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ALKYLATING AGENT INTERACTIONS WITH THE NUCLEAR MATRIX*

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Abstract—The interrelationship of DNA to the nuclear matrix is integral to the organization of chromatin within the nucleus and to the DNA replication process. The influence of nitrosourea and nitrogen mustard interactions with the nuclear matrix were studied in log phase HeLa cells. Alkylation of the nuclear matrix by chlorozotocin (CLZ) or 1-(2-chloroethyl-3-cyclohexyl)-1-nitrosourea (CCNU) was 1.58 and 1.27 pmoles drug/μg protein, respectively, whereas carbamoylation by CCNU was 32.5 pmoles/μg. These constituted approximately 30% of the total (nuclear) drug modifications. The structural matricin fibrillar components of the matrix were alkylated and carbamoylated twice as much as the ribonuclear protein elements (RNP). However, when alkylations are measured per microgram of protein, the ratio of covalently bound drug to RNP:matricin was 1.2 for both CLZ and CCNU. The RNP:matricin carbamoylation ratio for CCNU was 0.9. The importance of DNA and matrix protein alkylations to the process of reassociation was studied. Under control conditions, *in vitro*, approximately 80% of the DNA was associated with the matrix at a protein:DNA ratio (μg for μg) of 50:1. Direct alkylation or carbamoylation of the matrix proteins did not affect these DNA–protein interactions. However, using *in vitro* alkylated DNA (1 alkylation/10^2 base pairs), there was a 60% reduction of the alkylated nucleic acid bound to the matrix at the same protein: DNA ratio. The reduced binding of DNA to matrix may be a function of interference with the DNA recognition sites by alkylation of specific bases. The interference of DNA–matrix association by DNA alkylation may contribute to the cytotoxic activity of these antineoplastic agents.

Under physiological conditions, chloroethylnitrosoureas spontaneously decompose to yield alkyldiazohydroxide and isocyanate moieties [1]. This class of cytotoxic agents can participate in the following interactions with nuclear macromolecules [2]: (i) monofunctional adducts (alkylations) within nucleic acids or proteins; (ii) bifunctional cross-linkage of nucleic acids (inter- or intra-strand); or (iii) nucleic acid cross-links with proteins; (iv) DNA single-strand breaks; and (v) carbamoylation of proteins. Methylnitrosourea participates in similar reactions but does not form cross-links, whereas chlorambucil, a bifunctional nitrogen mustard, causes alkylation and cross-linkage of nucleic acids, but does not carbamoylate proteins. Although it is probable that DNA is the ultimate nuclear target for these compounds, each of these drug interactions has the potential to interfere with nuclear functional integrity and could contribute to cytotoxicity.

The potential for a cell to survive may be determined severally by the type, number or diversity of drug lesions, their locations within the chromatin, and the rate and fidelity of their repair. Our initial analysis of the importance of drug binding to specific nuclear sites for cytotoxicity suggests that there may be regions within the complex of functionally active chromatin that are critical for alkylation-induced cell death [3, 4]. In this context, it is of interest that the relevance of drug interactions with the nuclear matrix and its relationship to chromatin has not yet been explored. The nuclear matrix constitutes the major architectural framework of the nucleus and is composed of a nuclear pore-lamina complex, residual nuceolar matrix, and interchromatic matrix [5–8]. Chromatin is attached at multiple sites on the inner nuclear membrane [9, 10] and the interchromatinic matrix [11]. Such attachments must serve to organize chromatin in a non-random fashion within the nucleus. There is evidence that replication of DNA is initiated at these matrix attachments and that multiple replication loops are attached to the matrix scaffold during the replication process. In addition, preferential binding of estrogens and androgens to the nuclear matrices of steroid responsive cells [12] is consistent with the concept that the matrix is integral to nuclear functional processes as well as acting as a structural framework.

These investigations have utilized radiolabeled nitrosoureas and the bifunctional alkylating agent, chlorambucil, to determine the quantitative alkylation and carbamoylation of nuclear matrix components and to assess, by an *in vitro* assay, whether drug modification of the matrix or DNA interferes with DNA–nuclear matrix associations. The prevention of these associations may interfere with chromatin organization within the nucleus, and may account for drug-induced reductions in DNA synthesis [13] and chromatin disaggregation, observed following alkylation [14, 15].

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METHODS

HeLa cells were maintained in Eagle's, Minimum Essential Medium, (M.A. Bioproducts, Walkerville, MD) with 10% fetal calf serum (K.C. Biologicals, Lenexa, KS). Log phase cells were harvested by centrifugation (5 min, 500 *g*) and washed in media without serum.

Isolation of nuclear matrix. Purified nuclei were prepared from washed cell samples by a modification of the method of Sporn *et al.* [16]. The number of homogenization strokes was increased to 50 with both the tight and loose dounce. Solution 3 (0.32 M sucrose, 0.3% Triton, 1 mM $MgCl_2$, 0.2 mM PMSF* adjusted to pH 6.4 with 1 mM K_2PO_4) was adjusted to 0.6% Triton, and following the second homogenization the nuclei were washed with four successive changes of Solution 3. Resultant nuclei were free of cytoplasmic contamination. This was confirmed by electron microscopy.

