

## *Basic Science In Medicine*

### THE EFFECT OF MECHLOROETHAMIN ON RECONSTITUTED CHROMATIN

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#### ABSTRACT

In this study the effect of a methylated derivative of nitrogen mustard (mechchloroethamin) on solubilized and reconstituted chromatin was investigated using UV/Vis spectroscopy, DNA and SDS gel electrophoresis and densitometric scanning techniques.

The results indicated that the 210 and 260 nm absorbances of reconstituted chromatin were decreased in a dose-dependent manner after treatment with various concentrations of mechchloroethamin. Alkylated chromatin showed difficulty in producing nucleosomes in comparison to the control, therefore a 50% reduction in absorbances was obtained. The amount of both core histone protein and DNA in the supernatant was decreased with increasing drug concentrations.

It is suggested that mechchloroethamin alters the interaction of DNA protein in the chromatin by producing cross-links between DNA and protein or protein and protein.

**Keywords:** Mechchloroethamin, chromatin, reconstituted chromatin, core histone.

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#### INTRODUCTION

Alkylating agents are the oldest and, as a group, the most heterogeneous class of antineoplastic agents in clinical use.<sup>1</sup> Mustard compounds form a large group of alkylating agents and mechchloroethamin [bis-(2-chloroethyl) methylamin] is the simplest member of the nitrogen mustard family and the first clinically used antitumor substance.<sup>2</sup> Nitrogen mustard (HN<sub>2</sub>) reacts with DNA to form alkyl derivatives of the bases. Because of its two reactive chloroethyl groups, it can act as a bridge to cross-link two bases located on different strands of DNA.<sup>3</sup> Another suggestion is that alkylating

agents modify the association between DNA and protein.<sup>4</sup> A change in the association of nuclear proteins with alkylated DNA has also been reported.<sup>5</sup> In other words, cross-linking of DNA and protein by bifunctional alkylating agents is responsible for the increased binding of DNA to proteins.<sup>6</sup> At the molecular level, chromatin is composed of nucleosomes, each of which has two molecules of four types of histones—H2A, H2B, H3 and H4—surrounded by a 1-2 turn of approximately 145 base pairs of DNA.<sup>7</sup> These repeating units have a complex superhelical structure that is thought to be mainly stabilized by histone H1 interaction.<sup>8</sup>

Since chromatin is considered as an important level of organization of cellular DNA, it is necessary to understand more about the interaction of these drugs with chromatin.

In this study the effects of bis (2-chloroethyl)

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methylamine on solubilized and reconstituted chromatin was investigated to elucidate the mechanism of antitumor drug action at the cellular chromatin level.

### MATERIAL AND METHODS

Mechloroethamin was obtained from Delagrang, through Red Crescent Society of Islamic Republic of Iran. Calf thymus was obtained from Ziaran slaughterhouse and transferred to the laboratory in ice. After removing the membranes, tissues were immediately frozen in liquid nitrogen before use.

DNA was prepared from calf thymus according to the procedure of Kay et al.<sup>9</sup> DNA in 1 mg/mL concentration was dissolved in 10 mM tris-HCl buffer, pH 7.4, containing 1 mM EDTA (tris-EDTA), and the absorbance was measured at 260 nm.

#### Purification of histones

Calf thymus histones were prepared according to the method of Johns.<sup>10</sup> Histone H1 was removed from the core histones as follows; total protein was dissolved in cold 0.01 N HCl (5 mg/mL) and centrifuged for 20 min at 3500 rpm. 60% perchloric acid (PCA) was added to the supernatant to produce a 5% solution of PCA and stirred for 20 min at 4°C and centrifuged as above. The pellet (core histone) was washed once with acetone-0.1 N HCl (6:1), three times with acetone and dried under vacuum. The purity of the core histone was assayed by SDS polyacrylamide gel electrophoresis. The protein was dissolved in tris-EDTA buffer in a 1 mg/mL concentration (fresh protein solution was made in each experiment) and in each experiment the pH was adjusted to 7.4.

