Characterization of DNA–Protein Cross-Links Formed by Treatment of L1210 Cells and Nuclei with Bis(2-chloroethyl)methylamine (Nitrogen Mustard)†

Cornelius B. Thomas,‡ Kurt W. Kohn, and William M. Bonner*

ABSTRACT: Proteins cross-linked to DNA after nitrogen mustard (HN2) treatment of cells or isolated nuclei were purified in CsCl gradients. The protein–DNA cross-links could be cleaved by incubation in dilute acid and could be stabilized by alkali pretreatment. These results indicate that proteins cross-linked to DNA by HN2 are bound to alkylated purines. Analysis of the DNA-bound proteins on $NaDodSO_4$–polyacrylamide gels showed that primarily large nonhistone proteins are cross-linked to DNA in cells treated with HN2. Very little if any histone is cross-linked to the DNA. Comparison of DNA bound proteins from HN2-treated cells and HN2-treated nuclei showed that in general the same proteins are linked to DNA in both cases, but some qualitative and quantitative differences exist.

Bifunctional alkylating agents are known to produce DNA interstrand cross-links in a number of cell systems (reviewed by Ludlum, 1975). In addition several workers have generated evidence that these agents also cause protein–DNA associations that are not broken by various extraction procedures. Rutman et al. (1961) and Steele (1962) noted that treatment of cells with such compounds decreased the extractibility of DNA into the aqueous phase after agitation with phenol. These results were interpreted as evidence that the unextracted DNA was bound to proteins in the phenol layer. Goldner et al. (1964) showed similar results after phenol extraction of HN2-treated Ehrlich–Lettre ascites tumor cells. In addition they found a 5- to 20-fold increase in residual protein over the control when they looked at DNA remaining in the aqueous phase after phenol extraction. Klatt et al. (1969) examined the cesium chloride banding patterns of DNA from Ehrlich–Lettre ascites cells after exposure to HN2[1] and found a significant decrease in the quantity of DNA banding at the normal density in cells from a sensitive tumor line with little effect on the DNA from a resistant tumor line. Trypsin treatment largely abolished this difference. Sokolov et al. (1975) demonstrated that HN2 treatment of chromatin produced an increase in DNA-bound protein as assayed by gel filtration, ultracentrifugation, and hydroxylapatite chromatography. Recent investigations from this laboratory, using the technique of alkaline elution, have revealed proteinase-sensitive DNA cross-links in L1210 cells treated with HN2 (Ewig & Kohn, 1977).

There is no direct evidence on the chemistry of these protein–DNA cross-links or on the kinds of proteins that are bound to the DNA. This has led to speculations based on indirect data. Grunicke et al. (1973) found that treatment of cells with the monofunctional alkylating agent methylmethanesulfonate led to an increase in protein-bound DNA as determined by phenol extractibility. This has led to suggestions that the DNA-protein cross-links of bifunctional agents may not contain bisethylamine bridges (Thomas, 1976). Salser & Balis (1970), studying the binding of amino acids to DNA in cells treated with various agents found an increase in total bound residues and a change in amino acid composition, which was produced by actinomycin D and proflavin as well as by the bifunctional alkylators, nitrogen mustard and mitomycin D. They suggested that these bound amino acids might be the result of cellular response to drug damage.

In this paper we present a partial characterization of isolated DNA–protein complexes formed in HN2 treated cells and nuclei. Evidence is presented that HN2 binds a subset of nuclear proteins to DNA and that the binding is primarily through alkylated purine residues.

Materials and Methods

Cells. Static cultures of L1210 mouse leukemia cells were maintained in Roswell Park Memorial Institute Medium 1630 supplemented with 20% fetal calf serum without antibiotics. At weekly intervals subcultures were initiated, and the cells were propagated in suspension culture in the presence of penicillin and streptomycin. Cell densities were kept between 0.25 and 1.0×10^6 per mL with a doubling time of 12 to 14 h. Cultures were periodically tested for Mycoplasma and found to be uncontaminated.

