Phosphotriesters in Rat Liver Deoxyribonucleic Acid after the Administration of the Carcinogen *NN*-Dimethylnitrosamine *in vivo*

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After treatment with *NN*-di[¹⁴C]methyl nitrosamine, samples of DNA were isolated from rat livers by a conventional phenol procedure and examined for the presence of phosphotriesters. A method capable of detecting relatively small amounts of ¹⁴C-labelled phosphotriesters was developed and used to establish that these products account for 10–12% of the total methylation pattern found after treatment with this agent *in vivo*. The significance of the presence of phosphotriesters in DNA is discussed.

Although it is over 20 years since it was first proposed that phosphotriesters might be present in DNA after reaction with alkylating agents (Elmore *et al.*, 1948), uncertainty surrounded this issue until direct evidence was provided for the existence of alkyl phosphates in DNA, by Bannon & Verly (1972). They found that after treatment with methyl methanesulphonate or ethyl methanesulphonate *in vitro* alkylation of the phosphate residues accounted for 1 and 15% of the total reaction respectively. Lawley (1973) has shown that after treatment *in vitro* with *N*-methyl-*N*-nitrosourea 18% of the total products in DNA were present as alkyl phosphates.

Esterification of the phosphate residues in DNA by these alkylating agents produces triesters which are stable under conditions of mild hydrolysis (Bannon & Verly, 1972; Lawley *et al.*, 1973), but in RNA these products are unstable (Brown & Todd, 1955). Their presence in poly(A) after incubation with ethyl methanesulphonate was indicated by chain breakage, but this was not observed in similar experiments with methyl methanesulphonate (Ludlum, 1969). Measurements have been made of the frequency of chain fission in RNA from bacteriophage R17 which had been treated with a series of methylating and ethylating agents (Shooter *et al.*, 1974*a,b*). The outcome of these studies was to distinguish between agents of the S_N2 type such as methyl methanesulphonate and dimethyl sulphate, which produce very little alkylation of phosphate residues compared with reagents of the S_N1 type, e.g. the *N*-methyl- or *N*-ethyl-nitrosoureas and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, which give much higher yields of these triesters and *O*⁶-alkylguanine. Ethyl methanesulphonate also falls into this latter category on the basis of these reactions (Bannon & Verly, 1972; Orr, 1973).

Previous studies from our laboratories (O'Connor *et al.*, 1972) have shown that the methylation patterns

produced by methyl methanesulphonate or *NN*-dimethylnitrosamine in rat liver rRNA *in vivo* are similar to those found after alkylation of bacteriophage R17 RNA and rabbit reticulocyte RNA *in vitro*, either with dimethyl sulphate or with *N*-methyl-*N*-nitrosourea (Lawley & Shah, 1972). The same is true for the methylation sites formed in liver DNA by methyl methanesulphonate or *NN*-dimethylnitrosamine (O'Connor *et al.*, 1973) when compared with the alkylation of DNA in cells grown *in vitro* and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Lawley & Thatcher, 1970). It was to be expected therefore that treatment with *NN*-dimethylnitrosamine *in vivo* would lead to the alkylation of phosphate residues both in rRNA and in DNA. In fact an indication of this had already been obtained from analyses of rat liver rRNA and DNA in which conditions of strong acid hydrolysis were used. Chromatography of these hydrolysates on columns of Dowex 50 (H⁺ form) gave rise to an early-eluted peak of radioactivity which was present in much larger amounts after treatment with *NN*-dimethylnitrosamine *in vivo* compared with results obtained after treatment with methyl methanesulphonate (O'Connor *et al.*, 1972, 1973). As expected for phosphotriesters (Bannon & Verly, 1972) this material remained bound to isolated DNA during incubation at 37°C (Margison *et al.*, 1973). Further, the rapid formation of this material *in vivo* and its subsequent rate of loss indicated that it was derived by direct methylation and not from C₁ breakdown products incorporated via metabolic pathways (O'Connor *et al.*, 1973). Evidence is presented here for the formation of phosphotriesters in rat liver DNA after treatment *in vivo* with *NN*-di[¹⁴C]methyl nitrosamine.

