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N-(2-HYDROXYETHYL)-*N*-[2-(7-GUANINYL)ETHYL]AMINE, THE PUTATIVE MAJOR DNA ADDUCT OF CYCLOPHOSPHAMIDE *IN VITRO* AND *IN VIVO* IN THE RAT

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Abstract—The anti-cancer agent, cyclophosphamide, metabolises to the cytotoxic alkylating agent phosphoramide mustard, which can be dephosphoramidated to give nornitrogen mustard. A rat liver mitochondrial supernatant system was used to study the binding of [chloroethyl ^{3}H]cyclophosphamide to DNA. The reacted DNA was acid-hydrolysed and one major adduct was identified using Sephadex G-10 chromatography, followed by HPLC, using reversed-phase or ion-exchange systems. Further studies, using [^{14}C]guanine as reaction substrate for [chloroethyl ^{3}H]cyclophosphamide, phosphoramide mustard or nornitrogen mustard, demonstrated the main adduct from each reaction had identical chromatographic properties in these systems. The radiolabelled ratio in the [^{3}H]cyclophosphamide–[^{14}C]guanine reaction demonstrated a monoadducted product. From this evidence and from ^{1}H NMR data, the common adduct was putatively identified as a hydroxylated nornitrogen mustard adduct (*N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine).

In *in vivo* studies, rats were injected intraperitoneally with 2.775 MBq [^{3}H]cyclophosphamide. Total organ [^{3}H] content and DNA binding levels were ascertained. Maximal levels of [^{3}H] binding to DNA were seen between 1–4 hr with the highest binding levels observed in the bladder. The *in vivo* adduct was shown, using various HPLC systems, to co-chromatograph with the *in vitro* adduct and thus the main *in vivo* adduct was putatively identified as *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine.

Cyclophosphamide, a cyclic derivative of the alkylating agent nornitrogen mustard, is one of the most widely used anti-cancer agents. Although originally synthesised [1] to be selectively toxic to cells containing high levels of the enzyme phosphoramidase, it has been established that activation proceeds via the mixed function oxidases of the liver oxidising the cyclophosphamide [2] to 4-hydroxycyclophosphamide. This product equilibrates with its acyclic tautomer, aldophosphamide, which is further metabolised by aldehyde oxidase and aldehyde dehydrogenase, or converted nonenzymatically to the metabolites 4-ketophosphamide, carboxyphosphamide and aldophosphamide. Alternatively, the products acrolein and phosphoramide mustard can be spontaneously formed [3, 4]. Both acrolein [5] and phosphoramide mustard [6] are cytotoxic, with phosphoramide mustard implicated as the main alkylating agent generated by cyclophosphamide metabolism [7]. The cyclophosphamide metabolite nornitrogen mustard, detected in human urine samples [8], is produced by cleavage of the phosphoramide residue from the phosphoramide mustard [9]. The cytotoxic products of cyclophosphamide metabolism are shown in Fig. 1.

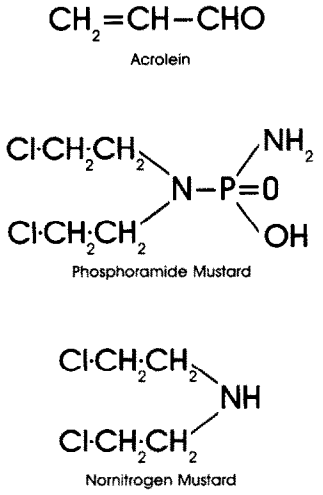

Fig. 1. Cytotoxic products of cyclophosphamide metabolism.

The reaction products of phosphoramide mustard with nucleosides and nucleotides have been characterised using a variety of *in vitro* systems [8, 10–13]. From these studies, the preferred site of nucleic acid base reaction has been characterised as the N-7 position of guanine. The alkyl moiety involved in the binding of cyclophosphamide-derived alkylating species to DNA [14] and polynucleotides [11] is likely to be phosphoramide mustard [11]. However, binding to proteins is mainly through acrolein [15].