Labeling. On the day prior to use, cells were centrifuged and placed into leucine-free medium supplemented with 20% fetal calf serum. Proteins were labeled by a 17-h incubation with 0.1 or 1.0 μCi per mL of [U-^{14}C]-L-leucine (New England Nuclear, 275–325 mCi/mmol). In some experiments DNA was labeled with 0.01 μCi per mL of [*methyl*-^{14}C]thymidine (New England Nuclear, 53 mCi/mmol) or [*methyl*-^{3}H]thymidine (New England Nuclear, 20 mCi/mmol). Under these conditions the fetal calf serum provided enough leucine to sustain normal cell proliferation while permitting satisfactory incorporation of radioactivity.

Isolation of Nuclei. Nuclei were isolated at 0–2 °C by a detergent method (Berkowitz et al., 1969). Pellets containing up to 1.5×10^8 cells were washed once with 10 mL of buffer A (1 mM potassium phosphate, 1 mM $CaCl_2$, 0.32 M sucrose, pH 6.4) and centrifuged for 5 min at 170*g*. The cells were resuspended in 1 mL of buffer A and lysed with the addition of 9 mL of buffer A containing 0.3% Triton N-101 (Sigma Chemical Co.). Nuclei were pelleted at 675*g* for 5 min and washed once with the same buffer.

† From the Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received January 3, 1978.*

‡ Current address: Box 3225, Duke University Medical Center, Durham, N.C. 27710.

[1] Abbreviations used: HN2, nitrogen mustard (bis(2-chloroethyl)-methylamine); PCA, perchloric acid.

Alternatively, in some experiments nuclei were isolated by a physiological ionic strength method using the above protocol except that buffer B (1 mM potassium phosphate, 5 mM $MgCl_2$, 150 mM NaCl, pH 6.4) was substituted for buffer A. Both procedures yielded clean nuclei without cytoplasmic tags or other debris as judged by trypan blue staining and phase microscopy.

HN2 Treatment. Nuclei or cells (5×10^7) were suspended in 2 mL of the desired buffer. The appropriate concentration of 2-chloroethyldimethylamine (Eastman) or HN2 (Merck, Sharp and Dohme) was added for a 30-min incubation at 37 °C. The reaction mixtures were then cooled to 4 °C, and unbound compound was removed by washing as described below. Other results from this laboratory indicate that under these conditions, most of the HN2 cross-links form during the 30-min incubation at 37 °C and no further cross-link formation is apparent after this time (Ross et al. 1978). Except for the RNase incubation, all further steps are at 0–4 °C.

Cesium Chloride Banding. Aliquots containing 10^6 [^{3}H]- or [^{14}C]thymidine-labeled cells were washed twice by centrifugation in phosphate buffered saline at 4 °C. The cell pellet was lysed by a 1-h incubation at 37 °C in 2 mL of buffer C (0.1 M $NaHCO_3$, 0.05 M Na_2EDTA, 0.3% Sarcosyl (Ciba-Geigy), pH 10.8). One milliliter of buffer D (1.0 M sodium trichloroacetate, 0.1 M $NaHCO_3$, 0.05 M Na_2EDTA, pH 10) was added for a further 30-min incubation at 37 °C. Equal portions (3.0 g each) of ^{14}C- and ^{3}H-labeled samples were combined and mixed with 6.10 g of CsCl. The solution was then overlayered with mineral oil and centrifuged at 40 000 rpm for 65 h in a Beckman type 65 rotor at 20 °C. Alternate 5-drop fractions were counted for radioactivity. Uncounted fractions at the DNA peak were pooled and dialyzed 16 h against buffer C minus Sarcosyl to remove the CsCl. The solution was divided into 0.2-mL aliquots and added to 3.8 mL of buffer C with or without 2 mg per mL of proteinase K (EM Biochemicals) for a 1-h incubation at 37 °C. Two milliliters of buffer D and 6.10 g of CsCl were added. Equilibrium centrifugation in CsCl was then repeated.