The nuclear matrix was isolated following the method outlined by Berezney and Coffey [17]. Centrifugation time at all steps was decreased by half. For reassociation studies, matrices were resuspended in 2 M NaCl, 5 M urea, freeze-thawed (× 3) to disperse the matrix proteins, and dialyzed for 48 hr against 0.1 M NaCl, 0.1 mM EDTA, 10 mM Tris (pH 7.4). Protein determinations were carried out using Bio-Rad Protein Assay I (BioRad Laboratories, Richmond, CA) with bovine gamma globulin as a standard.

For measurements of drug binding to the matrix components (Tables 1 and 2), log phase HeLa cells were treated with 50 μCi of radiolabeled drug for 2 hr prior to nuclear matrix isolation. For reassociation experiments, isolated matrix and extracted DNA were treated with drug. In some cases 10 μCi of [^{14}C-cyclohexyl]CCNU was incubated with the isolated matrix for 2 hr followed by a 48-hr dialysis (0.1 M NaCl, 0.1 mM EDTA) [final specific activity 778 dpm/μg matrix protein (42 pmoles/μg)]. When necessary, bulk nuclear matrix proteins were labeled by treating log phase HeLa cells with 25 μCi of 1-[U-^{14}C]lysine monohydrate (340 mCi/mmole, Amersham-Searle, Arlington Heights, IL) for 24 hr prior to matrix isolation.

Isolation of DNA. DNA was extracted from a total of 5×10^8 log phase HeLa cells, which had been preincubated with 2 pulses (24 and 12 hr prior to harvesting) of 25 μCi of [methyl-1',2'-^{3}H]thymidine (94.4 Ci/mmole) (Amersham-Searle). A crude nuclear pellet was prepared by vortexing the cells in 10 ml of 10 mM Tris (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 1% Triton. The resulting pellet was resuspended in 10 ml of nuclear lysis buffer [30 mM Tris (pH 8.0), 100 mM NaCl, 10 mM EDTA, 1% Triton) and incubated at 37° for 18 hr with 5 μg Proteinase K (EM Biochemicals, Elmsford, NY). The suspension was further treated with 20 Kunitz units of RNase A (Sigma Chemical Co., St, Louis, MO) and 10 Kunitz units of T2 RNase (Sigma) for 30 min at 37°. After the addition of a further 0.5 mg Proteinase K (30 min), the mixture was subjected to a modified phenol extraction of DNA (five phenol washes; three chloroform extractions). The purified DNA was dialyzed against 1 M NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA (×2) and 10 mM Tris (pH 7.4), 1 mM EDTA (×2). The DNA was then treated with 200 μg S1 nuclease (Calbiochem-Boehringer, La Jolla, CA) to remove the single-stranded DNA for 1 hr at 37° in 3 mM $ZnCl_2$, 0.03 M sodium acetate, 0.01 M NaCl (pH 4.5). A 24-hr dialysis was then carried out against multiple changes of 10 mM Tris (pH 7.4), 1 mM EDTA. Isolated DNA was cleaned by spermine precipitation [18]. Spermine hydrochloride (Sigma) was added at a final concentration of 2.0 mM, and the mixture was left on ice for 15 min prior to centrifugation for 15 min at 10,000 rpm. The pellet was rinsed with 70% ethanol, 300 mM Na acetate, 10 mM Mg acetate. This suspension was left on ice for 1 hr and centrifuged as for the spermine precipitation. The resultant pellet was washed and resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA.

[^{14}C-Cyclohexyl]CCNU (8.37 Ci/mole), [^{14}C-chloroethyl]CCNU (10.9 Ci/mole) and [^{14}C-chloroethyl]CLZ (13.73 Ci/mole) were supplied by Drs. Harry Wood and Robert Engle, Drug Development Branch, NCI, Bethesda, MD. [^{14}C-Chloroethyl]chlorambucil (1 Ci/mole) was the gift of Dr. Beryl Hartley-Asp, AB Leo Laboratories, Sweden. [^{3}H-Methyl]MNU (2 Ci/mmole) was obtained commercially from the New England Nuclear Corp., Boston, MA.

For reassociation experiments, isolated DNA was treated with 50 μCi of [^{14}C-chloroethyl]CCNU or [^{14}C-chloroethyl]CLZ in 1 ml for 2 hr. The DNA was exhaustively dialyzed against 10 mM Tris (pH 7.4), 1 mM EDTA. To test for possible differences between *in vivo* and *in vitro* treatment of DNA, in some cases, cells were concentrated into 20 ml and treated with 250 μCi of [^{3}H]MNU for 2 hr or 250 μCi of [^{14}C]chlorambucil for 6 hr. DNA was isolated from these drug-treated cells by the procedures outlined previously.

DNA was quantitated by the method of Burton [19].

