ABSTRACT

The stability of phosphoramide mustard, a metabolite of cyclophosphamide was studied at pH 7.2 and 37°C using 31P nuclear magnetic resonance. The phosphorus signal of phosphoramide mustard disappeared with a half-life of 8 min indicating rapid conversion to other species. The final product, inorganic phosphate, appeared with a half-life of 105 min indicating that phosphoramide mustard was easily deporphosphamidated.

A rat liver microsomal system was used to study the binding of [chloroethyl-3H]cyclophosphamide to DNA. DNA was hydrolyzed in 0.1 N HCl:0.5 N NaCl at 80°C for 20 min, conditions known to convert phosphoramide mustard to nornitrogen mustard with liberation of the phosphoramide residue. After such treatment three adducts were detected by high-performance liquid chromatography using several elution systems. They were all 7-substituted guanine adducts of nornitrogen mustard; two were monoalkylation products with an intact N-(2-chloroethyl)-N-[2-(7-guaninyl)ethyl]amine or an hydroxylated mustard arm N-(2-hydroxyethyl)-N-[2-(7-guaninyl)ethyl]amine; the third adduct was a cross-linked product N,N-bis[2-(7-guaninyl)ethyl]amine. The relative abundance of these adducts depended on the length of the microsomal incubation. After 2 h, N-(2-chloroethyl)-N-[2-(7-guaninyl)ethyl]amine was the main product but after 6 h N-(2-hydroxyethyl)-N-[2-(7-guaninyl)ethyl]amine was most abundant, and at this time the cross-linked product represented 12% of the total adducts.

The adducts in DNA depurinated readily and after 24 h at pH 7.0 and 37°C 70% of them had been liberated. The rate of depurination was decreased in the presence of 0.5 N NaCl. After short-term depurination in 0.1 N HCl at 25°C the primary alkylating species was phosphoramide mustard rather than nornitrogen mustard.

In in vivo studies mice were given injections i.p. of 100 µCi of cyclophosphamide. Maximal levels of radioactivity had been incorporated into DNA between 2-7 h after injection; the specific activity of DNA from the kidney and lung exceeded that from the liver. While the level of radioactivity found in kidney DNA was rapidly reduced the rate of fall was lower in the lung. Between 24 and 72 h the specific activity of lung DNA exceeded that from the kidney and liver DNA by a factor of 3:8. Lung is the principal target tissue for tumor formation in mice after an i.p. injection.

INTRODUCTION

Few attempts have been made to characterize the DNA adducts produced by alkylating anticancer agents (1) even though their effect is thought to result from interaction with DNA (2). Cyclophosphamide presents a particular challenge since it acts in contrast to most other alkylating agents used after metabolic activation (3-5). Cyclophosphamide is initially oxidized by the hepatic cytochrome P-450-dependent mixed-function oxidases into 4-hydroxycyclophosphamide, which is thought to exist in equilibrium with its ring-opened tautomer, aldoxophosphamide. These are converted nonenzymatically or metabolized by aldehyde dehydrogenase and aldehyde oxidase to the relatively nontoxic metabolites 4-ketophosphamide, carboxyphosphamide, and aldoxophosphamide. Alternatively they can spontaneously form the cytotoxic species acrolein and phosphoramide mustard (6-9). Phosphoramide mustard undergoes cleavage of the phosphoramide residue to form NOR3 (8, 10), a metabolite of cyclophosphamide which has been detected in human urine (11). The structures of cyclophosphamide, phosphoramide mustard, and nornitrogen mustard are shown in Chart 1.

Model studies have been carried out in vitro to characterize the reaction products of phosphoramide mustard with nucleosides and nucleotides (10,12-16). Particularly the work of Mehta et al. (12), Mehta and Ludlum (13), and Vu et al. (10) has helped to understand the reactions of PAM with nucleic acid constituents. The preferred site of reaction is the N-7 position of guanine and a considerable labilization of nucleoside products has been noted (12,14-16). Cyclophosphamide can generate alkylating species capable of binding to DNA (17, 18) and polynucleotides (13) in the presence of a microsomal system. The alkyl moiety was thought to be phosphoramide mustard (13). In another study using a microsomal system, binding to nucleic acids was the result of reaction with phosphoramide mustard, whereas with acrolein (19) most of the binding was to protein.