Materials and Methods

Materials

NN-Di[¹⁴C]methyl nitrosamine (10mCi/mmol), [¹⁴C]methylamine hydrochloride (22.4mCi/mmol) and [¹⁴C]methanol (58mCi/mmol) were purchased

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from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled *NN*-dimethylnitrosamine for dilution of the labelled material and for injection was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Some of the labelled nitrosamine used in this study was prepared by Dr. M. J. Capps at a specific radioactivity of 3.34 mCi/mmol as previously described (O'Connor *et al.*, 1972) and was used without further dilution. Labelled *N*-methyl-*N*-nitrosourea (1.2 mCi/mmol) was prepared from [¹⁴C]methylamine hydrochloride as described by Murray & Lloyd-Williams (1958). For column chromatography (Dowex 50W-X4-400 mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; DEAE-cellulose DE52 and paper for chromatography were from H. Reeve Angel and Co., London E.C.4, U.K. and DEAE-Sephadex A-25 was from Pharmacia (G.B.) Ltd., London W.5, U.K.

Pancreatic deoxyribonuclease (purified) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. *Escherichia coli* alkaline phosphatase (type IIS), venom phosphodiesterase (type II) and the marker compounds 3-methyladenine and 7-methylguanine were from Sigma (London) Chemical Co., London S.W.6, U.K. Cyclo Chemicals, Los Angeles, Calif., U.S.A., provided the marker 7-methyladenine; *O*⁶-methyldeoxyguanine and 3-methylguanine were obtained from Dr. P. D. Lawley, Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, U.K.

Methods

Animals. Male Wistar rats (210–230 g) were allowed access to food and water at all times. *NN*-Di[¹⁴C]-methyl nitrosamine (2 mg/kg) was given as a single intraperitoneal injection between 10:30 and 11:00 h and the animals were killed by decapitation 3 h later.

Purification and hydrolysis of DNA. Rat liver DNA was purified as described previously (O'Connor *et al.*, 1973). The pre-hydrolysis of DNA was carried out as described (Bannon & Verly, 1972) by heating for 90 min at 100°C in 0.1 × SSC*. The heat-depurinated DNA was precipitated by the addition of 2 vol. of cold ethanol in the presence of either 1 M-KCl or 1 M-NaCl. DNA was then redissolved and reprecipitated twice and finally dissolved for dialysis against 2 litres of cold water over a 24-h period.

The hydrolysis of DNA with HClO₄ (72%, w/v), 0.1 M-HCl, or enzymically with venom phosphodiesterase and alkaline phosphatase, was carried out as detailed by O'Connor *et al.* (1973), except for the use of the commercially purified sample of venom phosphodiesterase. Enzyme hydrolyses were tested

for completeness before column chromatography by spotting a portion of the digest on strips of Polygram CEL 300 UV (Macherey-Nagel and Co., Düren, Germany) for development in propan-2-ol-aq. NH₃ (sp. gr. 0.880)–water (7:1:2, by vol.).

The alkaline hydrolysis of enzymic digests was carried out by adjusting the concentration of the solution to 0.1 M-NaOH (pH 12.6) with 10 M-NaOH and incubating the mixture at 37° or 59°C for 20 h. The digest was then adjusted to pH 8.4 with 1 M-HCl.

Chromatography. Details of column chromatography on the Dowex-50 ion-exchange resins are given in the legends to the Figures and Tables. The procedures used and those for paper chromatography are as detailed elsewhere (O'Connor *et al.*, 1972). DEAE-cellulose and DEAE-Sephadex were exchanged before use and were then equilibrated with the eluting ion before packing the column. Urea (AnalaR) was purified further by passing an 8 M solution through a pad of DEAE-cellulose (OH⁻ form) to remove traces of u.v.-absorbing materials.

Determination of radioactivity. Samples from the column effluents were counted for radioactivity in Triton X-100–toluene phosphor systems, which have been described previously (O'Connor *et al.*, 1973). When alkaline solutions were to be counted 0.2 ml of 5 M-HCl was added to each vial to avoid fluorescence and to obtain stable background counting. The appropriate internal standards were used to estimate the efficiency of counting, which was 60–70% depending on the nature of the solution to be counted.