Electrophoresis of DNA was carried out in slab gels of 1% agarose. ϕX174 Hpa I standards were used as molecular weight markers. Samples were run for 3 hr at 100 V, stained with ethidium bromide, and photographed. Each of the tracks was then cut into 0.5 cm fractions and oxidized in a Packard Tri-Carb Tissue Oxidizer (Packard, Downers Grove, IL). The ^{3}H was oxidized into Monophase and the ^{14}C into Carbosorb and Permaflour, and the samples were counted in a Mark III Scintillation Counter (Searle-Analytic Inc., Des Plains, IL).

Reassociation methodology. Various concentrations of matrix proteins were added to 1 μg of DNA in a total volume of 1.5 ml TN buffer [0.1 M NaCl, 0.1 mM EDTA, 10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 0.1 mM dithiothreitol and 1 mM PMSF] and incubated for 1 hr at 22° (in some cases 4° or 37°). The samples were layered onto 25 mm HAWP 0.45 μm Millipore filters which had been presoaked in TN buffer. Suction was applied at a rate which required 5–10 sec for sample filtration; this was followed by

* Abbreviations: PMSF, phenyl methyl sulfonyl fluoride; CCNU, 1-(2-chloroethyl-3-cyclohexyl)-1-nitrosourea; CLZ, chlorozotocin; MNU, 1-methyl-1-nitrosourea; and dThd, thymidine.

Table 1. Comparative nitrosourea interactions with the nuclear matrix*

	Drug bound (pmoles/μg matrix protein)		% of Total nuclear drug binding to the matrix
	Total	Acid precipitable	
CLZ alkylation	1.58 ± 0.32	1.60 ± 0.27	26.7 ± 1.4
CCNU alkylation	1.27 ± 0.22	1.28 ± 0.19	31.3 ± 2.7
CCNU carbamoylation	32.5 ± 3.7	38.0 ± 6.9	33.1 ± 3.0

* Each point is the mean ± S.D. of at least six experiments. The matrix proteins constituted 4.7 to 5.0% of the total nucleoprotein constituents. HeLa cells in culture were treated with radiolabeled nitrosoureas (as described in Methods) prior to the isolation of nuclear matrix fractions.

a single wash of 5 ml of TN buffer (in some cases 500 mM NaCl or 1 mM sodium bisulfite was added). Filters were transferred to glass vials, and the DNA bases, were hydrolyzed by boiling in 0.5 ml of 0.5 N HCl for 10 min. The vials were cooled, and the filters were dissolved in 1 ml ethyl acetate (dissolution time 5 min) and counted in a Mark III Scintillation Counter following the addition of 10 ml of ACS (Amersham-Searle). Each experiment was carried out in duplicate. The background DNA-filter binding was determined from a sample to which no protein had been added. Experimental results were expressed as percent reassociation of DNA expressed as a function of varying matrix protein concentrations.

RESULTS

The levels of alkylation and carbamoylation of the matrix fraction following treatment of log phase HeLa cells with nitrosourea are shown in Table 1. The matrix constituted approximately 5% of the total nuclear protein (225 μg/l × 10^8 cells). Pulse labeling with [^{3}H]uridine or [^{3}H]thymidine demonstrated that 5% of the total nuclear RNA (nRNA) and 1–2% of the total DNA were associated with the matrix. Drug binding to the matrix for both CLZ and CCNU represented approximately 30% of the total nuclear interactions, essentially all of which were acid precipitable. Carbamoylation by CCNU was approximately twenty times greater than the corresponding alkylation, a value consistent with a similar ratio for the whole nucleus. The basic sponge-like structure of the HeLa nuclear matrix was confirmed by electron microscopy. Prior treatment of HeLa cells with CLZ or CCNU for short time periods (2 hr) altered neither the gross morphological appearances of these framework structures nor the polypeptide pattern achieved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (data not shown).

Further characterization of the drug binding to the matrix was achieved through separation of the fibrillar matricin components from the ribonuclear protein elements using a sonication technique described by Berezney [20]. Table 2 demonstrates that the major portions (65–78%) of both alkylation and carbamoylation were localized in the structural matricin fibrillar fraction, with approximately 30% within the ribonuclear protein complex. Since the fibrillar components represent a larger proportion of the matrix, drug binding, expressed as pmoles/μg protein, is approximately equivalent.