Three DNA adducts, all 7-substituted guanine adducts of nornitrogen mustard [16] with either intact or hydroxylated mustard arms, and one cross-linked product, have been identified. It is not known if these nornitrogen mustard adducts arise as a result of phosphoramide mustard reaction with DNA, and subsequent phosphoramide loss, or directly through nornitrogen mustard reaction.

The present study was undertaken to characterise the DNA adduct produced when cyclophosphamide was activated *in vitro*, using a liver-mixed function oxidase system in the presence of DNA and the *in vivo* adduct in the rat, after intraperitoneal injection of cyclophosphamide.

MATERIALS AND METHODS

Chemicals and biochemicals. Specific reagents were obtained from the following sources: nornitrogen mustard, Aldrich Chemical Company Ltd, Gillingham, Dorset, U.K.; NADP, glucose-6-phosphate, Boehringer-Mannheim Ltd, Lewes, Sussex, U.K.; cyclophosphamide, Koch-Light Laboratories, Haverhill, Suffolk, U.K.; calf thymus DNA, guanine, sodium dodecyl sulphate (SDS), RNase T, RNase A and Proteinase K, Sigma Chemicals (London) Ltd, Dorset, U.K. [^{3}H]Cyclophosphamide (specific activity = 555 GBq/mM) labelled in the dichloroethyl side-chains was obtained from Amersham International, plc, Amersham, Bucks, U.K.; and [^{14}C]guanine (specific activity = 2.05 GBq/mM) was obtained from New England Nuclear plc, Southampton, U.K. Phosphoramide mustard was synthesized from nornitrogen mustard by the method of Freidman and Seligman [17]. All other chemicals were of laboratory reagent grade.

DNA-[^{3}H]cyclophosphamide preparation in vitro with rat liver post-mitochondrial supernatant. Fifty milligrams of calf thymus DNA was dissolved in 10 ml 150 mM NaCl–15 mM sodium citrate (SSC), to which was added 30 ml 500 mM PO_4 buffer, pH 6.8, 1.25 ml 1 M $MgCl_2$, 62.5 mg NADP, 300 mg glucose-6-phosphate and 30 ml phenobarbitone-induced rat liver S-9 (25% in 150 mM KCl). The volume was made up to 100 ml with SSC and the mixture made 5 mM with respect to cyclophosphamide. 1.85 MBq [^{3}H] cyclophosphamide was added and the resulting mixture was divided between four 250 ml conical flasks, which were then incubated in a shaking water bath, at 37°, for 90 min. The DNA was recovered by adding an equal volume of acetone, pelleting by centrifugation and resuspending the pellet in 50 ml 1% w/v SDS, 0.5 M NaCl. This was extracted once with an equal volume of phenol (saturated with 50 mM Tris–HCl, pH 8.0), twice with equal volumes of chloroform–isoamyl alcohol (24:1) and the DNA was then precipitated from the aqueous layer by the addition of two volumes of ice-cold ethanol. The recovered DNA was resuspended in distilled water at 5 mg/ml and hydrolysed by heating at 70° for 20 min with the solution made 0.1 N in HCl. The hydrolysed DNA solution was divided into 5 ml aliquots, lyophilised and stored at −90° until required.

[^{3}H]Cyclophosphamide–[^{14}C]guanine preparation in vitro using rat liver post-mitochondrial supernatant. Five milligrams guanine were dissolved in 5 mM KH_2PO_4 pH 4.5, to give a concentration of 0.5 mg/ml. To this was added 5 ml 500 mM PO_4, pH 6.8, 0.15 ml 1 M $MgCl_2$, 6.25 mg NADP and 30 mg glucose-6-phosphate. 9.25 KBq [^{14}C]guanine, 92.5 kBq [^{3}H]cyclophosphamide and 7.5 mg cold cyclophosphamide were then added, followed by 5 ml phenobarbitone-induced rat liver S-9 (25% w/v homogenate in 150 mM KCl). The volume was made up to 20 ml with SSC and the solution was incubated at 37° in a shaking water bath, in a 250 ml conical flask, for 90 min. The protein was then precipitated out with an equal volume of acetone, the supernatant recovered and the acetone removed by gentle rotary evaporation. The resulting aqueous layer was extracted once with phenol (saturated with 50 mM Tris–HCl, pH 8.0), twice with chloroform–isoamyl alcohol (24:1), then divided into 2 ml aliquots, lyophilised and stored at −90° until used.