#### Reconstitution of chromatin

Reconstitution experiments were carried out at a histone sonicated DNA ratio of 1:2.5 (w/w).<sup>13</sup> DNA was sonicated for 20 times, each time with 30 second periods of sonication and 30 second intervals. Histone and sonicated DNA were mixed in 2M NaCl, 10 mM tris HCl, and 1 mM EDTA (pH 7.4) in the presence of polyglutamic acid (polyglutamic acid: DNA ratio was 1:10), followed by sequential dialysis against decreasing concentrations of NaCl (1.6/1.4/1.0/0.8/0.6/0.3 and 0.15 M) and finally against 0.05 M NaCl in tris-EDTA buffer. After centrifugation for 5 min at 10000 g and 4°C, the reconstituted nucleoprotein was loaded onto a linear sucrose gradient of 5-25% prepared in 10 mM tris-HCl and 1 mM EDTA (pH 7.4) and run for 6 hours at 55000 g (sw rotor 41). Two milliliter fractions were collected, and two peaks corresponding to the reconstituted chromatin and free DNA were obtained which were cut according to their optical density profile, pooled and dialyzed overnight against 500 mL of tris-EDTA buffer and then used on day (freshly).

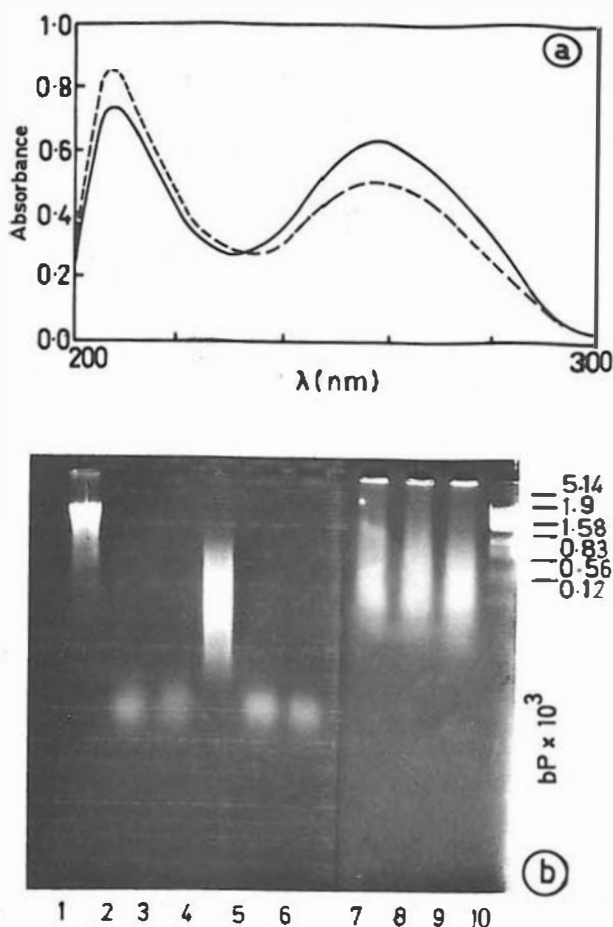


Fig. 1-a, Comparative UV absorption spectra of reconstituted chromatin (---) and calf thymus DNA (—). b. DNase-I digestion of DNA, sonicated DNA and reconstituted chromatin. (1): native DNA. (2,3): 5 and 15 min digested DNA, respectively. (4): undigested sonicated DNA. (5,6): 5 and 15 min digested DNA, respectively. (7): undigested chromatin. (8,9): 5 and 15 min digested chromatin, respectively. (10): standard molecular weight.