Phenol extracted DNA was isolated either from HeLa cells receiving no prior drug treatment or from cells pretreated with 100 μCi of [^{14}C-ethyl]chlorambucil or [^{14}C-methyl]MNU. Figure 1 shows a 1% agarose gel of the DNA from both extractions. The DNA ranged from 2000 to 7000 base pairs in length, with the majority of the [^{3}H]dThd found at 3500–4000 base pairs as demonstrated by the tritium profile in Fig. 2. Treatment of DNA *in vitro* with [^{14}C]CCNU and [^{14}C]CLZ was necessitated by the low specific activities of these drugs. Pretreatment of HeLa cells with [^{3}H]dThd (see Methods) resulted in a specific activity of approximately 10,000 dpm of [^{3}H]dThd/μg DNA irrespective of subsequent drug treatments. DNA incubation with [^{14}C]CLZ *in vitro* produced drug binding of 1010 dpm/μg DNA (35 pmoles/μg); with [^{14}C]CCNU, it was 780 dpm/μg DNA (25 pmoles/μg). These values were equivalent to approximately 40–

Table 2. Drug binding to nuclear matrix sub-fractions*

	Matricin fibrils		Ribonuclear protein	
	(pmoles/μg protein)	% of Total drug bound to matrix	(pmoles/μg protein)	% of Total drug bound to matrix
CLZ alkylation	3.96	77.8	4.73	22.2
CCNU alkylation	5.05	65	5.85	35
CCNU carbamoylation	51.5	70	44.4	30

* Matricin fibrils and ribonuclear protein elements were separated from isolated nuclear matrix fractions as described in [20]. Values are the mean of three experiments except for the CCNU carbamoylation values which are the mean of two experiments.

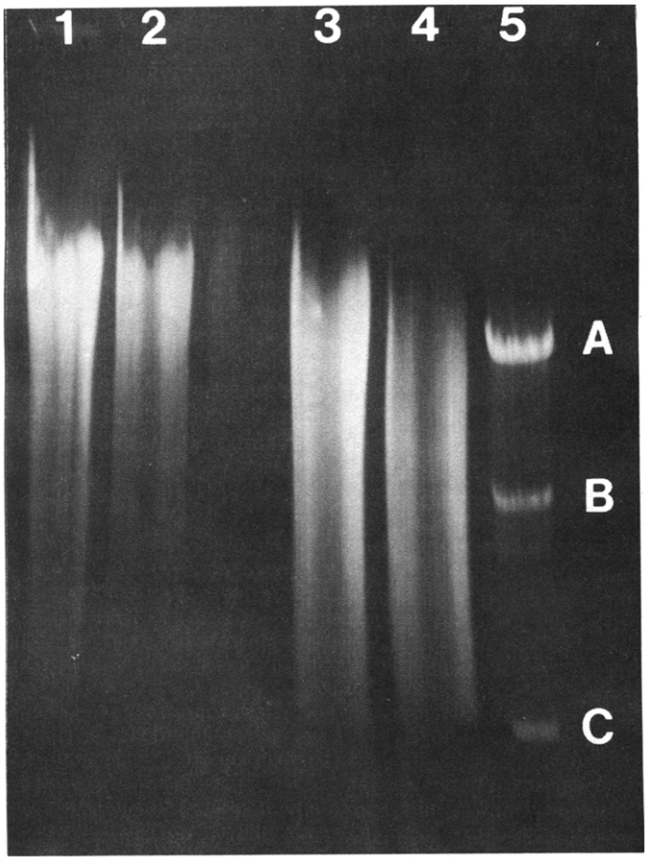

Fig. 1. 1% Agarose gel electrophoresis of isolated HeLa cell DNA, ethidium bromide stained. DNA isolated from cells pretreated with dThd was extracted as described in Methods. Lane 1: DNA treated *in vitro* with 25 μCi of [^{14}C-chloroethyl]CCNU and spermine precipitated to remove free drug and oligonucleotides. Lane 2: DNA, spermine precipitated with no drug treatment. Lane 3: DNA treated *in vitro* with 50 μCi of [^{14}C-chloroethyl]CCNU without spermine precipitation. Lane 4: DNA with no drug treatment and no spermine precipitation. Lane 5:ϕX174, Hpa molecular weight standards: A = 3730 base pairs (bp); B = 1264 bp; C = 392 bp. Two micrograms of DNA was added to each lane.

60 alkylations/3000 base pairs. Lower levels of alkylation were obtained by *in vivo* treatment of HeLa cells with [^{3}H]chlorambucil or [^{3}H]MNU which resulted in DNA with approximately 100 dpm/μg for both chlorambucil and MNU. This is approximately equivalent to 2.5 alkylations/3000 base pairs.

The proteins used in the reassociation assays were prepared by sequential extractions as described previously [17] and were primarily the 65–70 K matrix proteins with other minor non-histone protein species.

Figure 3 shows the reassociation kinetics of [^{3}H]dThd from control and drug-treated HeLa cells. In this experiment, DNA was incubated with 5 mM unlabeled CLZ and CCNU for 2 hr to achieve relatively high levels of alkylation. There were no significant differences in the reassociation kinetics of either control or drug-treated DNA.

A comparison of the reassociation kinetics of alkylated regions of DNA with the nuclear matrix is shown in Fig. 4, following *in vivo* treatment with chlorambucil or MNU or *in vitro* exposure to CCNU or CLZ. The reassociation patterns were similar for CCNU, MNU, and CLZ, where no saturation in the reassociation kinetics was apparent. For chlorambucil, saturation occurred at a 50:1, protein:DNA ratio; however, the plateau was at 60% as opposed to 90% for dThd (top curve). Since both ^{14}C and ^{3}H were present on the same DNA, these data suggest that either some of the alkylated products were unstable and dissociated, or that specific regions of DNA exist which are alkylated and prevent the protein–DNA recognition and reassociation.