Isolation of DNA–Protein Complexes. Nuclear pellets were resuspended in 0.3–0.4 mL of buffer A or B containing 0.3% Triton N-101 and dialyzed for 2 h at 4 °C against 200 volumes of 0.02 M EDTA, 0.05 M Tris-HCl, pH 7. Where indicated the dialyzed sample was incubated at 37 °C for 10 min in the presence of 0.2 mg per mL of RNase A (Worthington). The samples were sonicated for 5 s at setting 1 of a Branson S125 sonifier equipped with a microtip. Debris was removed with a 5-min centrifugation at 170*g*, and the supernatant was adjusted to 0.3% Sarcosyl. The samples in a volume of 0.42 mL were layered over 3.0-mL cushions of 3.0 M CsCl solution (36.7% CsCl by weight, 0.02 M EDTA, 0.05 M Tris-HCl, 0.3% Sarcosyl, pH 7) in polyallomer tubes and centrifuged at 40 000 rpm for 23 h at 4 °C in a Beckman SW-56 rotor. The bottom 0.7 mL (density range 1.43 to 1.51 g/cm^3) was removed with a Beckman tube slicer. Samples were counted for radioactivity or dialyzed 16 h against H_2O for further studies.

For resedimentation experiments samples in a volume of 0.42 mL were centrifuged through 3.0 mL of the above 3.0 M CsCl solution under the same conditions. The top 2.5 mL and the bottom 0.92 mL were separated with a Beckman tube slicer and assessed for radioactivity. In experiments involving hot PCA treatment, samples were neutralized with Tris base prior to resedimentation.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Samples from the bottom of CsCl gradients were dialyzed 16 h against H_2O, buried in powdered sucrose to concentrate them, and redialyzed for 4 h to remove sucrose. For protein release from DNA, the samples were adjusted to contain 0.3% Sarcosyl, 50 mM sodium bisulfite, 30 mM sodium citrate (pH 4.3) and incubated at 48 °C for 6 h. Over 80% of the leucine radioactivity was released by this method. The samples were then adjusted to contain 2% $NaDodSO_4$, 30 mM Tris-HCl (pH 6.8), 10% glycerol, and 5% mercaptoethanol. Sample aliquots containing 6000 cpm were electrophoresed on $NaDodSO_4$–polyacrylamide slab gels 1.5 mm thick, 13 cm wide, 25 cm long containing 15% acrylamide, 0.09% bis(acrylamide) in the resolving gel. Other parameters were as previously described (Laemmli, 1970). After electrophoresis for 16 h at 20 mA, the gels were stained with 0.1% Coomassie Blue R-250 in 40% EtOH, 10% HOAc, then destained in 20% EtOH, 10% HOAc. Gels were then prepared for fluorography (Bonner & Laskey, 1974) and exposed to flashed Eastman Kodak Royal X-Omat R film (Laskey & Mills, 1975). Densities of film images were traced on a Joyce-Lobel densitometer.

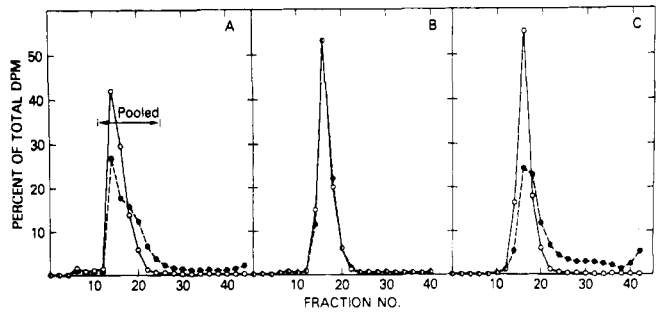

FIGURE 1: Cesium chloride banding of DNA from nitrogen mustard treated cells. (A) One million [^{3}H]thymidine-labeled cells (●—●) were treated in 1 mL of medium with 0.5 mM HN2 at 37 °C for 15 min, processed as described in Materials and Methods, and centrifuged in CsCl. One million untreated [^{14}C]thymidine-labeled cells (○—○) were processed similarly and mixed with the treated cells just prior to banding. Fractions were collected from the bottom. Those containing the DNA peak were pooled and dialyzed, and aliquots were rebanded after incubation with (B) or without (C) proteinase K.