The present work was undertaken to characterize the DNA binding products of cyclophosphamide in a rat liver microsomal system. In vivo studies were also carried out to determine the level of binding in different tissues and to see if the level of binding could be shown to be related to the organotropic effects of this compound in animals.

MATERIALS AND METHODS

Preparation of Substituted-Guanine Standards. Noritrogen mustard-guanine standards were prepared by heating at reflux overnight a solution of cyclophosphamide, phosphoramide mustard, or nornitrogen mustard with sodium cyanoborohydride or potassium cyanoborohydride in 2-propanol. The reaction mixture was then diluted with water and extracted with chloroform. The organic layer was evaporated to dryness and the residue was dissolved in methanol. The solution was then applied to a column of silica gel and eluted with chloroform.

The fractions containing the desired product were collected and the solvent was evaporated to dryness. The residue was then dissolved in methanol and applied to a thin-layer chromatography plate. The plate was developed with developing solvent and the product was visualized with a UV lamp. The product was then scraped from the plate and weighed. The identity of the product was confirmed by thin-layer chromatography and high-performance liquid chromatography.

The abbreviations used are: NOR, noritrogen mustard; PAM, phosphoramide mustard; NOR-G, N-(2-chloroethyl)-N-[2-(7-guaninyl)ethyl]amine; NOR-OH, N-(2-hydroxyethyl)-N-[2-(7-guaninyl)ethyl]amine; N,N-bis[2-(7-guaninyl)ethyl]amine; PAM-G, N-[2-(chloroethyl)-N-[2-(7-guaninyl)ethyl]phosphorodiamid acid; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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solution of NOR (bis[chloroethyl]amine hydrochloride) (0.9 g; Aldrich), deoxyguanosine (0.3 g), and triethylamine (0.3 ml) in 25 ml of 2,2,2-trifluoroethanol. The solution was cooled and the precipitate was filtered off and washed with trifluoroethanol. The precipitate was taken up in 3 n HCl and chromatographed on a 2.5 × 25-cm Dowex 50WX2 cation exchange column and eluted with 3 n HCl (500 ml) followed by 5 n HCl. The fraction volumes were 10 ml. NOR-G eluted in fractions 45–55 and the yield was 20 mg. G-NOR-G eluted in fractions 75–80 in yields of about 2 mg. NOR-G-OH was prepared by boiling NOR-G in 50 mM ammonium formate, pH 4, for 1 h. NOR-G-OH was purified in the same cation exchange chromatographic system where it eluted in fractions 30–35.

PAM (cyclohexylammonium salt; a gift from Dr. Robert R. Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute) reaction products were prepared by incubating 1 g of PAM with 0.3 g deoxyguanosine in 25 ml of trifluoroethanol at 37°C overnight. Trifluoroethanol was evaporated under vacuum and the solids were taken up in 50 ml of methanol. After removal of insoluble material by centrifugation the supernatant was applied to a 2.6 × 100-cm LH-20 Sephadex column which was eluted in methanol; 10-ml fractions were collected. Materials exhibiting the UV spectroscope properties of 7-alkyldeoxyguanosine eluted in fractions 20–25, while unmodified deoxyguanosine eluted in fractions 30–40. The fraction containing 7-substituted deoxyguanosine was evaporated to dryness and suspended in 0.1 n HCl:0.5 n NaCl and kept at 25°C for 6 h. After neutralization the material was purified by HPLC using a Shersorb octadecyl silane-2 column (10 × 250 mm) eluted with a 0–50% methanol gradient which was 10 mu in sodium phosphate, pH 6.0. The gradient was formed by a gradient maker containing 100 ml of the appropriate solvent in each chamber. Fractions (3.6 ml) were collected. The 7-substituted guanine eluted in fractions 15–16.

The standards obtained were characterized by UV spectra in neutral, alkaline, and acid media using a Gilford Model 250 spectrophotometer. NMR spectra were recorded on a Nicolet NT-300 instrument operated in the Fourier transform mode at 300 MHz. Samples were dissolved in dimethyl-d$_2$ sulfoxide using 0.5% tetramethylsilane as an internal standard. Fast-atom bombardment mass spectrometry was performed on a VG Micromass ZAB-2F instrument. Samples were applied to the probe in glycerol.

$^{31}P$ NMR studies on the stability of PAM in an aqueous solution were carried out using 20 mg of PAM in 0.5 n HEPES, pH 7.2 (0.5 ml) at 37°C. Hexamethyphosphoramide was used as an external standard. The amounts of phosphorus-containing components were obtained by integration of $^{31}P$ signals. Data were obtained on a Varian XL-200 NMR spectrometer equipped with Advance Data System at 80 MHz.