#### DNase I digestion

Reconstituted chromatin, sonicated DNA and free DNA were digested in RSB buffer (10 mM tris HCl, pH 7.2, containing 15 mM NaCl, 3 mM MgCl<sub>2</sub>, 60 mM KCl, and 0.25 mM sucrose) at a DNA concentration of 1 mg/mL with 20 µg/mL DNase I (Sigma) for 5 and 15 minutes at 37°C. The reaction was terminated by chilling the samples on ice. Digested samples were analyzed by agarose gel electrophoresis as described below.<sup>14</sup>

#### Interaction of reconstituted chromatin with drug

Free DNA and reconstituted chromatin were divided into several equal portions each containing 50 µg/mL of DNA, and then mechloroethamin was added at 0, 20, 50, 100 and 200 µg/mL concentrations. The samples were incubated for 45 min at 37°C and the spectra of the samples

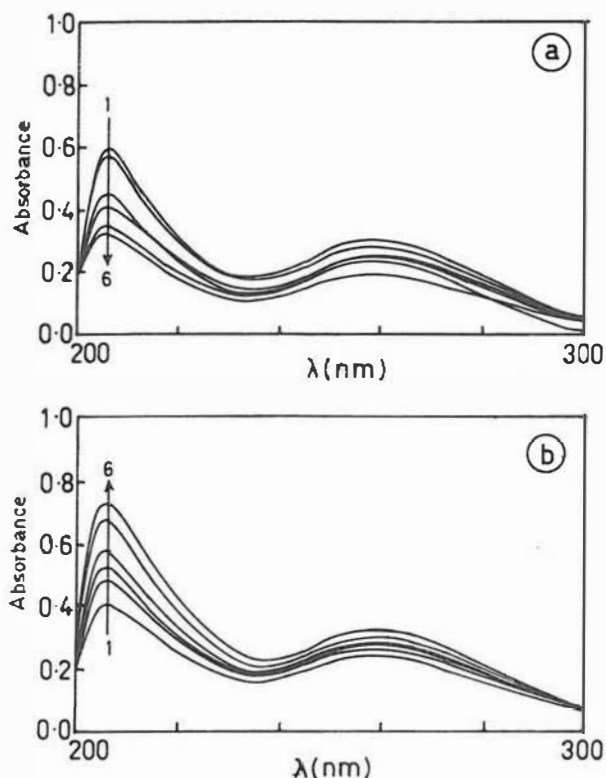


Fig. 2. UV absorption (a) and difference spectra (b) of drug treated samples in 200-300 nm. (1): control. (2-6) are 10, 20, 40, 80 and 100  $\mu\text{g/mL}$  mechloroethamin, respectively.

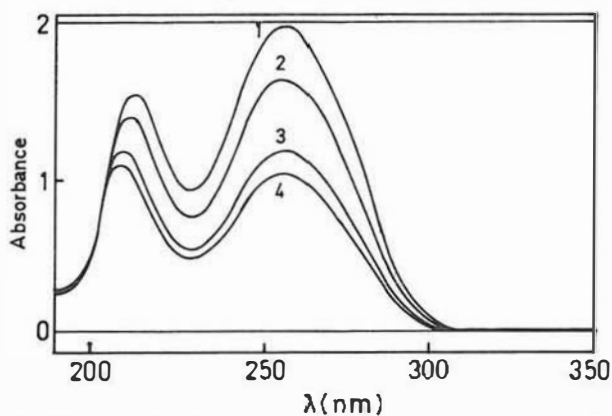


Fig. 3. UV spectra of reconstituted chromatin. (1): control. (2-4): 20, 50 and 100  $\mu\text{g/mL}$  - treated samples.

were drawn at 190-350 nm using a UV260 Shimadzu spectrophotometer.