Recovery of [¹⁴C]methanol. The behaviour of methanol when applied as a dilute solution to columns of DEAE-cellulose equilibrated with 0.01 M-NH₄HCO₃ was verified before these analyses were made. Complete recovery (99%) of a sample of [¹⁴C]methanol was obtained with no retention of the radioactive label, which is contrast with the much slower elution of the purine deoxynucleosides, e.g. deoxyguanosine (see the Results section). To estimate the amount of the [¹⁴C]methanol released by alkaline hydrolysis of enzyme-digested DNA, carrier methanol was added to the samples before they were applied to columns of DEAE-cellulose. The breakthrough and the 0.01 M-NH₄HCO₃ (pH 8.4) wash from these columns (usually about 100 ml, containing 10 ml of unlabelled carrier methanol) were combined. The mixture was distilled by using a fractionation column of single-turn glass helices, and the first 25 ml (b.p. 94–98°C) was collected for redistillation (b.p. 65–68°C). The methanol content of this second distillate (7–8 ml) was estimated to be about 50–55% from the density of the mixture and from a calibration curve constructed for the refractive index of methanol–water mixtures. The radioactivity of the entire second distillate was then measured in a Triton X-100–toluene (1:1, v/v) counting

* Abbreviation: SSC, standard saline citrate (0.15 M-NaCl in 0.015 M-sodium citrate, pH 7.1).

medium; a non-volatile internal standard ($[^{14}\text{C}]$ -adenine) was used to estimate the efficiency of counting. The identity of the $[^{14}\text{C}]$ methanol was checked further by distilling off the methanol-toluene binary azeotrope after washing out the vial with toluene. The binary azeotrope distilled at 63–65°C [cited as 63.7°C; Handbook of Chemistry and Physics (1966–1967)] and the composition of the mixture was verified by density and refractive-index measurements as described above. The recovery of the methanol by this procedure was about 70%. In a typical analysis the specific radioactivity of the methanol from the second distillation was 440 d.p.m./10ml and after recovery as the toluene-methanol azeotrope was 431 d.p.m./10ml. In these analyses the counting rate was about 140–200 c.p.m., compared with a background of 33–35 c.p.m.

Preparation of methyl phosphate. Methyl phosphate was prepared by the reaction of methanol with excess of POCl_3 under reduced pressure to remove the HCl evolved. Distillation of the mixture under reduced pressure gave POCl_3 , methyl phosphodichloridate and higher products (Kosolopoff, 1950). The methyl phosphodichloridate fraction (b.p. 50°C; 14 mmHg) was then hydrolysed in an aqueous suspension of BaCO_3 to give a precipitate containing methyl phosphate. This crude material was purified by precipitation from water by the addition of 0.1 vol. of ethanol followed by chromatography on DEAE-Sephadex A-25 (for conditions see Fig. 4), and finally the methyl phosphate was reprecipitated as the barium salt. Paper chromatography (Whatman no. 1) in the solvents butan-1-ol-acetic acid-water (5:2:3, by vol.) ascending, and propan-2-ol-aq. NH_3 (sp.gr. 0.880)-water (7:1:2, by vol.) descending, gave R_F values of 1.1 and 1.8 respectively, relative to P_i . A molybdate spray was used for detection of these materials (Bandurski & Axelrod, 1951), and after exposure to u.v.-light (254 nm) methyl phosphate produced a blue coloration.

Radioactively labelled methyl phosphate was prepared by the reaction of 0.1 ml of 1 M-potassium phosphate buffer (pH 7.6) with 2 mg of N - $[^{14}\text{C}]$ methyl- N -nitrosourea at 37°C until the evolution of N_2 had ceased. The mixture was diluted and applied to a column (1.5 cm $\times$ 20 cm) of DEAE-cellulose for elution with a linear gradient of 0.01–0.3 M- NH_4HCO_3 (pH 8.5); four radioactive products were obtained. The radioactive material which was eluted in the position of a monophosphate was shown to co-chromatograph on paper with the unlabelled methyl phosphate, and both products were found to be sensitive to alkaline phosphatase.

Results

It has been shown that the 3- and 7-alkyl substituted purine deoxyribonucleosides in DNA are heat-labile

(Bannon & Verly, 1972; Lawley *et al.*, 1973). These constitute most of the radioactive material, and their removal by this simple procedure was employed to allow a more precise measurement of the residual products of DNA alkylation.