Microsomal System. Microsomes were prepared from 6-week-old male Sprague-Dawley rats given 0.1% phenobarbital in drinking water for 6 days prior to sacrifice. Livers were homogenized in 3 vol of 0.25 M sucrose:50 mM Tris, pH 7.5. The homogenate was centrifuged at 1,000 × g for 15 min. The supernatant was passed through cheesecloth and centrifuged at 10,000 × g for 15 min. The supernatant was again filtered through a cheesecloth and centrifuged at 105,000 × g for 60 min. The precipitate was resuspended in sucrose:Tris and centrifuged again. The microsomal precipitate was resuspended in sucrose:Tris at 1.4 ml/g liver and stored in liquid nitrogen.

Binding of [3H]cyclophosphamide to DNA was studied in a microsomal system containing a volume of 10 ml:30 mg calf thymus DNA, 20 mM potassium phosphate buffer, pH 7.5, 2.5 mM MgCl$_2$, 0.1 mM EDTA, 0.7 mM NADPH, 10 mM glucose-6-phosphate, 0.14 units of glucose-6-phosphate dehydrogenase, 2.5 mg microsomal protein, and 25 μCi [chloroethyl-$^3$H]cyclophosphamide (specific activity, 1 Ci/mmol; Amersham). Incubations were carried out at 37°C for the indicated periods of time. After incubation 10 ml of 0.5 n NaCl and 1 ml of 20% sodium dodecyl sulfate were added. Phenol extraction was carried out with an equal volume of redistilled phenol saturated with water. This was followed with three extractions with equal volumes of chloroform:isoamyl alcohol (1:3). An aliquot was used to calculate the level of water-soluble labeling, which was obtained after two additional extractions with equal volumes of ethyl acetate.

DNA was spooled from the final water phase after addition of 1 vol of cold ethanol. DNA was dissolved two times in 20 ml of 0.5 n NaCl and precipitated with an equal volume of ethanol. The final precipitate of DNA was washed with cold absolute ethanol.

7-Alkylguanines were liberated from DNA suspended in 0.1 n HCl:0.5 M NaCl at 80°C for 20 min. The solution was neutralized and DNA was precipitated with 2 vol of cold ethanol. The ethanol was removed from the supernatant under nitrogen and the resulting solution was analyzed by HPLC as described above.

In Vivo Experiments. NIH Swiss mice were given injections i.p. of 100 μCi of [3H]cyclophosphamide (1 Ci/mmol) in 300 μl of 0.1 M NaCl. Two animals were used for each analysis. Livers, kidneys, and lungs were removed and washed in cold 50 mM NaCl:0.2% Triton-X-100 mM Tris buffer, pH 7.2. The tissues were minced and then homogenized by hand in 20 ml of the medium using 20 strokes of a Teflon-glass homogenizer. Aliquots were taken for the determination of total radioactivity and the homogenates were centrifuged at 2,000 × g for 5 min. The supernatants were poured into 1 ml of 20% sodium dodecyl sulfate and extracted with phenol and chloroform:isoamyl alcohol, as described above, to obtain RNA. To precipitate out the RNA, 2 vol of ethanol at −20°C were added and allowed to stand for at least 2 h. Before measurement of radioactivity RNA was dissolved three times in 0.5 n NaCl followed by ethanol precipitation.

The nuclear pellets were rehomogenized in 40 ml of the NaCl:Triton:
Tris medium and passed through cheesecloth. The nuclei were collected by centrifugation and suspended in 2 ml of 0.2 n NaCl:10 mM Tris, pH 7.2:0.2 ml 20% sodium dodecyl sulfate, and after 0.2 ml proteinase K (Merck) 10 mg/ml in 10 mM Tris:10 mM EDTA, pH 7.0:0.25 mM NaCl had been added, the proteins were digested at 37°C for 1 h. Twenty ml of NaCl:Triton:Tris and 1 ml of 20% sodium dodecyl sulfate were added followed by extractions with phenol and chloroform:isoamyl alcohol as above. DNA was precipitated with an equal volume of cold ethanol and the resuspension solubilization cycle in 0.5 n NaCl with ethanol precipitation was repeated three times. In some experiments DNA was treated with pancreatic RNase, but since no appreciable release of nucleotides was observed the treatment was not used routinely. For HPLC DNA was treated with 0.1 n HCl:0.5 n NaCl at 80°C for 20 min followed by neutralization, ethanol precipitation, and removal of ethanol. DNA and RNA were quantified from readings at A260. Absorbance at A260 was used to assess the purity of the preparations.