#### Preparation of chromatin in the presence of drug

Reconstituted chromatin was prepared in the presence of mechloroethamin according to the method mentioned above. Firstly, DNA was interacted with various concentrations of

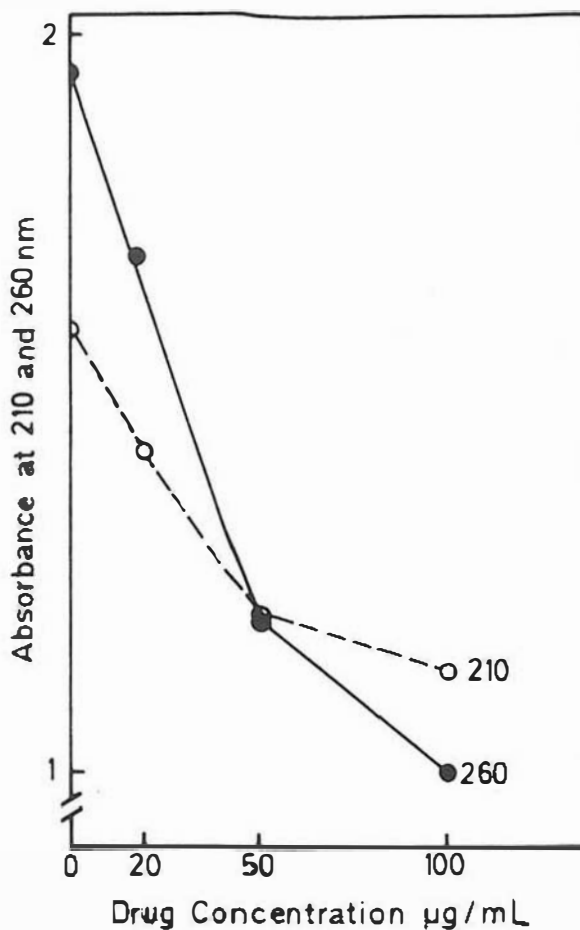


Fig. 4. Change in the absorbance of chromatin solutions in the presence of various concentrations of mechloroethamin.

the drug and then core histone protein was added; secondly, various concentrations of drug were added to the DNA and histone mixture. All the samples were centrifuged to remove aggregated material (10 min, 10000 g) and the spectra of the supernatants were drawn at 190-350 nm.

The supernatants were produced in 12% trichloroacetic acid (TCA) by respect to 70% TCA and the precipitates were then collected by centrifugation at 6000 g for 5 min, dissolved in SDS sample solvent and loaded onto the gel.

#### Gel electrophoresis

SDS polyacrylamide gel electrophoresis was essentially carried out as described by Lammeli.<sup>11</sup> Coomassie Brilliant Blue stained gels were scanned by a Backman model R-112 densitometric gel scanner.

The procedure of Fangman<sup>12</sup> was employed for agarose gel electrophoresis. Agarose (2%) was dissolved in 0.01M phosphate buffer (pH=7) by heating the solution in a boiling water bath. After cooling to 50°C, the solution was poured on to the plate. Then the samples were loaded and the gel was run for 1 hr at 80-100 V. After electrophoresis the gel

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was stained with 50  $\mu\text{g/mL}$  ethidium bromide, destained with distilled water and photographed.

### RESULTS

UV absorption spectroscopy and the DNase I digestion procedure were used to analyze the reconstituted chromatin to confirm the purity of the working material. Fig. 1 illustrates the comparative spectrum between reconstituted chromatin and calf thymus DNA and their DNase I digested patterns. It was shown that although native and sonicated DNA were particularly sensitive to digestion by DNase I, the reconstituted chromatin was resistant. Also, both samples showed nearly the same spectrum pattern, with a slight absorbance reduction at 260 nm for calf thymus DNA (Fig. 1-a). After incubation of the reconstituted chromatin with different concentrations of mechloroethamin (37°C) the UV difference spectra of the samples were drawn between 200-300 nm.