Origin and distribution of the radioactivity contained in the heat-labile and heat-stable fractions of labelled DNA

Rat liver DNA from animals treated with NN -di $[^{14}\text{C}]$ methylnitrosamine was dissolved in SSC solution and pre-hydrolysed by heating in a boiling-water bath. The progressive change in bound radioactivity is shown in Fig. 1, and after 90 min 24% of the radioactivity remained bound to the DNA. This result is similar to that obtained for DNA treated *in vitro* with ethyl methanesulphonate (Bannon & Verly, 1972).

A portion of the heat-depurinated DNA was then evaporated to dryness by vacuum desiccation, hydrolysed to the constituent bases with 72% HClO_4 and analysed by chromatography on Dowex 50 (H^+ form). Table 1 (line *a*) shows that a large part of the radioactivity present in this heated DNA was eluted early from the column. Table 1 also shows (line *b*) that the free alkyl purines, when themselves subjected to hydrolysis with 72% HClO_4 , do not break down to produce a significant amount of radioactivity that is eluted early. One possible source of the early-eluted material could be the degradation products derived from the strong acid hydrolysis of *O*-methylation products in DNA. This was found to be the case (Table 1, line *c*) when *O*⁶-methylguanine

Fig. 1. Heat-depurination of DNA isolated from animals injected with NN -di $[^{14}\text{C}]$ methylnitrosamine (2 mg/kg)

The DNA was dissolved in $0.1 \times \text{SSC}$ and heated at 100°C. Samples were taken for precipitation by the addition of 2 vol. of ethanol in the presence of 1 M-KCl. Note that the small discrepancy from 100% at zero time is due to trace depurination during solution of the DNA at 4°C.

Table 1. Analysis of ^{14}C -labelled products

All samples were analysed by ion-exchange chromatography on columns (28 cm $\times$ 1 cm) of Dowex 50 (H^+ form) after digestion in 72% HClO_4 for 1 h at 100°C and were derived as follows. (a) ^{14}C -labelled DNA, which had been isolated from animals killed 3 h after an injection of NN -di[^{14}C]methylnitrosamine, was heat-depurinated and precipitated with ethanol in the presence of 1 M-KCl. (b) The corresponding alkyl purine fraction was treated with HClO_4 to remove salt and reduced to dryness by vacuum desiccation before the addition of HClO_4 for hydrolysis. (c) ^{14}C -labelled O^6 -methylguanine was obtained by column chromatography on Dowex 50 (NH_4^+ form) of a mild acid hydrolysate of calf thymus DNA treated with N -[^{14}C]methyl- N -nitrosourea *in vitro*. The isolated base was freeze-dried to remove water and ammonium formate before the addition of HClO_4 for hydrolysis. (d) ^{14}C -labelled methyl phosphate was obtained as described in the Materials and Methods section. (e) ^{14}C -labelled methyl phosphate was hydrolysed in the presence of 4 mg of calf thymus DNA. For (d) and (e) 2×10^6 d.p.m. were applied to the column. In both cases the major early-eluted peak was followed by a second minor peak containing about 1.0% of the total radioactivity eluted.

^{14}C -labelled samples	Distribution (% of radioactivity eluted from columns)		
	Early-eluted	3- and 7-alkyl purines	Other peaks of radioactivity
(a) Heat-depurinated DNA	54	<0.5	45*
(b) Free alkyl purines	<0.5	97.4	2
(c) O^6 -Methylguanine	99	—	—
(d) Methyl phosphate alone	100	—	—
(e) Methyl phosphate + calf thymus DNA	99.8	0.2	—

* Derived from the metabolic labelling of thymine, common purines and traces of 3-methylcytosine and 1-methyladenine.

was treated in this manner. However, in a sample of NN -di[^{14}C]methylnitrosamine-treated DNA this could account for only 5–6% of the total methylation products, of which 20–25% normally appear in the early-eluted fractions (see O'Connor *et al.*, 1973). Another possible base-derived source of this material would be O^4 -methylthymidine, but this is unlikely to account for more than 1% of the total products by reference to the methylation pattern produced by the reaction of N -methyl- N -nitrosourea with DNA *in vitro* (Lawley *et al.*, 1973). Thus, of the total methylation products that are eluted from columns of Dowex 50 (H^+ form) after strong acid hydrolysis, there remains about 20% of these that could be attributed to the presence of methyl phosphate in DNA.