The binding of both untreated and alkylated DNA was reversible following washes with 500 mM NaCl (Fig. 5), where less than 10% of the DNA remained bound to the matrix at a 200:1, protein:DNA ratio. The possible involvement of enzymatic activity in the destabilization of the matrix–DNA binding was evaluated by carrying out the reassociation experiments at 4°. In addition, 5 mM sodium bisulfite was used as a phosphatase inhibitor in some assays to check for possible enzymatic degradation of phosphate groups, with the concomitant liberation of alkylated phosphotriesters. Neither of these techniques yielded results which differed from the normal reassociation kinetics (Fig. 5). Overall, the results suggested that the DNA–matrix interactions, as

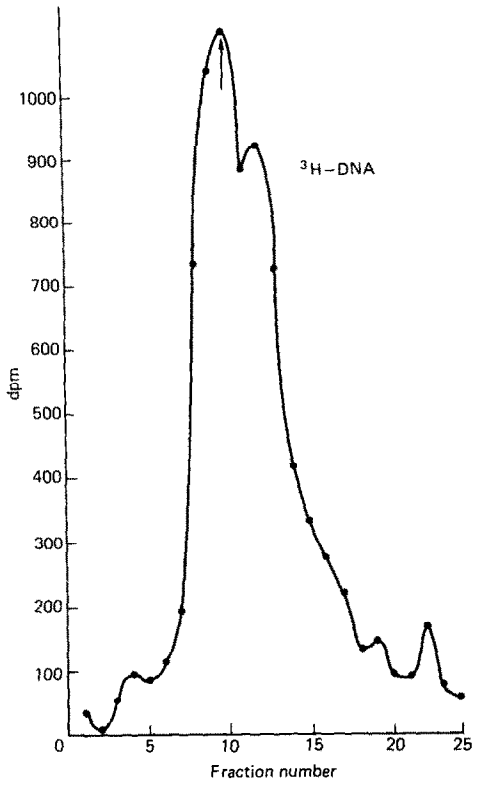

Fig. 2. Profile of [^{3}H]dThd-labeled DNA (non-drug treated). Lane 2 of the 1% agarose gel (Fig. 1) was cut into 0.5 cm strips and oxidized prior to scintillation counting. Arrow represents 3730 bp marker.

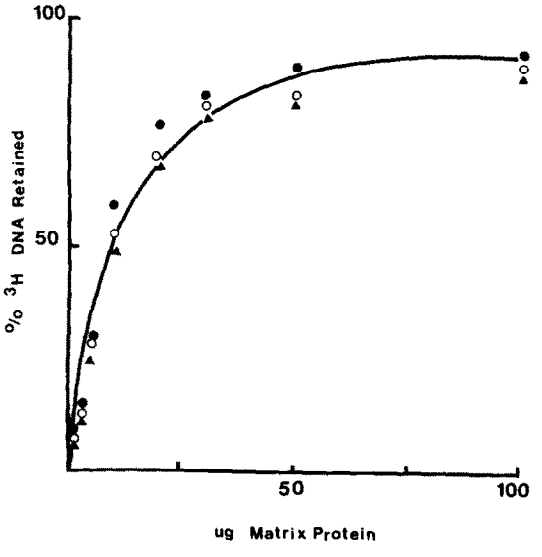

Fig. 3. Nuclear matrix protein: DNA reassociation assay, showing the filter retention of [^{3}H]dThd-labeled DNA through binding with various amounts of matrix proteins. Treatment of DNA *in vitro* with unlabeled CCNU (●—●) or chlorozotocin (▲—▲) at concentrations of 1 mM did not alter the kinetics of reassociation from those of DNA receiving no drug treatment (○—○). Data are the mean of three duplicate assays. S.D. < 10% of mean. Background filter retention of DNA < 2%.