Phosphoramide mustard and nornitrogen mustard–[^{14}C]guanine reaction. Guanine was dissolved at 0.5 mg/ml in 5 mM KH_2PO_4, pH 4.5 (10 ml) and 15 mg of phosphoramide mustard or nornitrogen mustard were added. 9.25 KBq [^{14}C]guanine were added to each incubate and the resulting solution incubated at 37°, with stirring, for 3 hr. The solutions were then divided into 2 ml aliquots, lyophilised and stored at −90° until used.

Preliminary separation of reaction products on Sephadex G-10. Aliquots of the recovered products of the [^{3}H]cyclophosphamide–DNA, [^{3}H]cyclophosphamide–[^{14}C]guanine, phosphoramide mustard–[^{14}C]guanine and nornitrogen mustard–[^{14}C]guanine reactions were applied to a Sephadex G-10 column, 2.5 × 40 cm, equilibrated with 100 mM ammonium formate. Elution was performed with this buffer at 60 ml/hr and the eluted peaks were collected and lyophilised. Each lyophilate was then redissolved in 5 mM KH_2PO_4 pH 4.5 and measured for radioactive content.

HPLC analysis. Samples containing radioactivity from the preliminary Sephadex G-10 separations were analysed on HPLC, using both reversed-phase and ion-exchange techniques. A 0.6 × 25 cm column, packed with Whatman Partisil 10-ODS-2 for reversed-phase studies, or with Partisil 10-SCX for ion-exchange studies, was used in conjunction with a Pye-Unicam LC-XP gradient programmer, LC-XPD pump and LC-UV detector, with analysis of HPLC runs carried out automatically on a Pye-Unicam video chromatography control centre (Pye-Unicam, Cambridge, U.K.).

For reversed-phase studies, the gradient run was 1 mM formic acid for 20 min, followed by 0–100% methanol in 1 mM formic acid over 15 min, at a flow rate of 2 ml/min, monitored at 265 nm. Aliquots (1 ml) were collected directly for radioactive determination, in Optiphase scintillant (Fisons plc, Loughborough, U.K.) and counted on a Packard 300C liquid scintillation counter. Cpm were converted to dpm by external standard using a previously prepared quench curve. For ion-exchange studies on Partisil 10-SCX, the reaction product was studied using a 10 mM ammonium formate (pH 4.5) over 60 min, at a flow rate of 2 ml/min, and the eluent

monitored at 265 nm. 1 ml samples were collected and counted for radioactivity as above.

In vivo [^{3}H]*cyclophosphamide DNA binding studies*. Four groups of four male Wistar rats were each injected intraperitoneally with 2.775 MBq [^{3}H]cyclophosphamide (37 GBq/mmol, 140.6 MBq/mg) in ethanol. At timed intervals after the initial injection, one group of four animals was killed and exsanguinated by severing the jugular vein. The liver, kidneys, lungs, heart, spleen and bladder were removed from each animal and weighed. The DNA from each organ was then isolated.