This possibility was investigated as follows. Table 1 (line d) shows that hydrolysis of [^{14}C]methyl phosphate by HClO_4 yielded products which were eluted as a single early peak during chromatography on Dowex 50 (H^+ form). However, since both negatively charged and uncharged species would not be retained by this cationic resin some of this material was subjected to chromatography on DEAE-cellulose [HCO_3^- form]. In this system approximately half of the radioactive material was eluted as uncharged materials, followed immediately by a minor peak (about 1%) and the remainder as methyl phosphate, with a trace (about 0.3%) of impurities with a higher net negative charge. This is in agreement with the observation that, at 100°C, methyl phosphate is more readily hydrolysed at pH 4 than at pH 1 and below (Hudson, 1965). Formic acid (Bannon & Verly, 1972)

also failed to degrade alkyl phosphates quantitatively to the corresponding alcohol.

From the above it was likely that methyl phosphate would be present in strong-acid digests of DNA methylated *in vivo* by agents such as NN -dimethyl-nitrosamine. An early-eluted peak of radioactive material obtained by chromatography of such a digest on Dowex 50 (H^+ form) was therefore freeze-dried and applied to a column of DEAE-cellulose (HCO_3^- form). A single peak of radioactivity was eluted from the column in the position of methyl phosphate and was shown to be sensitive to alkaline phosphatase. Volatile products were presumably removed by the freeze-drying process, as they were not detected as uncharged materials in the elution profile.

One question arising from the presence of methyl phosphate in strong acid hydrolysates of DNA is the extent to which it may function as a methylating agent. Thus acid hydrolysis of calf thymus DNA in the presence of [^{14}C]methyl phosphate (Table 1, line e) demonstrated that its capacity as a methylating agent was very low, as expected. However, 0.2% of the radioactivity eluted from the column was coincident with the elution of marker 7-methylguanine. Re-chromatography of this material on a column of Dowex 50 (NH_4^+ form) and on paper demonstrated that the radioactivity cochromatographed with 3-methyladenine and not with 7-methylguanine.

Although it is evident (see above) that DNA isolated from animals treated with NN -dimethylnitrosamine does contain some methyl phosphate, the data in Table 2 show that it is unlikely that phosphotriesters

Table 2. *Distribution of radioactivity in the heat-labile and heat-stable fractions of ^{14}C -labelled DNA isolated from animals killed 3 h after an injection of NN-di ^{14}C methyl-nitrosamine*

DNA was depurinated by heating for 90 min at 100°C before precipitation with cold ethanol in the presence of KCl. The heat-stable fraction was reprecipitated, dialysed against water for 24 h and then evaporated to dryness before hydrolysis with HClO_4 and analysis by column chromatography on Dowex 50 (H^+ form).

Fraction of DNA	Total radioactivity (%) (a)	Radioactivity due to methylation (%) (b)
DNA (total)	100	100*
Heat-labile	76	84.3
Heat-stable		
(a) due to methylation	14.2	24.0
(b) metabolic incorporation	9.8	
		15.7
		—

* Equivalent to 90.2% of the total radioactivity after correction for radioactivity incorporated via metabolic pathways (see column a).

amounting to 20% of the total products are present in DNA. The heat-stable fraction contained only 24% of the total radioactivity, of which about 10% was due to metabolic incorporation into thymine, guanine and adenine (Table 2, column a). Now if we consider only the radioactivity due to direct methylation (column b) 5–6% of this can be accounted for as *O*⁶-methylguanine, leaving no more than about 10% of the total methylation products to be attributed to the esterification of the phosphate.

Table 2 (column b) also shows that about 85% of the total products ascribed to methylation are heat-labile, but the 3- and 7-alkyl-substituted purines together account for no more than 75% of these (see O'Connor *et al.*, 1973); thus a further fraction of up to 10% remains unidentified as a heat-labile product. An analysis of the heat-labile products from a sample of heat-depurinated NN-di ^{14}C methylnitrosamine-treated DNA is given in Fig. 2. The elution profile shows that an early-eluted product had been released from the DNA together with the 3- and 7-methyl-purines.