Results

The formation of protease-sensitive DNA complexes by HN2 treatment was verified in initial isopycnic banding studies (Figure 1). DNA from cells treated with HN2 banded anomalously in cesium chloride gradients, with a large fraction of the DNA equilibrating at densities lower than that of reference DNA from untreated cells (Figure 1A). When the DNA was removed from the gradients, pooled, incubated with proteinase K, and recentrifuged under identical conditions, the HN2-treated DNA now banded identically with the control (Figure 1B). Without prior incubation with protease, another aliquot of HN2-treated DNA again banded atypically (Figure 1C). These results suggested that HN2 treatment resulted in the formation of DNA–protein complexes resistant to separation by exposure to high ionic strength and detergent.

For characterization of these DNA–protein complexes, a dissolution procedure was developed based on centrifugation of lysed nuclei through 3.0 M CsCl (see Materials and Methods). At this molarity of CsCl, DNA sediments while protein bands in the upper third of the gradient. In the absence of HN2 treatment, the pellet (bottom 0.7 mL of the gradient) contained 91% of the [^{3}H]thymidine-labeled material and 0.1% of the [^{14}C]leucine-labeled material. Thirty-three percent of the [^{14}C]leucine-labeled material was soluble in hot PCA, indicating that part of the ^{14}C radioactivity present in the pellet was not protein but nucleic acid.

When HN2-treated nuclei were centrifuged through 3.0 M CsCl (density 1.38 g/cm^3), [^{14}C]leucine-labeled material

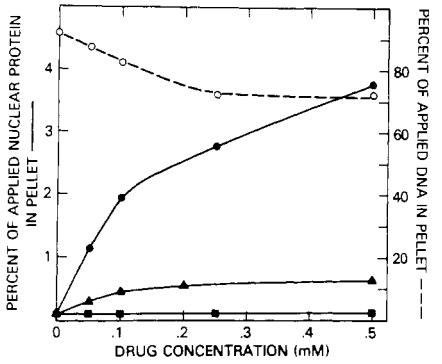

FIGURE 2: Effect of alkylation on sedimentation of DNA and protein through CsCl. [^{14}C]Leucine-labeled cells, 5×10^7 (▲—▲), were reacted with HN2 in 2 mL of Hanks balanced salt solution for 30 min at 37 °C. Treatment was terminated by addition of 8 mL of ice cold buffer. Cells were pelleted and resuspended for nuclear isolation. Nuclei were isolated, RNase treated, lysed, and centrifuged through 3.0 M CsCl as described in Materials and Methods. Radioactivity which sedimented to the tube bottom was expressed as percent of total applied nuclear dpm. [^{14}C]-Leucine-labeled nuclei (5×10^7) prepared as described in Materials and Methods were reacted with HN2 (●—●) or 2-chloroethylmethylamine (■—■) in 2 mL of 1 mM potassium phosphate (pH 6.8), 1 mM $CaCl_2$, 0.32 M sucrose. Treatment was terminated by addition of 8 mL of ice cold buffer. Treated nuclei were manipulated as were nuclei prepared from treated cells except that they were not RNase digested. DNA recovery was measured from [^{3}H]thymidine-labeled nuclei which were treated with HN2 (○ - - - ○) in parallel with the [^{14}C]leucine-labeled nuclear sample.