[^{3}H]*Cyclophosphamide–DNA purifications from rat organs*. Each liver was suspended in 50 ml 1% SDS–1 mM EDTA, other organs in 15 ml 1% SDS–1 mM EDTA and homogenised. The figures in parenthesis show the amounts used in the smaller incubate. Twenty-five milligrams (7.5 mg) of proteinase-K in 2.5 ml (0.75 ml) was added, the mixture was incubated at 37° for 30 min and, following the addition of 2.5 ml (0.75 ml) 1 M Tris–HCl, pH 8.0, extracted with an equal volume of phenol (all organic solvents used were saturated with 50 mM Tris–HCl pH 8.0). The aqueous layer was recovered by centrifugation and extracted sequentially with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). Five millilitres (1.5 ml) of 5 M NaCl was then added to the aqueous layer and the DNA precipitated by the addition of 2 vol. of ice-cold ethanol. The recovered DNA was washed twice in an equal volume of 70% v/v ethanol and then redissolved in 10 ml (3 ml) 1.5 mM NaCl:0.15 mM trisodium citrate:1 mM EDTA. One hundred microlitres (30 μl) of 1 M Tris–HCl pH 7.4 were added, with 750 units (225 units) of RNAse T1 and 1.5 mg (0.45 mg) of RNAase A [in 1.5 ml (0.45 ml) water, heat treated at 90° for 20 min previously]. The mixture was incubated at 37° for 15 min. Subsequent to the addition of 625 μl (187.5 μl 1 M Tris–HCl, pH 8.0, the DNA solution was sequentially extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). Then 1.5 ml (0.45 ml) 5 M NaCl was added and the DNA precipitated by the addition of 2 vol. of ice-cold ethanol. The recovered DNA was then sequentially washed in 20 ml (6 ml) ethanol, 20 ml (6 ml) ethanol:acetone (1:1) and, finally, 20 ml (6 ml) acetone. The sample was dried under reduced pressure, redissolved in distilled water and the DNA concentration measured using a diphenylamine assay. Purity was assessed using the OD_{260}/OD_{280} ratio. Aliquots of the DNA solutions were hydrolysed by making them 0.1 N in HCl and incubating at 70° for 20 min. These were counted for radioactivity and analysed by HPLC.

RESULTS

Sephadex G-10 separation of reaction products

Sephadex G-10 chromatography of the acid hydrolysed [^{3}H]cyclophosphamide–DNA reaction products, formed after liver mono-oxygenase activation of the cyclophosphamide, gave rise to four distinct peaks, as monited at 265 nm. These were, in order of elution, a large peak running with the void volume composed of residual proteins and oligonucleotides, an unidentified radioactive peak at 100 min, guanine at 140 min and adenine at 200 min. Reaction products, formed by liver mono-oxygenase activation of [^{3}H]cyclophosphamide in the presence of [^{14}C]guanine, were resolved into three distinct peaks on Sephadex G-10 chromatography, a peak running with the void volume, composed of residual proteins, an unknown radioactive peak at 100 min and guanine at 140 min (Fig. 2).

In both these separations the bulk of the bound radioactivity to DNA bases was present in the peak separating at 100 min. In the DNA reaction, the bulk of the bound [^{3}H] counts were present in this peak. In the [^{14}C]guanine reaction, the presence of both [^{3}H] and [^{14}C] in the 100 min peak indicated a cyclophosphamide-derived guanine product.

In each case, the reaction between phosphoramide mustard and [^{14}C]guanine and the reaction between nornitrogen mustard and [^{14}C]guanine, gave two distinct peaks on Sephadex G-10, the uncharacterised peak at 100 min and guanine at 140 min. In both separations the 100 min peak contained [^{14}C], although only a small amount of this peak was present from the nornitrogen mustard–[^{14}C]guanine reaction, reflecting the weak alkylating activity of nornitrogen mustard at physiological pH [13]. The

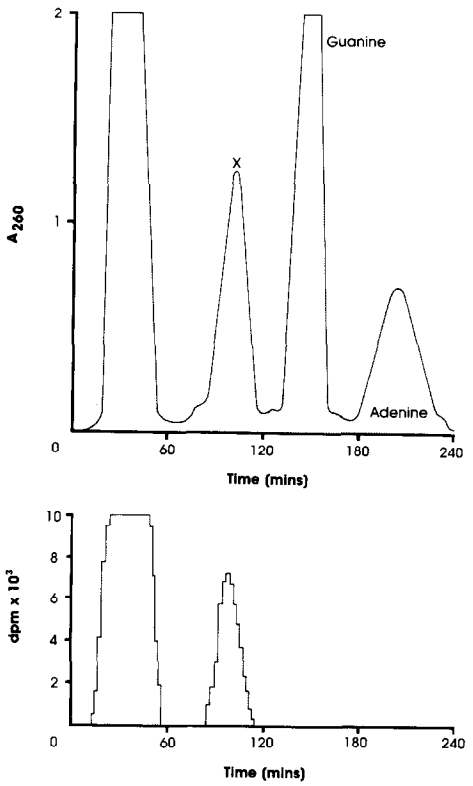

Fig. 2. G-10 separation of [^{3}H]cyclophosphamide–DNA reaction products. Acid-hydrolysed cyclophosphamide-reacted DNA was applied to a G-10 column, previously equilibrated in 0.1 M ammonium formale and eluted at 60 ml/hr with the same buffer. The upper figure shows the UV trace with time, at 260 nm, the lower shows the radioactive content of 1 ml aliquots collected over the elution period.