Stability of phosphotriesters in DNA to hydrolysis by venom phosphodiesterase

Alkyl phosphates in DNA are stable to heating at 100°C at neutral pH, and therefore the heat-stable fraction of heat-depurinated DNA can be used for the isolation of these products from DNA (Bannon & Verly, 1972; Lawley, 1973). Samples of the heat-stable fractions were digested with venom phos-

Fig. 2. *Ion-exchange chromatography on Dowex 50 (NH_4^+ form) of the heat-labile products from a heat-depurinated sample of NN-di ^{14}C methylnitrosamine-treated DNA*

The column was developed with 0.3 M-ammonium formate (pH 8.9) and 7 ml fractions were collected for radioactivity counting and measurement of u.v.-absorbance. Positions of the 3- and 7-substituted marker compounds are indicated.

Fig. 3. *Chromatography on DEAE-Sephadex (A-25) of a sample of heat-depurinated DNA which had been digested with venom phosphodiesterase*

The digestion was carried out for 23 h at 37°C in the presence of 1 mM- MgCl_2 and 10 mM-Tris-HCl (pH 8.0) to minimize the activity of the contaminating traces of 5'-nucleotidase activity. After 1 and 2 h NaOH was added to readjust the digest to pH 8.0. The column was developed with a linear gradient of LiCl (0–0.2 M) in the presence of 7 M-urea and 5 mM-Tris (pH 7.5) and 6 ml fractions were collected. ●, Radioactivity; ····, u.v. absorption at 254 nm; ----, salt gradient. For details see the text.

phodiesterase at pH 8.0. Fig. 3 shows that the greater part of the radioactive products in this digest were eluted from Sephadex A-25 by LiCl in the presence of 7 M-urea close to the positions of deoxynucleotide monophosphates, implying that the products exhibited two net negative charges. The four major nucleotides were only partially separated by this system and were eluted as a typical double peak of u.v.-absorbing

Fig. 4. Chromatography on DEAE-Sephadex (A-25) of a sample of heat-depurinated DNA which had been digested with venom phosphodiesterase in the presence of alkaline phosphatase for 19 h

Details of the procedure and symbols are given under Fig. 3, but in this case the column was developed in the absence of urea.

materials; the first contained dpC, dpT and dpA, the second was dpG. Part of the radioactivity was due to metabolic incorporation into the common bases and part was due also to the presence of traces of stable methylated bases. Some of the radioactive material preceded the u.v.-peak in a way similar to that described by Bannon & Verly (1972) for phosphotriesters present in enzymic digests of DNA treated with ethyl methanesulphonate (*in vitro*). Phosphotriesters eluted at this molarity must presumably carry a 5'-phosphate group. Digestion of a further sample with venom phosphodiesterase in the presence of alkaline phosphatase resulted in the early elution of all the radioactivity from the column, which would be expected of deoxynucleosides and dideoxynucleoside phosphotriesters, which carry no net negative charge (Fig. 4).

Measurement of phosphate alkylaton in DNA

Phosphotriesters in DNA are labile in the presence of strong alkali (Reese & Saffhill, 1968; Bannon & Verly, 1972; Lawley, 1973). With a dideoxynucleoside methyl phosphate ester the hydrolysis would be expected to yield 1 mol of methanol and 1 mol each of 5'-Me-dN and 3'-Me-dN. The new phosphodiester would then carry one net negative charge, but when these were chromatographed on a column of Sephadex A-25 only minimal retention of these species was observed (see Bannon & Verly, 1972). DEAE-cellulose, however, would be expected to retain species carrying a single net negative charge (Tomlinson & Tener, 1963), and this was confirmed by using the dinucleosides C—C, C—A and C—G. The most weakly retained of these, C—C, remained bound to the column during the elution of deoxy-

Fig. 5. Separation of negatively charged and uncharged species by ion-exchange chromatography on DEAE-cellulose (HCO_3^- form)