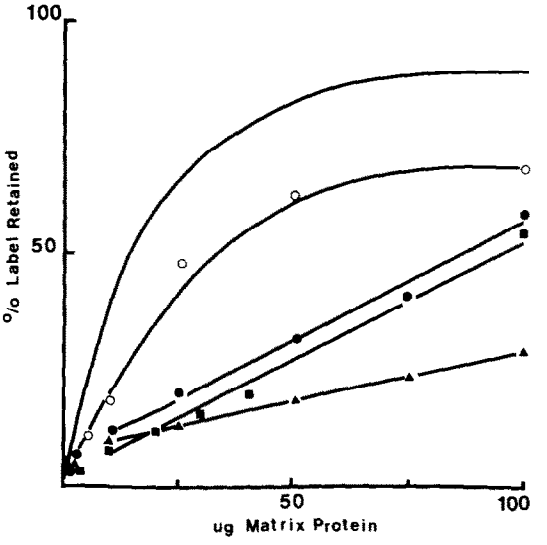

Fig. 4. Reassociation kinetics using DNA labeled at the sites of drug interactions. The assays were performed as for Fig. 3, except that DNA was treated with radiolabeled alkylating agents. The retention of the radiolabel represents that proportion of the alkylated DNA which undergoes binding with the matrix proteins. The top curve is the dThd reassociation curve which is unaffected by drug treatments (see Fig. 3). Key: (○) DNA extracted from HeLa cells treated *in vivo* with 250 μCi of [^{14}C-ethyl]chlorambucil; (●) DNA extracted from HeLa cells and then treated *in vitro* with 50 μCi of [^{14}C-chloroethyl]CCNU; (■) DNA extracted from HeLa cells treated *in vivo* with 250 μCi of [^{3}H-methyl]MNU; and (▲) DNA extracted from HeLa cells and then treated *in vitro* with 50 μCi of [^{14}C-chloroethyl]chlorozotocin. Data are the mean of at least three duplicate experiments. S.D. < 10% of the mean.

measured in these studies, are primarily non-covalent.

Since the nuclear matrix *per se* was heavily modified by chloroethylnitrosoureas, the importance of protein carbamoylation for DNA–matrix binding was assessed. Figure 6 shows that between 50 and 60% of the matrix protein (labeled with [^{14}C]lysine) remained on the nitrocellulose filter independent of the initial protein concentration. Treatment of the isolated matrix with [^{14}C-cyclohexyl]CCNU gave a measure of the carbamoylated matrix products which were retained on the filter. In this case, drug treatment resulted in the binding of 42 pmoles/μg matrix. The retention patterns were similar for CCNU and [^{14}C]lysine. In addition, the reassociation of DNA was similar to the pattern using non-drug treated matrix, suggesting that carbamoylation of the proteins did not alter the DNA–matrix interactions.

To determine whether there was a preferential retention of a specific-sized DNA, the filtrate of the reassociation assay was collected, concentrated, and separated by electrophoresis as shown in Fig. 7. The major band of ethidium bromide stained DNA occurred at approximately 4000 base pairs (lanes 7 and 8). By measuring the size of the DNA in the filtrate (lanes 2–6), it was found that the major bands appeared to have the same mobility as the unreassociated DNA (lanes 7 and 8).

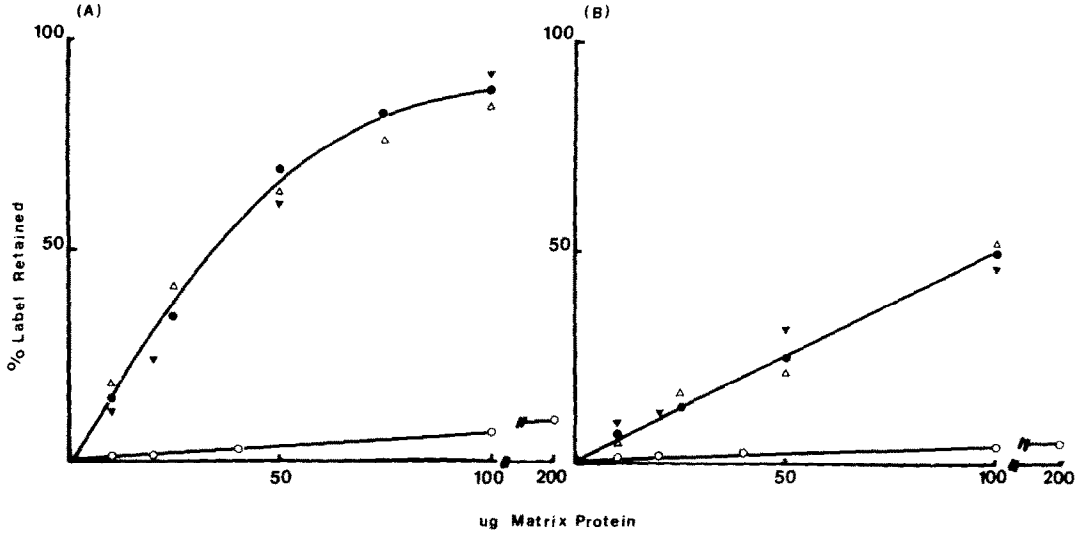

Fig. 5. Effect of varying buffer conditions and temperature on the reassociation process. Panel A: Reassociation of [^{3}H]dThd-labeled DNA. Panel B: Reassociation of [^{14}C-chloroethyl]CCNU-labeled DNA. All conditions are as for Fig. 4 except: (●) 37°; (△) 4°; (▼) + 1 mM sodium bisulfite; and (○) +500 mM sodium chloride. All experiments are the mean of three duplicate experiments. S.D. < 10% of the mean.