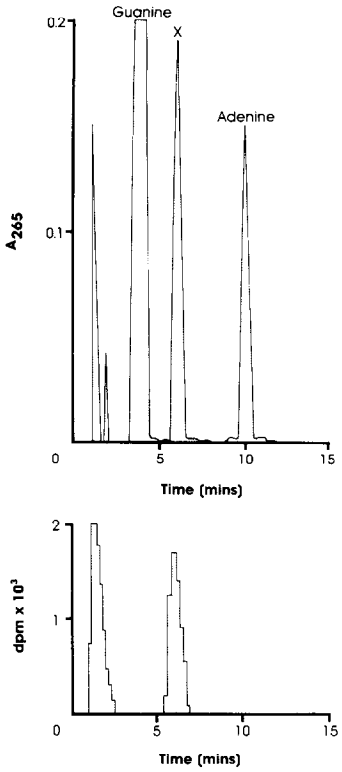

Fig. 3. HPLC analysis of [^{3}H]cyclophosphamide-reacted DNA products. The isolated peak X from the preliminary Sephadex G-10 separation was applied to a reversed-phase HPLC system as detailed in Materials and Methods. The subsequent elution profile, monitored at 265 nm, is shown in the upper figure, whilst the lower figure shows the radioactive profile of the eluent over the time period. Peak X was purified by repeated HPLC runs, lyophilised and stored for further analysis.

percentage recovery of radioactivity from each separation was in the order of 99–99.5% of applied radioactivity.

In view of the above data, the uncharacterised peak eluting at 100 min from all four reaction mixtures was further analysed on HPLC.

HPLC analysis

The peak eluting on Sephadex G-10 at 100 min from the four samples was further analysed on reversed-phase HPLC. The elution profile of the Sephadex G-10-derived [^{3}H]cyclophosphamide–DNA peak demonstrated a major radioactive peak eluting at 6.5 min, whilst the elution profile of the Sephadex G-10-derived [^{3}H]cyclophosphamide–[^{14}C]guanine peak also demonstrated a major radioactive peak at 6.5–7 min, containing both [^{3}H] and [^{14}C] in a calculated 1 : 1 ratio, indicating the presence of a monoadducted guanine derivative (Fig. 3). Although the bulk of the guanine and adenine had been separated, small amounts were still present in the sample, due to breakthrough on the Sephadex G-10. Re-applying the isolated sample removed much of these contaminants; however, this was not done routinely as the radioactive peak was easily separable on HPLC.

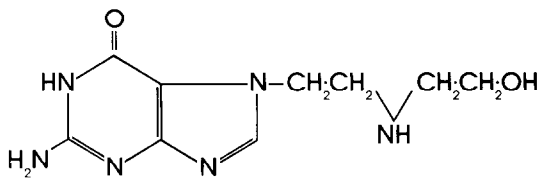

Fig. 4. Proposed structure of the major *in vitro* cyclophosphamide-derived DNA adduct.

The Sephadex G-10-derived peaks from both the phosphoramide mustard–[^{14}C]guanine and the nornitrogen mustard–[^{14}C]guanine reaction also yielded the major radioactive peak at 6.5–7 min.

This major radioactive peak from each reaction mix, observed at 6.5–7 min, was collected and purified using repeated HPLC runs, aliquoted, lyophilised and stored at −90° until used.

The lyophilised reverse-phase purified products were re-dissolved and subjected to ion-exchange HPLC. All four reactions yielded a single peak which co-chromatographed at 33 min. Increasing the elution buffer concentration from 10 to 15 mM ammonium formate resulted in a decrease in retention time to 18.5 min, consistent for all four products.