The profiles are presented in Fig. 2. Fig. 2-a indicates that when the spectra were drawn against tris-EDTA buffer, as the drug concentration increased, the absorbance at both 260 and 210 nm decreased. The difference spectra of the samples which were drawn against the same concentration of drug at each point are presented in Fig. 2-a. The peaks showed hyperchromicity as the concentration of drug was increased. In the second step, reconstituted chromatin was prepared in the presence of various concentrations of mechloroethamin. DNA was first incubated with different amounts of drug, and protein and polyglutamic acid were added to the mixture, followed by sequential dialysis against decreasing concentrations of salts as described in the *Methods* section. After addition of the components, precipitation occurred in the drug-treated samples. Analysis of the supernatants by measuring the absorbances at 260 and 210 nm and changes of their spectra (Fig. 3) indicated that alkylated chromatin had encountered difficulties in producing nucleosomes in comparison to the control (untreated DNA). As shown, the amount of soluble chromatin diminished with increasing drug concentrations so that the absorbances at 210 and 260 nm were lower than the control, therefore nearly a 50% reduction in the absorbances were obtained (Fig. 4). The SDS polyacrylamide gel electrophoresis of the supernatants is given in Fig. 5-a. Fig. 5-b shows the scan of the gel. The amount of all proteins decreased with increasing drug concentrations. The amount of DNA in treated and control supernatants was determined on agarose gel (Fig. 6). As shown, although the control produced a band at the free DNA position, mechloroethamin treated samples did not show any band.

### DISCUSSION

In order to understand the pharmacology of DNA binding

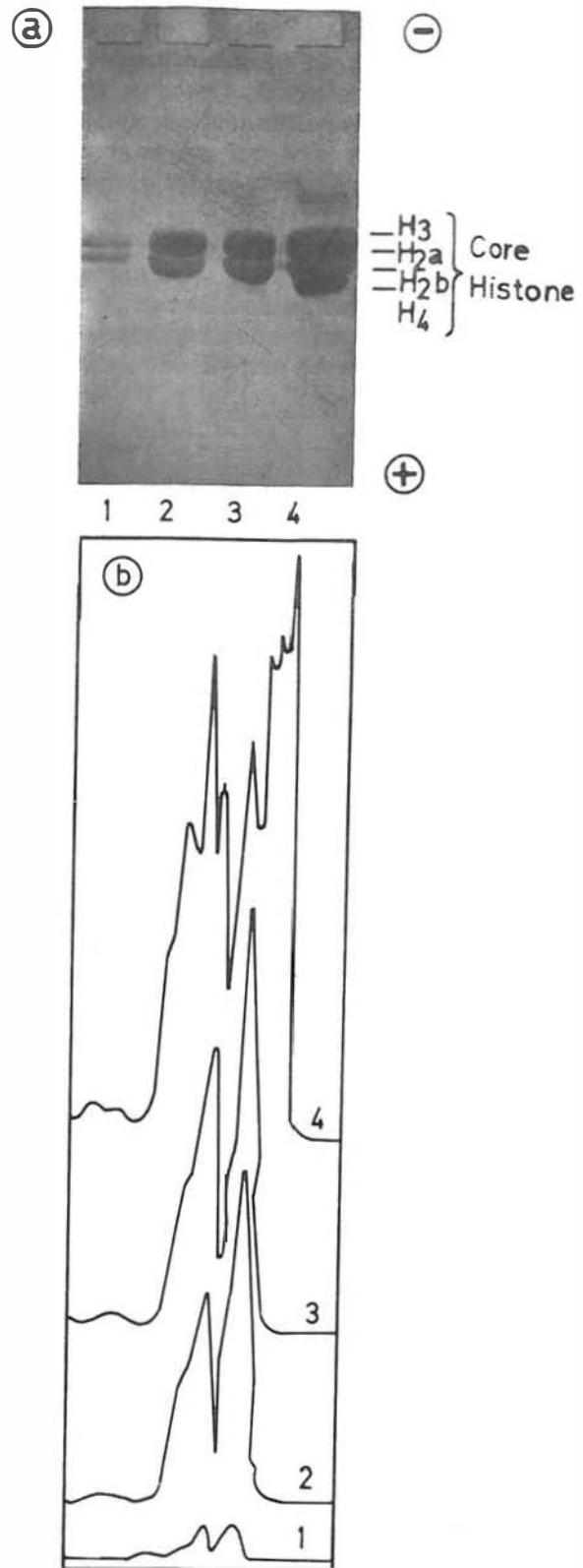


Fig. 5. SDS polyacrylamide gel (a) and scan (b) of the same gel. (1-3): 100, 50, and 20  $\mu\text{g/mL}$  drug treated samples, (4): control.