DISCUSSION

The interactions of nitrosoureas and other classes of alkylating agents with nucleophilic sites of biological molecules can result in a wide range of covalent and ionic products. To understand the mechanism of nitrosourea cytotoxicity, it is necessary to determine the full spectrum of drug–cell interactions and to characterize them with respect to their independent contribution to cell death. The nuclear membrane and associated matrix skeleton have, to this time, received only cursory consideration [21] as potentially important targets of nuclear reactant drugs. The present data indicate that approximately 30% of the total nuclear nitrosourea binding occurs on the nuclear matrix. Since the matrix constitutes only 5% of the total nuclear protein, 5% of the RNA, 1–2% of the DNA and trace amounts of phospholipids, such interactions would appear to be preferential.

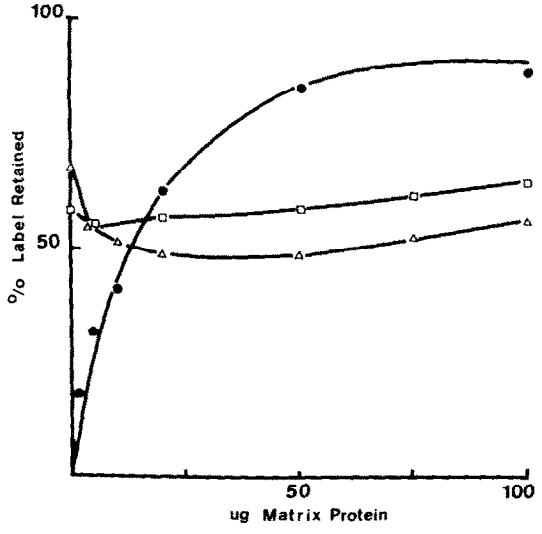

Fig. 6. Filter retention of matrix proteins during DNA reassociation. Log phase HeLa cells were split into two aliquots; and one was pretreated for 24 hr with 25 μCi of [^{14}C]lysine. Nuclear matrices were prepared from both cultures. The non-^{14}C-labeled matrix fraction was treated for 2 hr with [^{14}C-cyclohexyl]CCNU to carbamoylate the proteins (final specific activity 778 dpm/μg protein) prior to reassociation with the DNA. Reassociation conditions were as for Fig. 3. Key: (●) [^{3}H]dThd retention (DNA); (□) [^{14}C]lysine matrix protein retention; and (△) [^{14}C-cyclohexyl]CCNU (carbamoylated protein) retention. Each point is the mean of three duplicate assays. There is no significant difference between the retention of [^{14}C]lysine proteins (□) and [^{14}C-cyclohexyl]CCNU carbamoylated proteins (△) (P = >0.1).

It has been proposed that the matrix plays an important role in replication, transcription and chromatin organization through loop attachments [11, 22]. As a consequence, the extent of drug modification may adversely influence the capacity of the matrix to mediate these nuclear functions. It is likely that most of the nucleophilic macromolecules of the matrix are subject to electrophilic attack by chloroethylnitrosoureas. Some RNA species, such as HnRNA [23] and snRNA, are matrix components and these may have structural functions, since many are metabolically stable and resistant to RNase [24]. It is possible that a proportion of the drug bound to the matrix reacted with these macromolecules prior to their incorporation into the matrix. The importance of these drug modifications to ultimate cytotoxicity will prove to be dependent upon the turnover rate, relative pool sizes, and relative duplication of the RNA species involved. Similarly, the importance of drug modification of the fibrillar non-histone complement of the matrix relates to the same biological parameters.

