

## THE EFFECT OF ADRIAMYCIN ON SALT FRACTIONATED CHROMATIN

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### ABSTRACT

In this study calf thymus chromatin was fractionated into active ( $S_1$  and  $S_2$ ) and inactive ( $P_2$ ) chromatin. Then the interaction of an anthracycline antibiotic, adriamycin, with these fractions was investigated employing absorption and difference UV/Vis spectroscopy and SDS and agarose gel electrophoreses.

The results suggest that the binding of adriamycin to the  $S_1$  fraction is slight but  $S_2$  and  $P_2$  were considerably affected by drug action. In both cases the absorbances at 260 and 480 nm were decreased. As drug concentration is increased in the mixture, core histones are aggregated and coprecipitate with the chromatin. The extent of condensation in  $P_2$  is higher when compared to the  $S_2$  fraction.

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### INTRODUCTION

The differential sensitivity of chromatin to nuclease digestion is used to separate transcriptionally active from inactive regions.<sup>1,2</sup> There is considerable biochemical and cytological evidence to support that active and inactive chromatin may be structurally different, including increased sensitivity to nuclease digestion, higher frequency of nuclease hypersensitive sites, difference in solubility, decreased level of DNA methylation, the absence of histone  $H_1$ , the presence of non-histone proteins and changes in protein composition (histone acetylation, ubiquitination).<sup>3-6</sup>

The anthracycline antibiotic daunomycin and the closely related adriamycin are currently widely used in cancer chemotherapy. Daunomycin is used for the treatment of acute-leukemia, while adriamycin (14-hydroxyl-daunomycin) is used in treating various solid tumors.<sup>7-9</sup>

Numerous studies suggest that the anthracyclines function primarily at the DNA level by blocking the processes of

replication and transcription.<sup>10,11</sup> In the cell nucleus, DNA is complexed with special sets of chromosomal proteins named histones which together make nucleosomes.<sup>12</sup> Reports on the binding of anthracycline antibiotics to chromatin and  $H_1$  depleted nucleosomes suggest a higher affinity of these drugs to naked DNA rather than to nucleosomes.<sup>13,14</sup>

In the chromatin, active parts differ from inactive chromatin in a wide variety of features that might affect the binding of drugs to them. Therefore in this study the effects of adriamycin on salt fractionated active and inactive chromatin was investigated.

### MATERIALS AND METHODS

Calf thymus was obtained from Ziaran slaughter house, transferred to the lab in liquid nitrogen and stored at  $-170^{\circ}\text{C}$  until use. Adriamycin was purchased from Sigma Chemical Co. and used without further purification. It was dissolved in distilled water at a stock concentration of 1 mg/mL,

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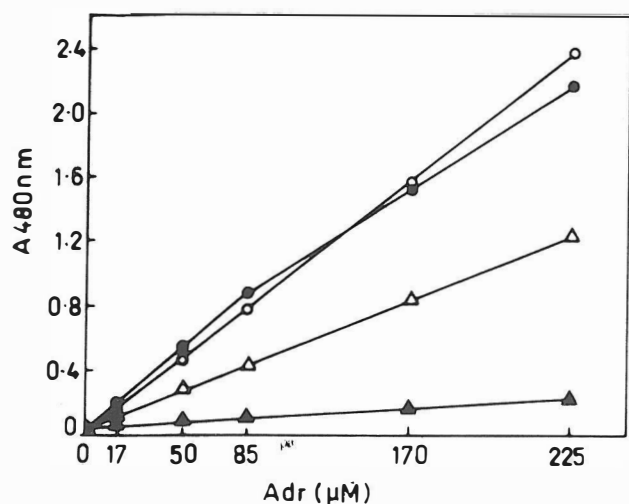


Fig. 1. Absorbance changes at 480 nm of supernatant fractions  $S_1$  (●-●);  $S_2$  (△-△) and the pellet  $P_2$  (▲-▲) after interaction with various concentrations of adriamycin. ○-○ is the serial concentration of adriamycin in Tris buffer pH= 7.5. Results are means of three experiments ( $p < 0.01$ ).

divided into equal portions and stored at  $-20^\circ\text{C}$  prior to use. Dilutions of drug stock into appropriate buffers were prepared immediately before use and their concentrations determined spectrophotometrically.