Internal standards were used in HPLC in most instances. However, depurination conditions sometimes release of large amounts of guanine and adenine which interfered with visualization of markers. In such instances calibration of elution was based on measurements of guanine and adenine.

RESULTS

Characterization of Guanine Standards. Reasonable amounts of NOR-G, G-NOR-G, and NOR-G-OH (Chart 2) were prepared in nonaqueous conditions using 2,2,2-trifluoroethanol as solvent. These products showed similar UV spectra under neutral (pH 7.0), acid (0.1 n HCl), and alkaline (0.1 n NaOH) conditions. The respective maxima were 285 (shoulder at 247), 251, and 280 nm, which is in agreement with data for similar nitrogen mustard adducts (20). The only difference in shapes of 251, and 280 nm, which is in agreement with data for similar amounts of NOR-G, G-NOR-G, and NOR-G-OH (Chart 2) were shown). The half-time for appearance of phosphate was 105 min under these conditions.

The instability of the PAM-G adduct prompted us to examine the stability of PAM in aqueous solution by 31P NMR spectroscopy. Chart 3 shows that the phosphorus resonance for intact PAM appears at -16.6 ppm upfield from a hexamethylphosphoramide standard (0.0 ppm). The PAM sample was initially homogeneous. On incubation in 0.5 mM HEPES buffer at pH 7.2 and 37°C the initial resonance at -16.6 ppm disappeared with a half-life of 8 min and a new phosphorus resonance appeared at -16.9 ppm (Chart 3). The half-time for appearance of this signal was 8 min. This signal subsequently disappeared (t90 = 50 min) and a resonance at -28.4 ppm (coincident with the resonance for P1) increased in intensity with time (data not shown). The half-time for appearance of phosphate was 105 min under these conditions.

The stability of PAM was also investigated by 31P NMR spectroscopy under conditions that are usually used to depurate DNA and release 7-alkylpurines. The treatments were (a) 0.1 n HCl:0.5 n NaCl at 25°C, overnight, (b) 0.5 n NaCl:0.1 m sodium phosphate buffer at pH 7.0 and 100°C for 10 min, and (c) 0.1 n HCl:0.5 n NaCl at 70°C for 20 min. The resonance for intact PAM phosphorus was absent in all cases and the respective amounts of liberated Pi were 50, 72, and 65% of the total phosphorus signal. When PAM was incubated in 2 n HCl at 25°C overnight >95% of phosphorus was present as free phosphate. These results indicate that conventional methods of treating DNA to liberate 7-alkylpurines derived from reaction with PAM can liberate most of the phosphoramide residues as P1 and produce nitrogen mustard analogues.

Microsome-mediated Binding of Cyclophosphamide to DNA. Binding of radioactivity from [chloroethyl-3H]cyclophosphamide to DNA was examined in a microsomal system using phenobarbitat-induced microsomes. The appearance of water-soluble metabolites, an indicator of microsomal activity, in-
CYCLOPHOSPHAMIDE BINDING

Table 1

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<th>Mustard protons</th>
<th>Guanine protons</th>
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<td>N-7-CH$_2$</td>
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<td>N-8H</td>
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Chemical shifts in ppm (multiplicity, no. of protons)

NOR-G: 4.64 3.54 9.58 3.39 3.92 8.72 7.37 12.2 256
G-NOR-G: 4.63 3.64 9.58 3.64 4.63 8.72 7.27 11.9 371
NOR-G-OH: 4.61 3.64 9.02 3.51 3.03 8.52 7.06 11.6 238

*a* triplet; s, singlet.

Chart 3. 31P NMR kinetics of the decomposition of PAM in 0.5 n HEPES buffer, pH 7.2 at 37°C. The spectra were acquired at 2-min intervals. The phosphorus resonance for intact PAM appears at -16.6 ppm. Hexamethylphosphoramide was used as standard (0 ppm). The half-time (t$_{1/2}$) for the disappearance of the PAM phosphorus signal was 8 min and half-time for appearance of the resonance at -16.9 ppm was 8 min.