UV spectra

The UV spectra of aliquots of each of the four purified adducts were obtained under acid (pH 1), neutral (pH 7.0) and alkaline (pH 13) conditions. The adducts showed identical UV spectra with absorption maxima at 248 nm, 282 nm (shoulder at 245 nm) and 278 nm. Alkaline treatment effects were irreversible, indicating ring opening of the imidazole ring [18]. These results showed good agreement with previously reported N^7-guaninyl-nitrogen mustard products [19].

NMR studies

Spectra of the isolated adducts were obtained using a Bruker WH 360 ^{1}H Fourier transformed NMR spectrometer with dimethyl sulphoxide as solvent. Signals consistent with the mustard methylenes appeared between 3.1 and 4.6 ppm, with the 1′ N-7CH$_2$, adjacent to the N-7 of guanine at 4.6 ppm (singlet) and the 4′ N-7-CH$_2$ at 3.08 ppm (triplet). The 2′ N7-CH$_2$ and 3′N-7CH$_3$ were both masked by H$_2$O under DMSO, but both showed as triplets at 3.63 ppm and 3.50 ppm respectively in D$_2$O, with the 1′ N-7-CH$_2$ also appearing as a triplet in D$_2$O. The N-7-NH proton appeared at 9.01 ppm (singlet) under dimethyl sulphoxide, disappearing in D$_2$O. No evidence of a phosphoramide group was detected and the data show good agreement with those obtained for hydroxylated nornitrogen mustard-guanine in a previous study [16]. The proposed structure of the major *in vitro* adduct is shown in Fig. 4.

In vivo *studies*

Rats injected intraperitoneally with [^{3}H]cyclophosphamide were killed at time intervals of 1, 4, 8 and 24 hr, after initial injection, and the total radioactivity/gram of tissue and the DNA-bound radioactivity were determined. The tissue radio-

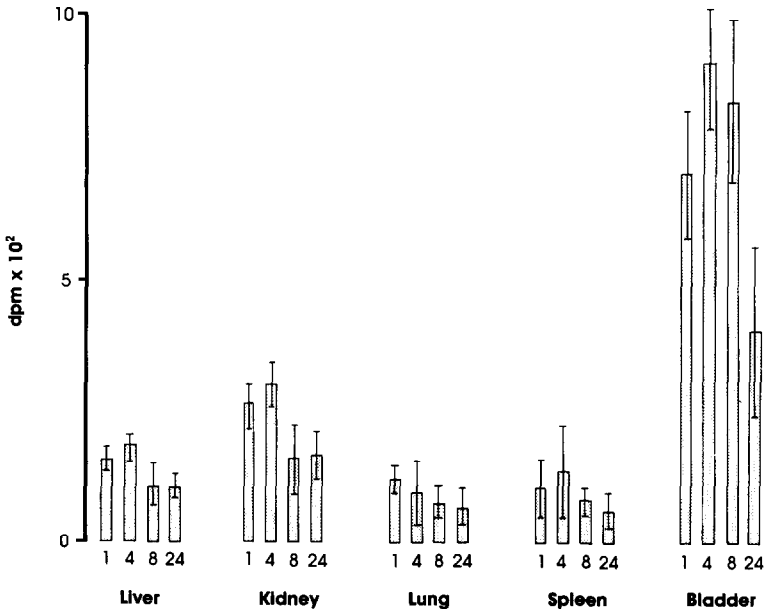

Fig. 5. [^{3}H] binding to organ DNA at time points after initial injection. DNA was isolated from the organs specified and measured for radioactivity. The figures below each column indicate number of hours post-injection with the organ of the DNA samples shown for each group of four time points.

activity levels were highest at 1 hr post-injection, with the total [^{3}H] content of each organ falling over the time course of the experiment. However, the rate of loss of radioactivity content varied with the organ studied; liver radioactivity levels had a half life of $t_{\frac{1}{2}} = 2.9$ hr, whilst kidney and lung levels were of the order of $t_{\frac{1}{2}} = 1.7$ hr. Bladder radioactivity levels were of the order $t_{\frac{1}{2}} = 0.95$ hr; however, the levels noted in the bladder may be affected by the excretion of radioactive metabolites in the urine, which would be greatest in the early stages of the experiment.