### Nuclei preparation

Nuclei from frozen calf thymus were prepared essentially according to the procedure of Burgoyne et al.<sup>15</sup> All steps were carried out at  $4^\circ\text{C}$ . Thymus tissue was homogenized in 0.34 M sucrose in buffer A (60 mM KCl, 15 mM NaCl, 1 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , Tris-HCl 15 mM, pH = 7.4) containing 2 mM EDTA and 0.5 mM EGTA, and layered on 1.37 M sucrose in buffer A containing 10 mM EDTA and 0.25 mM EGTA followed by centrifugation at 16000 g for 15 min ( $4^\circ\text{C}$ ). The pellet was dispersed in 2.4 M sucrose, 0.1 mM EDTA and 0.1 mM EGTA in buffer A layered over an equal volume of the same sucrose solution and centrifuged for 45 min at 75000 g. Pure and intact nuclei were washed twice with 0.34 M sucrose in buffer A and then suspended in buffer A. Aliquots of 0.1 mL was taken, mixed with 3 mL of NNaOH and DNA content was determined by measuring the absorbance at 260 nm.

### Digestion of nuclei and fractionation of chromatin

Nuclei were digested as described by Leby & Dixon.<sup>16</sup> Nuclei were resuspended in digestion buffer (0.34 M sucrose, 0.05 M Tris-HCl (pH= 7.3), 0.001 M  $\text{CaCl}_2$ ) containing 5 mM PMSF and preincubated for 2 min at  $37^\circ\text{C}$ . Nuclei were digested with 10 units of micrococcal nuclease per mg of DNA for 20 min at  $37^\circ\text{C}$ . The reaction was stopped by chilling the sample on ice and immediately centrifuged for 10 min at 2000 g. The supernatant was designated  $S_1$ . The

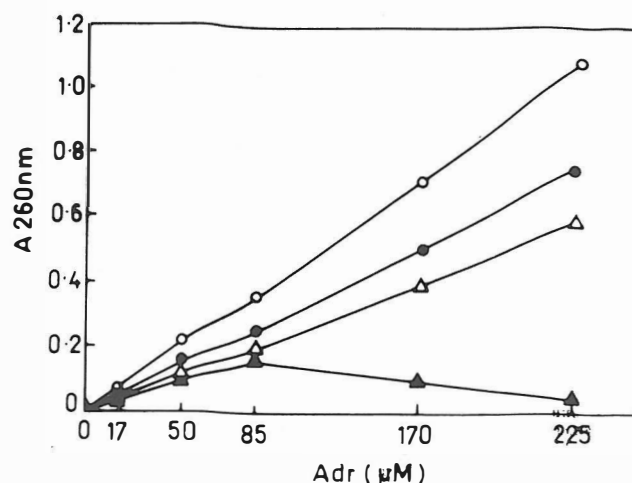


Fig. 2. Absorbance changes at 260 nm of supernatant fractions  $S_1$  (●-●);  $S_2$  (△-△) and  $P_2$  (▲-▲). ○-○ is the serial concentration of adriamycin in Tris buffer pH= 7.5. Results are means of three experiments ( $p < 0.01$ ).

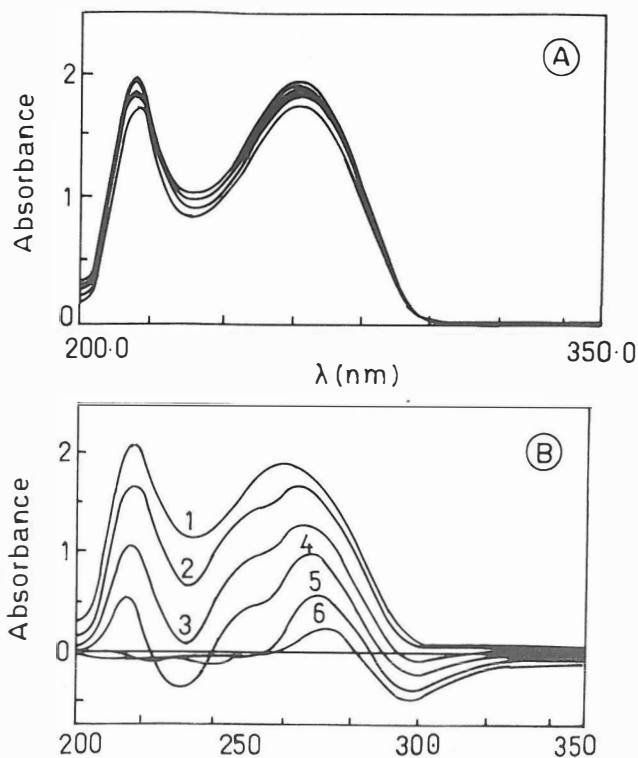
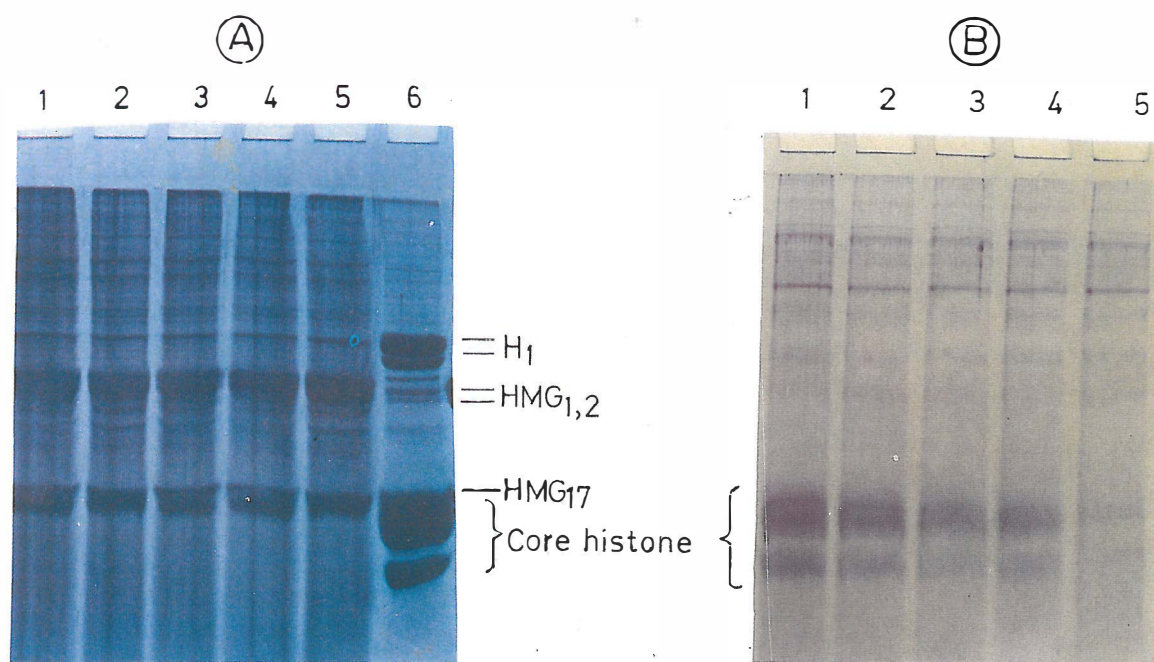