Chart 4. Time course of microsomal metabolism of [3H]cyclophosphamide to water soluble metabolites, expressed as [3H]cyclophosphamide converted to water soluble metabolites (○) and DNA bound metabolites (○). --- ---, proportion of DNA binding products released by treating with 0.1 n HCl/0.5 n NaCl at 80°C for 20 min. The data are the mean of two experiments.

increased sharply for 3 h and then started to level off (Chart 4). At 6 h over 60% of [3H]cyclophosphamide had been converted to water-soluble metabolites. The binding to DNA followed similar kinetics but the extent of binding leveled off after 3 h. The specific radioactivity of DNA attained was 3000 dpm/mg DNA, which corresponded to 0.6 alkyl residues/10⁶ nucleotide units of DNA. The radioactivity in DNA accounted for 0.3% of the water-soluble radioactivity. When the DNA was thoroughly washed to remove any noncovalently bound material and treated with 0.1 n HCl to remove 7-alkylpurines, about 90% of the radioactivity was liberated (Chart 4).

Radioactivity liberated from DNA by 0.1 n HCl was analyzed by HPLC using a 0–17% methanol gradient in 10 mM ammonium formate, pH 5.3 (Chart 5). Four peaks of radioactivity were detected. The second, third, and fourth peaks coeluted with NOR-G-OH, NOR-G, and G-NOR-G, respectively.

To confirm the identity of these DNA binding products of [3H]cyclophosphamide the radioactive adducts were chromatographed together with the synthetic standards in three different HPLC systems (data not shown). The first system eluted isocratically with 10 mM sodium phosphate buffer, pH 3.5, and the radioactivity coeluting with the NOR-G and NOR-G-OH standards was pooled. The two fractions pooled were then separated in a second HPLC system eluted with 0–50% methanol:10 mM sodium phosphate buffer, pH 6.0. The radioactivity coeluting with the NOR-G and NOR-G-OH markers was pooled again and subjected to a third HPLC system eluted with 0–17% methanol:10 mM ammonium formate, pH 5.3. The radioactivity still...
Cyclophosphamide binding

coeluted with the synthetic markers indicating that microsomes metabolized [3H]cyclophosphamide into products that were detected as NOR-G and NOR-G-OH. A sequential HPLC experiment was not carried out with G-NOR-G because of the relatively low level of labeling. However, the comigration of the labeled material with the standard was shown in five different HPLC systems.

Whereas NOR-G was the most abundant species there was almost no G-NOR-G in DNA isolated from a 2-h microsomal incubation (cf. Chart 5). However, the length of microsomal incubation did influence the relative proportions of these products. At 2 h the proportion of NOR-G-OH to NOR-G was 28:100 but at 6 h the proportion was 195:100 (data not shown). The cross-linked product (G-NOR-G) was always a minor adduct; at 6 h it represented only 12% of the total adducts. It was evident that in addition to primary alkylation secondary reactions such as hydroxylation of the unreacted chloroethyl group and cross-linking were taking place. However, the secondary reactions were probably not mediated by NOR-adducts in DNA, because NOR-G was very stable in neutral pH. For example, when NOR-G was incubated in 5 m Tris, pH 7.4, at 37°C only about 10% conversion to NOR-G-OH was observed after 2 days. Between pH 3.5 and 6.0 this product was less stable and both NOR-G-OH and polymers were found. To investigate the stability of the modified DNA obtained in this microsomal system after 2, 4, and 6 h DNA was isolated, rinsed, and allowed to stand in neutral medium at 37°C for 24 h. A considerable degree of depurination took place in all the DNA samples (Chart 6). DNA incubated with microsomes for 2 h was depurinated to a lesser extent than were the 4- and 6-h DNA samples. In all cases the presence of 0.5 N NaCl retarded the rate of depurination. When the supernatant was analyzed by HPLC after treatment with 0.1 N HCl the major species were NOR-G-OH and NOR-G, which indicates that true depurination rather than dealkylation had taken place.