The neutralized alkali-degraded sample of a phosphodiesterase-phosphatase digest of heat-depurinated DNA was diluted 20-fold with water containing 10 ml of unlabelled methanol and applied to the column. The column breakthrough was collected and combined with a 20 ml wash of 0.01 M- NH_4HCO_3 (pH 8.4) for distillation of the [^{14}C]methanol as detailed in the Materials and Methods section. The elution was continued with the same buffer and the change to 0.7 M- NH_4HCO_3 (pH 8.4) is indicated by the arrow; 3 ml fractions were collected.

guanosine, which is the most strongly retained of the four deoxynucleosides; the R_F values of these dinucleosides relative to C—C were 1.11 and 1.22 respectively. Elution with strong salt gathers these materials into a single peak.

In this system, heat-depurinated enzyme-digested DNA, when subjected to alkaline hydrolysis (0.1 M-NaOH) at pH 12.6, yielded labelled nucleosides, which were eluted with the low-salt wash, and charged species, which were retained on the column and were eluted with high salt (Fig. 5). The column breakthrough combined with the wash was shown also to contain [^{14}C]methanol after recovery of the alcohol by distillation and additionally by redistillation as the methanol-toluene azeotrope (see the Materials and Methods section). If breakdown of the deoxynucleoside methyl phosphates occurred it would yield either [^{14}C]methanol and unlabelled deoxynucleoside phosphates or [^{14}C]methyl phosphate and unlabelled deoxynucleosides. In both cases the products would be eluted either in the methanol fraction or as charged species with the high-salt wash. The proportion of [^{14}C]methanol and ^{14}C -labelled charged species (Table 3) was about 33 and 66% respectively, indicating that alkaline hydrolysis of the phosphotriester bonds had in fact been non-selective. An analysis was made after alkaline hydrolysis at 59°C and this again showed non-selective cleavage of the phosphotriester bonds.

The total amount of alkyl phosphate obtained from these analyses (Table 3) proved to be 10–12% of the

Table 3. *Distribution of radioactivity contained in ^{14}C -labelled DNA isolated from animals treated with $\text{NN-dimethyl-}^{14}\text{C}$ -methylnitrosamine*

The molar proportions of the methylation products are expressed as a percentage of the total radioactivity in DNA after correction for metabolic incorporation into thymine, guanine and adenine. For further details see the text.

Fraction of DNA	Sample analysed by alkaline hydrolysis	
	at 37°C	at 59°C
DNA (total products)	100	100
Heat-labile fraction	78.9 (82.6)*	80.3 (84.0)*
Heat-stable fraction	21.1	19.8
Alkyl phosphate	11.4	10.2
(a) as methanol	3.8	3.4
(b) as charged species	7.6	6.8
Other methylated bases†	9.7	9.7

* Numbers in parentheses are corrected for 7-methylguanine carried over in the heat-stable DNA when precipitated in the presence of NaCl.

† Same analysis was used for determination of other methylation products (which in this case include 3.7% 7-methylguanine).

total reaction products. It should be noted in Table 3 that a small amount of 7-methylguanine was found associated with the heat-stable fraction, which was not the case when KCl was used for precipitation of the heat-depurinated DNA (see analyses given in Tables 1 and 2).

Discussion

Alkyl phosphate residues formed in DNA after treatment with *NN*-dimethylnitrosamine *in vivo* constitute an important part of the overall methylation pattern. They account for about 10% of the total products contained in liver DNA isolated by the phenol procedure. However, there still remains unidentified a further fraction of up to 10% of the radioactivity which is released from DNA together with the 3- and 7-methyl-substituted purines when DNA is heated in neutral solution. Although the fraction may be derived from the alkylation of some other material which remains associated with DNA during these purification procedures, it remains a possibility that it may be released from some highly labile site in DNA. The failure to detect this material after HClO_4 precipitation and freeze-drying of the alkyl purine fraction (Table 1, line *b*) suggests that it is volatile after heating in neutral solution. This is supported by the failure to detect uncharged materials when early eluted products from chromatography on

Dowex 50 (H^+ form) were freeze-dried and rechromatographed on DEAE-cellulose (HCO_3^- form).