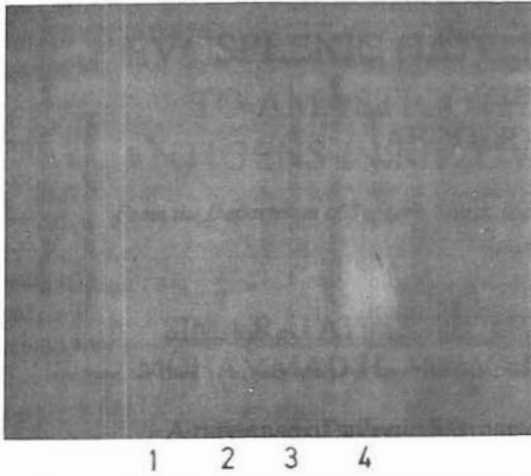


Fig. 6. Agarose gel electrophoresis of DNA in alkylated chromatin. (1-3): 100, 50 and 20 µg/mL drug treated samples, (4): control.

protein to DNA during the reaction seems to be necessary for protein-DNA cross-linking. Mechloroethamin is a potential reagent for mapping the proximity relations between DNA and protein in the nucleohistone complex. The altered binding of chromatin proteins has no effect on the chemotherapeutic action of nitrogen mustard.

The SDS polyacrylamide gel electrophoresis results show that the amount of protein and DNA that cross-link and precipitate out increase by increasing drug concentrations, indicating that reconstitution has been suppressed. Since DNA is regarded as a critical target for alkylating antitumor agents and it is extensively covered by proteins, the effect of drugs on the binding of DNA to proteins is often considered as part of their therapeutic mechanism. Our study raises questions about the mechanism of drug action, for example the alkylation of core histones individually and the interaction of alkylated histones with DNA will be the subject of future studies.

drugs in higher organisms, it is necessary to understand the interactions of these drugs with nuclear chromatin. In most cells, chromatin is a dynamic substance undergoing various alterations of structure in different conditions, such as reversible folding and infolding of the chromatin fiber. In this study, difference spectra obtained from the reconstituted chromatin treated with increasing concentrations of mechloroethamin showed hyperchromicity at 260 and 210 nm. It is therefore suggested that mechloroethamin can bring about extensive changes in chromatin structure. These alterations result from either unfolding of the ordered structure of chromatin or release of core particles, both leading to increasing absorbance at 210 nm. When absorption spectra are made against the buffer, hypochromicity is observed, because mechloroethamin has negative absorption at 210 nm. The results can possibly be explained as an alteration of chromatin structure by drug action causing a change in DNA-protein binding capacity. For this purpose when chromatin is reconstituted with alkylated DNA treated with mechloroethamin, the binding capacity of DNA to histone proteins is reduced as compared to the untreated samples. Therefore, it is postulated that if the ligand binds to nucleic acid at the same site of protein, it will replace these proteins and thus may affect nucleosome formation. It has been reported that DNA and proteins in core particles are more susceptible to alkylation than DNA itself.<sup>17</sup> Alkylating anti-tumor agents can alter the binding of DNA to protein. This is an essential part of the mechanism by which these drugs inhibit cell multiplication.<sup>18</sup>

Several authors<sup>4-19</sup> reported that alkylating anti-tumor agents bring about the formation of DNA protein complexes which are resistant to deproteinization by phenol-salt extraction procedures. Therefore protein-DNA cross-linking is an important parameter in drug action.

Another factor for drug effect is proximity. Proximity of

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