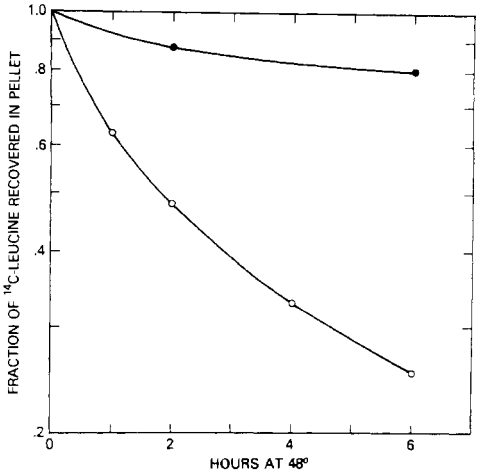

FIGURE 3: Release of protein from the sedimenting complex by dilute acid. Isolated nuclei from [^{14}C]leucine-labeled cells were treated at low ionic strength with 50 μM HN2 for 30 min at 37 °C and the protein sedimenting through 3.0 M CsCl was isolated as described in Materials and Methods. After dialysis against H_2O for 16 h, aliquots containing 2200–2600 dpm were adjusted to contain 0.3% Sarcosyl, 50 mM sodium bisulfite, and 30 mM sodium citrate, pH 4.3. After the appropriate incubation times at 48 °C the aliquots were neutralized with Tris base and centrifuged through 3.0 M CsCl as described in Materials and Methods. Radioactivity sedimenting to the tube bottom was expressed as a fraction of total recovered dpm (○ - - - ○). Numbers are corrected for the 31% of dpm released by the unincubated control in this experiment. Alkali treatment (● - - - ●) was achieved by incubation of samples with 0.05 M NaOH at 30 °C for 1 h followed by neutralization with HCl. Release of proteins at pH 4.3 from the alkali pretreated material was performed as above.

recovered in the tube bottom increased with increasing HN2 concentration (Figure 2). Similar treatment of nuclei with 2-chloroethyldimethylamine did not lead to any detectable binding of protein to DNA. Losses of DNA in this procedure were found to be about 10% over the untreated control at 0.1 mM HN2 treatment and reached 20% at 0.5 mM HN2. Increasing DNA losses can be expected as DNA–protein cross-linking increases because the complexes would become less dense as more protein is bound. In further studies, HN2 concentrations of 0.1 mM or less were used to minimize DNA losses.

When whole cells rather than isolated nuclei were treated with HN2, and the nuclei isolated after treatment, considerably less protein pelleted with the DNA. In addition, the amount of protein pelleted from nuclei of HN2-treated cells plateaued at HN2 concentrations above 0.1 mM, while the amount of protein pelleted when isolated nuclei were treated, continued to rise with HN2 concentration. Since cells were treated in Hanks balanced salt solution to prevent lysis, whereas nuclei were treated in 1 mM potassium phosphate, the effect of ionic strength on HN2 treatment was tested as a possible explanation for this difference. Nuclei, isolated and treated with 0.1 mM HN2 in an isotonic buffer (buffer B in Materials and Methods), had 0.47% of the nuclear protein in the CsCl pellet compared with 0.55% for cells treated in Hanks and 2.1% for nuclei treated in low salt (buffer A). This result indicates that the ionic environment may be a more important variable in HN2 treatment than whether or not the nuclei are in cells.

Further studies were carried out to document the physical association of DNA and protein in material isolated by pelleting through 3.0 M CsCl. Cells were labeled with [^{14}C]leucine, and the nuclei were treated with 50 μM HN2 for 30 min at 37 °C in a low ionic strength buffer (buffer A). Upon centrifugation through CsCl without prior RNase treatment, 1.0% of the applied nuclear ^{14}C was recovered in the pellet. Of this radioactivity 93.9% was precipitable after treatment with hot perchloric acid (PCA) and was presumably in protein. To test whether this protein pelleted because it was linked to nucleic acid, the DNA and RNA in aliquots of the isolated material were destroyed by various treatments and the residual protein was centrifuged through CsCl a second time. DNase I or hot PCA (0.5 M PCA, 70 °C, 20 min) treatment released respectively 91 and 94% of the protein from the sedimenting fraction as compared with 16% released in the control without enzyme or hot acid. A combination of DNase and RNase A released 99% of the protein on resedimentation. These results show that over 90% of the pelleted protein was bound to DNA. To remove any proteins which may be bound to RNA, RNase treatment was included in the isolation procedure in further experiments (see Materials and Methods).