Evidence that DNA replication occurs at an esti-

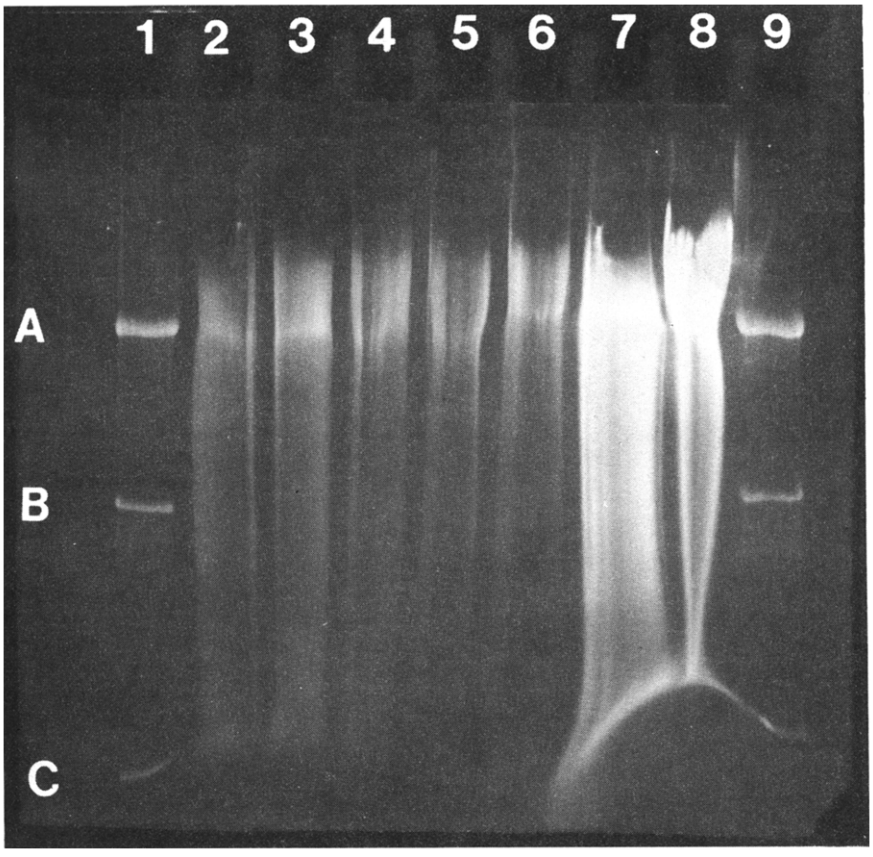

Fig. 7. 1% Agarose gel electrophoresis of DNA filtrate from reassociation assay. The reassociation assay was performed as in Fig. 3, and the filtrates at each incremental protein concentration were collected and lyophilized. Prior to reassociation, 10 μg aliquots of HeLa cell DNA, either before (lane 7) or after (lane 8) spermine precipitation, were saved. The lyophilized filtrates were resuspended in sample buffer and run under the conditions described in Methods. Lane 1: φX174 markers: A = 3730 bp; B = 1264 bp; C = 392 bp. Lane 2: Filtrate from 1 μg DNA reassociated with 2 μg matrix proteins (i.e. ratio 1:2). Lane 3: DNA:protein ratio, 1:5. Lane 4: Ratio 1:10. Lane 5: Ratio 1:25. Lane 6: Ratio 1:50. Lane 7: Whole DNA sample, no spermine precipitation. Lane 8: Whole DNA sample, spermine precipitated. Lane 9 as Lane 1.

mated 10,000 sites upon the matrix [11] suggests that chromatin organization is not random. Since the appearance of chromatin under the electron microscope can be radically altered by treatment with steroids and or nuclear-reactant drugs [4, 14], the nuclear matrix may exert a chromatin-organization function which permits "fluidity", while maintaining some restrictive influence upon chromatin arrangement. It was apparent from the reassociation experiments using alkylated DNA (labeled either by ^{14}C- or ^{3}H-ethylation) that the binding for those regions of DNA which contained alkylated bases was different from the data for the dThd-labeled DNA. This was the case for DNA treated either *in vitro* (CLZ or CCNU) or *in vivo* (chlorambucil and MNU), where reassociation was lower (cf. Fig. 4) than the corresponding [^{3}H]dThd values. A plausible conclusion from these data is that a proportion of the alkylated products was unstable and dissociated from the bulk DNA during the reassociation procedure. It is difficult to understand why such instability would not have resulted in the release of the alkylated products prior to the reassociation assay. Free drug and small alkylated products (which could be produced from adduct instability) were removed prior to the assay by dialysis and spermine precipitation.

Labilization of alkylated sites during the 1-hr reassociation incubation period was investigated by carrying out the procedure at 4°, by shortening the incubation period (data not shown), and by adding a phosphatase inhibitor to prevent possible phosphotriester release into the incubation mixture. None of these procedures altered the reassociation kinetics, indicating that enzymatic labilization of alkylated DNA products did not occur.

The possibility of preferential size retention of DNA was investigated by measuring the size of the DNA in the nitrocellulose filtrate (cf. Fig. 7). The DNA passing through the filter, at a number of protein:DNA concentration ratios, was the same size as that which was applied in the total reaction.

Carbamoylation of the matrix proteins, following nitrosourea treatment of the whole cells (cf. Table 1), occurred at a level equivalent to twenty times the alkylation; however, neither of these drug interactions influenced the reassociation kinetics of the DNA with the matrix. Similarly, drug treatment of

the isolated DNA did not affect the kinetics of the bulk [^{3}H]dThd reassociation with the matrix. The nature of the binding between the DNA and the matrix protein amino acids is unclear; however, a proportion of the drug binding was removed by treatment with 0.5 M sodium chloride, suggesting that it may be non-covalent in nature. This is in agreement with previous findings [25] and suggests that many ionic interactions may be of importance to the organization of DNA loops on the matrix protein structure. Other studies have demonstrated that these DNA–matrix associations are not the result of random adsorption of nucleic acids onto the matrix structures [11, 24]. The fact that a proportion of the alkylated DNA did not participate in matrix–DNA interactions could be a consequence of drug-induced alterations of the DNA bases which act at potential recognition sites for matrix attachment. The importance of these data relates to previous studies which have shown that DNA loop attachment sites on the matrix are not fixed and may be reversible, which would permit a "fluidity" in the nuclear organization of chromatin [22]. Although the binding assays are based on an *in vitro* methodology, it is plausible that such inhibition of DNA–matrix associations can occur *in vivo* with resultant interference with the fidelity of chromatin organization. The consequence of even a single interruption in the fidelity of DNA–matrix attachments could have potentially profound effects upon the integrity of nuclear function.

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