The Primary Alkylation Species. In all previous experiments DNA was hydrolyzed under conditions which converted most of the PAM adducts to NOR adducts. In view of the marked rate of depurination of the cyclophosphamide-treated DNA (see above), milder hydrolytic conditions were used to gain additional insight into the role of PAM in the alkylation. DNA labeled for 3 h in a microsomal system was isolated, washed, and suspended in 0.1 N HCl:0.5 N NaCl at 25°C for 6 h. The sample was neutralized and the DNA was precipitated with two vol of cold ethanol. The depurinated material was analyzed by HPLC using a 0–50% methanol gradient in 10 m sodium phosphate buffer, pH 6.0 (Chart 7). There was a marked difference in the distribution of radioactivity after HPLC as compared with the material treated under stronger hydrolytic conditions (cf. Chart 5). While NOR-G and NOR-G-OH were present (Chart 7, peaks c and d) two other prominent radioactive peaks were evident, one, fraction 7, possibly the hydroxylated derivative of PAM-G, and the other fractions 15–16 (Chart 7, peak b), which eluted with PAM-G. Such data suggest that PAM is the primary alkylation agent and NOR-G is a product of PAM-G after dephosphoramidation.

In Vivo Experiments. Mice were given injections i.p. of 100 µCi of cyclophosphamide and total tissue and covalently bound radioactivity in DNA and RNA were determined (Chart 8). The

![Chart 6](image-url)  
Chart 6. Liberation of radioactivity from DNA labeled with [3H]cyclophosphamide in 2-, 4-, and 6-h microsomal incubations. The washed DNA was dissolved in 10 m sodium phosphate buffer, pH 7.0 (C), and 0.5 N NaCl:10 m sodium phosphate buffer, pH 7.0 (O), and incubated at 37°C for 24 h. DNA was precipitated with 2 vol of cold ethanol and the radioactivity released was determined.

![Chart 7](image-url)  
Chart 7. HPLC separation of radioactivity liberated from [3H]cyclophosphamide DNA by treatment in 0.1 N HCl:0.5 N NaCl at 25°C for 6 h. The HPLC was developed with a 0–50% methanol gradient containing 10 m sodium phosphate buffer, pH 6.0. a–e, elution of guanine, PAM-G, NOR-G-OH, NOR-G, and G-NOR-G, respectively.

![Chart 8](image-url)  
Chart 8. Total radioactivity (A) and covalently bound radioactivity in DNA (B) and RNA (C) of mouse liver (○), kidney (□), and lung (▲) after administration of 100 µCi of [chloroethyl-3H]cyclophosphamide i.p. Each point, mean of 2–6 experiments.
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total tissue radioactive levels reached a maximum at 2 h and declined rapidly thereafter. Maximal binding to DNA was observed between 2 and 7 h and in RNA between 2 and 24 h. By 72 h a small amount of radioactivity remained in DNA and RNA.

The specific radioactivity of DNA and RNA were initially about equal in kidney and lung, and about 2-5 times higher in liver. However, while the radioactivity in kidney nucleic acid disappeared rapidly, the levels were more persistent in lung. The specific radioactivity of lung DNA was 3 times higher at 24 h and 7 to 8 times higher at 72 h compared with lung and kidney.

DISCUSSION

DNA binding studies of cyclophosphamide in biological systems represent a considerable challenge. As pointed out by Mehta and Ludlum (13) the products are likely to be labile and undergo secondary reactions. Even the preparation of nucleic acid derivatives for standards is a demanding task. The three standard compounds NOR-G, NOR-G-OH, and G-NOR-G have not been described previously in detail although some properties of guanine derivatives of NOR of (10, 21), of mustard (20-23), and of PAM (10, 12, 16) have been reported. The data of Vu et al. (10) demonstrated the NOR and PAM adducts of guanylic acid and a PAM cross-link of 2 guanylic acids.

PAM, the metabolite believed to be responsible for the cytotoxicity of cyclophosphamide (2, 9), is known to generate NOR spontaneously (8). Using 31P NMR we found the half-life of P1 formation from PAM in 0.5 n HEPES buffer, pH 7.2 at 37°C to be 105 min, which could explain why NOR can be detected as a human urinary metabolite of cyclophosphamide (11). The phosphoramid moiety of PAM is so unstable that any conventional depurination techniques applied to the DNA tends to cleave off the phosphoramid moiety. Enzymatic conversion to nucleosides is also unsatisfactory because of the inherent instability of the adducts. 31P NMR kinetic studies showed that the initial phosphorus signal disappears with a half-life of about 5 min at 37°C. The stability of PAM has been studied indirectly in earlier experiments by measuring its alkylation potential after various periods of incubation (16, 24); 31P NMR has been used in studies of cyclophosphamide metabolism involving steps prior to PAM (25, 26). The results on the lability of PAM imply difficulties in maintaining the unreacted mustard arm intact in biological samples. In view of these problems we used (a) fast purification of DNA in the cold wherever possible, (b) maintained high C1 concentrations to retard further reaction of the mustard, and (c) depurination in 0.1 n HCl, converting most PAM products if present into NOR products.