[^{3}H] Cyclophosphamide–DNA binding levels had reached their maximum by 4 hr, being similar, or slightly lower, at the 1 hour time point and falling in all cases after 4 hours. The fall of DNA binding levels over the 24 hr time period was approximately consistent for all the DNA studied. The time course of [^{3}H] binding in the DNAs studied is shown in Fig. 5.

HPLC analysis of DNA adducts after acid hydrolysis

The binding levels of [^{3}H] to the DNA samples were low, the highest binding obtained being 1030 dpm/mg DNA (0.206 alkyl residues/10^6 nucleotides). In initial HPLC analyses on unconcentrated samples, 80–85% of the applied radioactivity eluted at 6.5 min on reversed-phase HPLC (Fig. 6). This finding held true for DNA samples from each time point and each organ, demonstrating firstly that the adduct formed *in vivo* was the same in each case and, secondly, that the *in vivo* adduct co-chromatographed with the purified *in vitro* adduct. However, these observations were based upon a low [^{3}H] content per mg DNA applied, so repeated reversed-phase purifications were carried out, collecting the peak eluting at 6.5 min. The product was reduced in

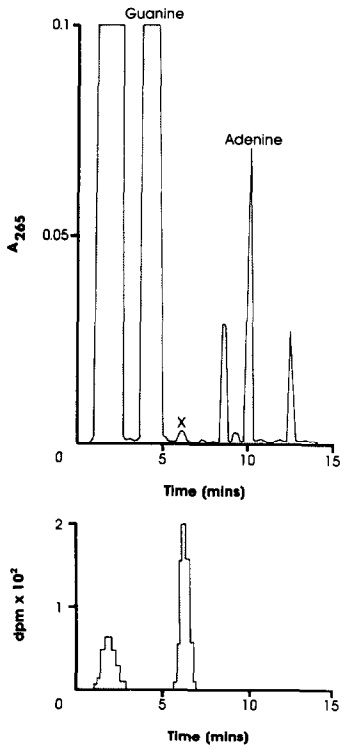

Fig. 6. HPLC analysis of *in vivo* [^{3}H]cyclophosphamide-treated rat kidney DNA. Rat kidney DNA, isolated and acid-hydrolysed as in Materials and Methods, was applied to a reversed phase HPLC system, as detailed in Materials and Methods. The UV profile, as monitored at 265 nm, is shown in the upper figure, whilst the radioactive profile is shown in the lower. Peak X co-chromatographed, under these conditions, with the adduct obtained *in vitro* (Fig. IV, V). Further ion-exchange HPLC confirmed this co-chromatography (see text).

volume and reapplied to the reverse-phase HPLC, where 90–95% of the applied [^{3}H] appeared at 6.5 min. This product was then applied to ion-exchange HPLC, under which conditions the *in vivo* adduct co-chromatographed with the *in vitro* adduct using either 10 and 15 mM ammonium formate as eluent.

DISCUSSION

The products of the metabolism of cyclophosphamide in the presence of DNA or guanine, in a biological system, are likely to be labile and possibly undergo secondary reactions [11]. Phosphoramide mustard [20, 21], the metabolite believed to be responsible for the cytotoxicity of cyclophosphamide, has been shown to generate nornitrogen mustard spontaneously [9, 16]. Indeed, the phosphoramide moiety of phosphoramide mustard is so unstable that any of the conventional techniques used to depurinate DNA cleave off this moiety. ^{32}P NMR kinetic studies have indicated a half-life of 5 min for the initial phosphorous signal of adducts at 37° [16].

Hemminki [16] demonstrated the *in vitro* metabolism of cyclophosphamide in the presence of DNA gave rise to three adducts, all 7-substituted guanine adducts of nornitrogen mustard. In the present work, we have attempted to determine structure and relative quantities of adducts produced *in vivo*.