There are a number of important implications which may result from the presence of phosphotriesters in DNA. Although esterification of the phosphate residues would not be expected to interfere directly with the hydrogen-bonding properties in base-pairing mechanisms, the phosphotriesters would be positioned outermost in the DNA molecule and would exert a steric hindrance on those enzymic processes moving sequentially along the double helix. In particular an effect on endonucleolytic cleavage mechanisms might be envisaged. Some support for this concept has come from the use of the phosphodiesterase from snake venom which will successfully degrade a wide variety of modified DNA substrates but cannot cleave those internucleotide linkages which are fully esterified. A blocking of diesterase action *in vivo* therefore would be expected to arise from the chemical modification of DNA after treatment with these carcinogenic agents.

Phosphotriester formation in DNA effectively neutralizes the net negative charge on the phosphate residues, and hence the formation of several phosphotriesters in close proximity might be expected to affect the binding of chromatin proteins to the DNA double helix and thereby possibly to affect the subsequent expression of coded information from those regions. By reference to a DNA model, modifications resulting from reaction with methylating agents can be grouped on the basis of the region of the DNA double helix which they occupy (see Table 4). Thus in addition to neutralization of net negative charges on DNA phosphate groups the distal position of the methyl groups bound to the phosphate residues would also be expected to hinder the binding of chromatin proteins to the phosphodiester backbone. On the other hand, base modifications which affect the base-pairing regions of the DNA molecule (Table 4) might be expected to be of greater significance for the mutational aspects of the action of these carcinogenic agents when base substitutions are envisaged. In fact two of these products, 3-methylcytosine and O^6 -methylguanine, are known to have a potential for miscoding *in vitro* (Ludlum, 1971; Gerschmann & Ludlum, 1973), and others, particularly O^4 -methylthymine, have yet to be examined in this respect. Whether miscoding or 'misreading' might arise from the presence of phosphotriesters in DNA has also to be established.

Studies *in vitro* have shown that alkylation of DNA by *N*-methyl-*N*-nitrosourea yields 18% of the total products as phosphotriesters (Lawley, 1973), and ethyl methanesulphonate produces 15% of these products, in contrast with methyl methanesulphonate, which yields only 1% (Bannon & Verly, 1972). In the present study only 10–12% of the total reaction products were isolated as phosphotriesters after

Table 4. *Position of the methylation products in the DNA double helix*

Outer phosphate-sugar helix	Narrow groove	Wide groove	Base-pairing regions
Phosphotriesters	3-Methylguanine 3-Methyladenine	7-Methylguanine 7-Methyladenine (5-Methylcytosine)*	1-Methylguanine 3-Methylcytosine 1-Methyladenine O ⁶ -Methylguanine O ⁴ -Methylthymine 3-Methylthymine N ⁶ -Methyladenine

* Naturally occurring methylated base.

reaction with *NN*-dimethylnitrosamine *in vivo*. Whether this represents the full extent of the potential phosphate reaction with *NN*-dimethylnitrosamine, or whether *in vivo* the association of chromosomal proteins with the phosphate groups in DNA results in a partial blockage of reaction at these sites, remains to be determined.

Previously we presented evidence for the loss of the early-eluted products from DNA *in vivo* (see the introduction). By 3 days only 40% of these materials remained bound to DNA and by 20 days there was only 10% of the initial amount (O'Connor *et al.*, 1973). From the data presented here, which show that after correction for radioactivity derived from O⁶-methylguanine about half the early-eluted material can be ascribed to methyl phosphates, and further, since O⁶-methylguanine was not detected in liver DNA 4 days after treatment (O'Connor *et al.*, 1973), it is evident that some loss of phosphotriesters is taking place *in vivo*, but the rate cannot be determined from the existing information.

It is now apparent that for agents of the methylating and ethylating series only those capable of producing significant amounts of O-alkylation products in cellular nucleic acids *in vivo* are found to be potent carcinogens. These facts support the view that there are critical reaction sites found *in vivo* which specifically initiate the carcinogenic process irrespective of whether the critical sites lie in the nucleic acids or in other cellular macromolecules. They do, however, underline the need for a continued extension of these studies to other types of carcinogen (e.g. aromatic amines, hydrocarbons etc.) to establish those reaction sites with miscoding potential, and their relative stability *in vivo*.

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