Although alkylating agents may react with DNA at many sites (Singer, 1975), Kohn & Spears (1967a) showed that most of the DNA-bound [^{14}C]HN2 residues can be released by heating at low pH while the DNA is not degraded. Pretreatment of the [^{14}C]HN2–DNA with alkali largely prevented the release of [^{14}C]HN2 by acid. These two properties, which are characteristic reactions of alkylated purines in DNA, indicate that the major reaction of HN2 with DNA is to form alkylated purines. To test whether the sedimenting protein was covalently attached to DNA through a nitrogen mustard bridge to purine residues, DNA–protein complexes were isolated from HN2-treated nuclei and heated in dilute acid under conditions shown to release [^{14}C]HN2 residues from DNA (Kohn & Spears, 1967a). After heating at 48 °C at pH 4.3 for various times, the extent of release of DNA-bound protein was determined by recentrifugation through CsCl. The kinetics of protein release (Figure 3) were similar to the kinetics of release of alkylated purines from HN2-treated DNA reported by Kohn & Spears (1967a). After 6 h at 48 °C, 75% of the labeled protein was released from the DNA, while under comparable conditions Kohn & Spears (1967a) reported that 83% of the bound HN2 was released.

Another possible explanation for this data is that the apparent release of protein is due more to the fragmentation of DNA which then failed to sediment rather than to the actual release of protein from the DNA. This possibility was tested by repeating the experiment with [^{14}C]thymidine-labeled cells. When the DNA was labeled, 99.7% of the DNA applied to the gradient after incubation at pH 4.3 was recovered from the pellet. This result shows that incubation at pH 4.3 leads to the release of protein from DNA but does not prevent the sedimentation of the DNA.

Another characteristic of HN2-DNA adducts is that alkali pretreatment largely prevents acid release of alkylated purines (Kohn & Spears, 1967b). Figure 3 shows that alkali pretreatment also greatly decreases the release of protein at pH 4.3.

The released proteins were analyzed by NaDodSO$_4$ gel electrophoresis (Figure 4). Two main conclusions are apparent. The first is that qualitatively the proteins cross-linked to DNA are similar whether nuclei in low or physiological salt or in cells are treated. The second is that few if any histones are cross-linked to DNA by HN2.

The qualitative similarity among the protein patterns of the different samples is apparent at all protein sizes. Except for a protein smaller than H4 and one at 28 000 daltons, there is a peak correspondence between treated cells and nuclei treated in physiological salt. Quantitatively however, proteins in the 125 000 to 200 000 daltons range are relatively more prominent in treated cells than in nuclei treated in physiological salt. In both treated cells and nuclei treated in physiological salt, little if any histone is cross-linked to DNA. The small peak that migrates between H2a and H2b could be H2a carrying a mustard guanine moiety from depurination. It is clear, however, that no measurable amount of H4 and H3 is seen.

Nuclei treated in low salt generate a pattern similar to nuclei treated in physiological salt except that proteins at 70 000 and 50 000 daltons are poorly represented or absent, and that more material is found in the histone region. Two broad peaks migrating approximately with H4 and H3 are seen in low salt nuclei but not in nuclei treated in physiological salt or cells. The larger proteins found cross-linked to DNA comigrate with proteins from control nuclei while the smaller proteins migrate slightly slower than corresponding proteins from control nuclei. Proteins released from DNA at pH 4.3 should be carrying a mustard purine moiety which would add about 230 to the molecular weight of the protein. This additional mass would be more significant to the mobility of the smaller proteins than to the larger and therefore might explain the visible migration differences observed for the small proteins but not for the larger.

One question of interest is whether any of the proteins that pellet through 3.0 M CsCl do so without HN2 treatment. In other words, are any of the proteins in the cross-linked fractions not really cross-linked by HN2 but just sediment with DNA through CsCl. To test this point, we analyzed the control by NaDodSO$_4$ gel electrophoresis. When an aliquot corresponding to the same amount of DNA as was taken for the cross-linked samples was analyzed, no bands were seen (data not presented). This result means that the presence of the proteins in the cross-linked fraction is dependent on HN2. When the control was concentrated so that equal amounts of radioactivity were loaded onto the gel, several bands were seen (Figure 4, bottom). As mentioned earlier one-third of this fraction is hot PCA soluble and therefore may be nucleic acid which in turn may account for the radioactivity at the top of the gel. No distinct protein bands are seen above 40 000 daltons. Three of the visible bands migrate faster than H4, one just ahead of H2a, one at 28 000 daltons and one at 38 000 daltons. With the possible exceptions of the protein at 38 000 daltons and the smallest protein, none of these are visible in the cross-linked fractions. Thus it is clear that HN2 cross-links a subset of proteins to DNA that is different from the subset that tends to sediment with untreated DNA.

