.* **135**, 203–213

Felix, K., Jilke, I. & Zahn, R. K. (1956) *Hoppe-Seyler's Z. Physiol. Chem.* **303**, 140–152

Jensen, D. E. & Reed, D. J. (1977) *Proc. Am. Assoc. Cancer Res.* **18**, 72

Khorana, H. G. (1959) *J. Am. Chem. Soc.* **81**, 4657–4660

Khorana, H. G. & Vizsolyi, J. P. (1961) *J. Am. Chem. Soc.* **83**, 675–685

Lawley, P. D. (1973) *Chem.-Biol. Interact.* **7**, 127–130

Lawley, P. D. & Shah, S. A. (1972) *Biochem. J.* **128**, 117–132

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

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

Lawley, P. D., Orr, D. J. & Jarman, M. (1975) *Biochem. J.* **145**, 73–84

Miller, P. S., Fang, K. N., Kondo, N. S. & Ts'o, P. O. P. (1971) *J. Am. Chem. Soc.* **93**, 6657–6665

Miller, P. S., Barrett, J. C. & Ts'o, P. P. O. (1974) *Biochemistry* **13**, 4887–4896

O'Connor, P. J., Margison, G. P. & Craig, A. W. (1975) *Biochem. J.* **145**, 475–482

Osterman-Golkar, S., Ehrenberg, L. & Wachtmeister, C. A. (1970) *Radiat. Bot.* **10**, 303–327

Schaller, H. & Khorana, H. G. (1963) *J. Am. Chem. Soc.* **85**, 3828–3835

Schaller, H., Weimann, G., Lerch, B. & Khorana, H. G. (1963) *J. Am. Chem. Soc.* **85**, 3821–3827

Shooter, K. V. & Merrifield, R. K. (1976) *Chem.-Biol. Interact.* **13**, 223–236

Sun, L. & Singer, B. (1975) *Biochemistry* **14**, 1795–1802

Swain, C. G. & Scott, C. B. (1953) *J. Am. Chem. Soc.* **75**, 141–147

Swenson, D. H., Farmer, P. B. & Lawley, P. D. (1976) *Chem.-Biol. Interact.* **15**, 91–100

Veleminsky, J., Osterman-Golkar, S. & Ehrenberg, L. (1970) *Mutat. Res.* **10**, 169–174

Walles, S. & Ehrenberg, L. (1969) *Acta Chem. Scand.* **23**, 1080–1084