In the present work microsomes rather than S-9 fractions were used to supply cytochrome P-450-dependent oxidizing capacity and to reduce the further metabolism of active intermediates by cytosolic reductases and oxidases (27, 28). Several HPLC systems showed that the adducts generated from [chloroethyl-3H] cyclophosphamide in the presence of microsomes could be detected as NOR-G, NOR-G-OH, and G-NOR-G. Some 0.3% of the water soluble radioactivity was bound to DNA. After short incubation times NOR-G was the main adduct but when incubation times were increased NOR-G-OH became the main product. At 6 h the cross-link adducts which may be intrastrand or interstrand, accounted for 12% of the total radioactivity identified. This is the first time that cross-links by cyclophosphamide have been shown by chemical means, even though evidence for cross-links has been acquired earlier by physical techniques (29). Nitrogen mustard produces about 25% cross-links in vitro and in vivo (23, 30, 31). The present microsomal system using pure DNA would not be appropriate for the detection of DNA-protein cross-links. These have been found using alkaline elution techniques with cultured cells (32).

The adducts derived from cyclophosphamide had a marked tendency to depurinate even at pH 7.0. Under low salt conditions about 70% of the adduct was depurinated at 37°C. High salt concentration retarded the rate of depurination. Moreover DNA adducts generated in a 2-h microsomal incubation depurinated more slowly than those generated at 4 and 6 h which also indicates a change in the types of adducts being produced. The rate of depurination of the adducts derived from cyclophosphamide is faster compared with sulfur mustard adducts, reported to have a half-life of 51 h at 37°C (21). Electron withdrawing effects of β-nitrogen attached to N-7 guanine appear to be associated with a proneness to depurination and imidazole ring opening because such reactions are remarkably fast in ethyleneimine adducts (33).

NOR is a poor alkylating agent in neutral pH (8, 16) even though it appears to form DNA-protein cross-links in cultured cells (32). When we incubated NOR-G at pH 7.0 for 2 days it appeared to be stable, suggesting that in neutral conditions NOR is not an active alkylating agent. Our results using mild acid treatment imply that PAM rather than NOR is the primary alkylating agent in the microsomal system. After a mild acid treatment the main adducts were depurinated and coeluted with the PAM-G marker. NOR-G adducts were much less abundant.

The results in vivo indicated relatively low levels of binding. When 100 μCi of cyclophosphamide (0.1 μmol, 3.3 μmol/kg) were administered to mice, the highest binding levels were about 100 dpm/A260, which is equivalent to about 0.4 alkyl residues/10⁶ nucleotide units of DNA. The level of binding was so low that unequivocal identification of the adducts in vivo was not readily possible. In a previous study cyclophosphamide (430 μmol/kg) was injected i.p. into rats. Liver DNA had a specific activity of about 20,000 dpm/mg (34). As in our study the level of binding to mouse liver was about 600 dpm/mg and we used less than 1% of the dose; the levels of binding were about 4 times higher in the mouse.

The levels of binding in DNA and RNA were higher in kidney and lung than in liver. Metabolic differences may underly such variation. Enzymatic conversion of 4-hydroxycyclophosphamide-aldophosphamide into nonalkylating metabolites may be more efficient in liver, while in kidney and lung more PAM may be formed. Radioactive levels in the kidney disappeared to about 30% in 24 h suggesting a rapid repair process. The radioactive levels in lung were more stable and between days 1 and 3 the specific radioactivity of lung DNA exceeded that of kidney and liver 3- to 8-fold.

It is of interest to compare the levels and stability of DNA binding to the probability of various organs developing tumors after cyclophosphamide administration. An IARC working group reviewed four studies where cyclophosphamide was administered to mice i.p. (9). One study examined only liver and bladder (35); another study only looked at lung adenomas and found cyclophosphamide positive (36). Two other studies found lung to be the most commonly affected organ (37, 38). This correlates...
with the stability of cyclophosphamide adducts in this organ. In rats and in humans the urinary bladder is the most common target organ of cyclophosphamide (9).

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Binding of Metabolites of Cyclophosphamide to DNA in a Rat Liver Microsomal System and \textit{in Vivo} in Mice

Kari Hemminki


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