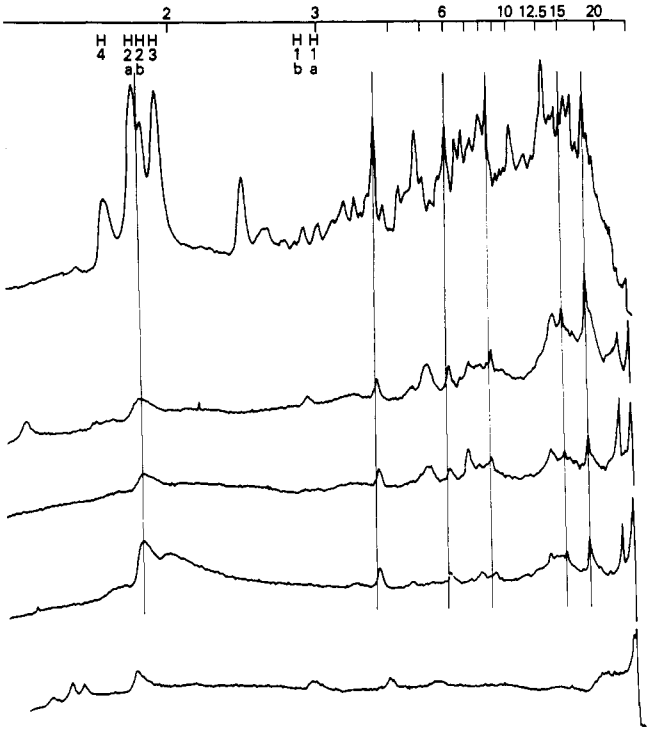

FIGURE 4: NaDodSO$_4$-polyacrylamide gel analysis of total ^{14}C-labeled nuclear proteins (top trace), and ^{14}C-labeled proteins released from DNA from HN2-treated cells (second trace), physiological salt nuclei (third trace), low salt nuclei (fourth trace), and ^{14}C-labeled proteins which pellet through 3.0 M CsCl without HN2 treatment (bottom trace). See Materials and Methods. ^{14}C (6000 cpm) was loaded onto each slot. The molecular weight scale at the top, in units of 10^4, is based on nonhistone standard proteins. Consequently, the scale does not reflect the true molecular weight of the histones, since these migrate more slowly than nonhistone proteins of similar molecular weight.

Discussion

We find that treatment of cells or nuclei with nitrogen mustard increases the extent of protein association with the nuclear DNA in sedimentation through 3 M CsCl. The protein was released from the sedimented pellet by degradation of the DNA either by DNase or by hot perchloric acid, indicating that the protein sedimented because it was attached to DNA.

The linking of protein to DNA appeared to require bifunctionality of the nitrogen mustard, since the monofunctional analogue, 2-chloroethylamine, did not have this effect. This finding argues against protein linking via aldehyde groups generated by depurination of alkylated DNA.

Further evidence indicated that the protein is linked through a nitrogen mustard bridge to guanine N-7, the predominant DNA alkylation site. Protein was found to be released from the DNA by mild acid at a rate similar to the rate of elimination of 7-alkylguanines reported by Brookes & Lawley (1961) and Kohn & Spears (1967a). In addition to the guanine N-7 position, however, nitrogen mustard alkylated to a smaller extent at adenine N-3, and the resulting alkylated base also is susceptible to elimination in mild acid (Brookes & Lawley, 1961). The alkylguanine moiety has been shown to be susceptible to alkali, which cleaves the imidazole ring and prevents

elimination of the alkylated residue by subsequent acid treatment (Brookes & Lawley, 1961; Kohn & Spears, 1967b). The finding that alkali pretreatment likewise prevented the release of protein by mild acid is consistent with guanine N-7 being a major site of linkage.

Nitrogen mustard provides a 7.5-Å link between two cross-linked moieties. If one end is linked to N-7 of guanine in the DNA major groove, studies with models show that the other end can reach the sugar phosphate backbone of the same strand but not the sugar phosphate backbone of the complementary strand. This indicates that the proteins cross-linked to DNA in these experiments are closely associated with certain regions of DNA.