The results from experiments studying the alkylation potential of phosphoramide mustard over various incubation periods [7, 13], and from ^{32}P NMR studies on cyclophosphamide metabolism [22, 23], have indicated difficulties in preserving the integrity of the phosphoramide mustard adduct. Thus, for the present studies, short incubation periods for cyclophosphamide in the biological systems were used, DNA was isolated rapidly and stored at −90°. Further, the use of acid hydrolysis for the production of DNA adducts ensured most phosphoramide mustard products not already changed would be converted to nornitrogen mustard products.

The HPLC systems used here showed that the major adduct generated by the cyclophosphamide–DNA, cyclophosphamide–guanine, phosphoramide mustard–guanine and nornitrogen mustard–guanine reactions behaved chromatographically identically in each case, with the isolated adducts from each having identical UV spectra. Previous studies on guanine derivatives of phosphoramide mustard [9, 10, 13], nornitrogen mustard [9, 19] and nitrogen mustard [18, 19] have shown mono-adducted N^7-substituted guanine derivatives. Our demonstration that the [^{3}H].[^{14}C] ratio of the [^{3}H]cyclophosphamide–[^{14}C guanine] isolated adduct to be 1:1 confirms the product as a mono-adduct. The adduct isolated from nornitrogen mustard–guanine reaction, although reflecting the poor alkylating properties of nornitrogen mustard [13, 20] by virtue of its trace amounts, nevertheless co-chromatographed with the isolated adducts from the other reaction mixtures, showing that the isolated adduct in each case was dephosphoramidated.

Whilst three types of DNA adduct derived from reaction with cyclophosphamide were identified in a previous study [16], a hydroxylated nornitrogen mustard–guanine, a nornitrogen mustard–guanine and a cross-linked product, the appearance of the latter two adducts was dependent upon length of reaction incubation [16], although in the HPLC analysis of the reaction products on [^{3}H]cyclophosphamide-DNA, trace amounts of radioactivity at two time points over the gradient course consistent with the adducts, demonstrated by Hemminki [16], were seen. These traces were, however, of such a low level as to preclude any study upon them, and we, therefore, could not reach any definitive conclusion concerning their nature. In our studies, the shorter reaction incubation was reflected in the production of the hydroxylated nornitrogen mustard–guanine adduct as the major adduct. ^{1}H NMR studies lend support to this identification.

The administration of [^{3}H]cyclophosphamide *in vivo* produced overall low levels of binding. The most persistent binding over the time course of the experiment was demonstrated in the liver; differences in the metabolism of cyclophosphamide, or of its metabolite, in different organs may account for the varying levels of persistence. The levels of binding were comparable with those of a previous study in the mouse; liver and kidney DNA levels in rat were 1–1.5 times those obtained in the respective mouse organ DNAs, with lung DNA binding levels in the rat being 3.5–4 times less than those obtained mouse [16]. No observations on bladder DNA were available from this study for comparison with rat bladder DNA binding levels. In both rat and human, the bladder is the most common target organ for cyclophosphamide [20] toxicity and the levels of DNA binding observed in these experiments in the bladder show good agreement with this.

On the basis of the studies reported here, the major adduct obtained *in vivo* is identical to that *in vitro*. Although DNA binding levels were low, repeated purification of the major *in vivo* adduct peak on reversed-phase HPLC allowed analysis, under differing HPLC regimes. In each case, the major DNA adduct obtained *in vivo* co-chromatographed with the adduct obtained *in vitro*. Therefore, whereas Hemminki found three adducts *in vitro*, namely *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl] amine, *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)ethyl] amine and *N*,*N*-bis-[2-(7-guaninyl) ethyl] amine, only the final-named was found *in vivo* in our study. No traces of the *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)ethyl] amine nor the *N*,*N*-bis[2-(7-guaninyl)ethyl amine were found *in vivo*.

In conclusion, the data we have generated show that the major *in vivo* DNA adduct isolated after cyclophosphamide reaction with DNA is *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl] amine. This adduct is produced following metabolism of cyclophosphamide to yield phosphoramide mustard. This reactive intermediate reacts with guanine in the DNA and the product of this reaction spontaneously dephosphoramidates.

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