When the proteins isolated from DNA–protein complexes were analyzed by $NaDodSO_4$ gel electrophoresis, the band pattern was similar whether nuclei were treated in physiological or low salt or whether cells were treated. This suggests that protein DNA stereochemistry in chromatin is similar in these conditions. However, certain differences were seen both in the relative amount of binding of different proteins and the lack of binding of certain proteins under different conditions. In these regards, nuclei treated in physiological salt were more similar to treated cells (also in physiological salt) than were nuclei treated in low salt. The total extent of reaction was similar in treated cells and in treated nuclei in physiological salt, while low salt nuclei bound 4–6 times as much protein under similar conditions.

One question of interest concerns the relative amounts of HN2 mediated protein–DNA cross-links and the more commonly studied DNA cross-links. Although the present study generated no findings on this point, other work from this laboratory (Ewig & Kohn, 1978) indicates that, with HN2, there are several protein–DNA cross-links for each DNA–DNA interstrand cross-link. Therefore protein–DNA cross-linking is an important parameter of HN2 action.

Another similar question relates to the relative rates of protein–protein cross-linking and protein–DNA cross-linking. If the former was very much faster cross-linked protein complexes should be seen on the gel; if the latter was very much faster then single polypeptide chains should predominate. The present data do not answer this question very satisfactorily because nuclei contain so many proteins that it is impossible to be certain whether a protein band from the complex that comigrates with a nuclear protein is in fact that protein or a HN2-linked protein polymer that happens to have the same R_f. Certainly fingerprinting of bands or high-resolution two-dimensional gel electrophoresis would go further to answer this question. However, inspection of the gel patterns suggests that most of protein bands from protein–DNA complexes are not cross-linked protein polymers and that protein–protein cross-linking is a slower process under these conditions than protein–DNA cross-linking.

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References

Berkowitz, D. M., Kakefuda, T., & Sporn, M. B. (1969) *J. Cell Biol. 42,* 851.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46,* 83.

Brookes, P., & Lawley, P. D. (1961) *Biochem. J. 80,* 496.

Ewig, R. A. G., & Kohn, K. W. (1977) *Cancer Res. 37,* 2114.

Ewig, R. A. G., & Kohn, K. W. (1978) *Cancer Res.* (in press).

Goldner, R. H., Martin-Guzman, G., Jones, J., Goldstein, N. O., Rotenberg, S., & Rutman, R. J. (1964) *Cancer Res. 24,* 964.

Grunicke, H., Bock, K. W., Becher, H., Gäng, V., Schnierda, J., & Puschendorf, B. (1973) *Cancer Res. 33,* 1048.

Klatt, O., Stehlein, J. S., McBride, C., & Griffin, A. C. (1969) *Cancer Res. 29,* 286.

Kohn, K. W., & Spears, C. L. (1967a) *Biochim. Biophys. Acta 145,* 720.

Kohn, K. W., & Spears, C. L. (1967b) *Biochim. Biophys. Acta 145,* 734.

Laemmli, U. K. (1970) *Nature (London) 227,* 680.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56,* 335.

Ludlum, D. B. (1975) *Handb. Exp. Pharmacol. 38,* 6.

Ross, W. E., Ewig, R. A. G. & Kohn, K. W. (1978) *Cancer Res.* (in press).

Rutman, R. J., Steele, W. J., & Price, C. C. (1961) *Cancer Res. 21,* 1134.

Salser, J. S., & Balis, M. E. (1970) *Biochem. Pharmacol. 19,* 2375.

Singer, B. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15,* 219.

Sokolov, N. A., Piker, E. G., Zakaryan, Blinova, G. G., & Tseitlin, P. I. (1975) *Biull. Eksp. Biol. Med. 79,* 42.

Steele, W. J. (1962) *Proc. Am. Assoc. Cancer Res. 3,* 364.

Thomas, J. O. (1976) in *Aging, Carcinogenesis, and Radiation Biology* (Smith, K. C., Ed.) p 193, Plenum Press, New York, N.Y.