Fig. 3. Difference spectra of supernatant fractions  $S_1$  (A) and  $S_2$  (B) after interaction with different concentrations of adriamycin. Lanes 1-6 are 17, 50, 85, 170 and 225  $\mu\text{mol}$  adriamycin.

pellet was resuspended by homogenization in two volumes of 1 mM EDTA and then centrifuged at 4200 g for 15 min. The supernatant was adjusted to 0.1 M NaCl and left at  $4^\circ\text{C}$  for 30 min and then centrifuged at 12000 g for 20 min. The supernatant was designated  $S_2$  and the pellet  $P_2$ .



**Fig. 4.** 15% SDS polyacrylamide gel electrophoresis pattern of supernatant fractions S<sub>1</sub>(A) and P<sub>2</sub>(B). Lanes 1-5 from A and B are 0, 17, 50, 85 and 170 μmol adriamycin, respectively. Lane 6 in A is standard whole histones.

#### Binding measurements

Fractions obtained from digestion (S<sub>1</sub>, S<sub>2</sub> and P<sub>2</sub>) were diluted with the reaction buffer (0.05M Tris pH= 7.5) and after brief homogenization divided into several portions, each containing 1 mg/mL of DNA. To the samples, adriamycin with serial concentrations of 10, 30, 50, 100 and 150 μg/mL (17-225 μM) were added and the volumes adjusted to 1 mL with tris buffer pH= 7.5. Treated and control samples were then incubated in the dark at 23-25°C for 30 min. After incubation, the samples were centrifuged for 5 min at 10000 rpm (Eppendorf) at 4°C. The supernatants obtained from the treated and the controls were subjected to spectroscopic analysis using a double beam Shimadzu UV 260 spectrophotometer.

Absorbances at 480 (the characteristic drug absorbance) and 260 nm were monitored and the difference spectra were recorded between 190 and 550 nm using buffer and the same concentration of drug at each point as a reference.

#### DNA and protein extraction

The proteins released in the supernatants were precipitated with 10-20% trichloroacetic acid (TCA) with respect to 100%. TCA and the pellets were dissolved in SDS sample buffer for electrophoresis (see below).

DNA was isolated from the supernatants by the method of Britten.<sup>17</sup> Briefly, to 400 μL of the samples, 0.1 vol of 5 M sodium percholate and 0.5 vol of phenol: chloroform: isoamylalcohol were added and vortexed for 5 min at room temperature. The samples were then centrifuged for 5 min at

2000 g at 4°C. The aqueous phases were separated and the extraction was repeated twice as above. To the final combined aqueous phases, 2 vol of ethanol was added and DNA pellets were collected by centrifugation, washed with ethanol followed by acetone and dried under vacuum.

#### Gel electrophoresis

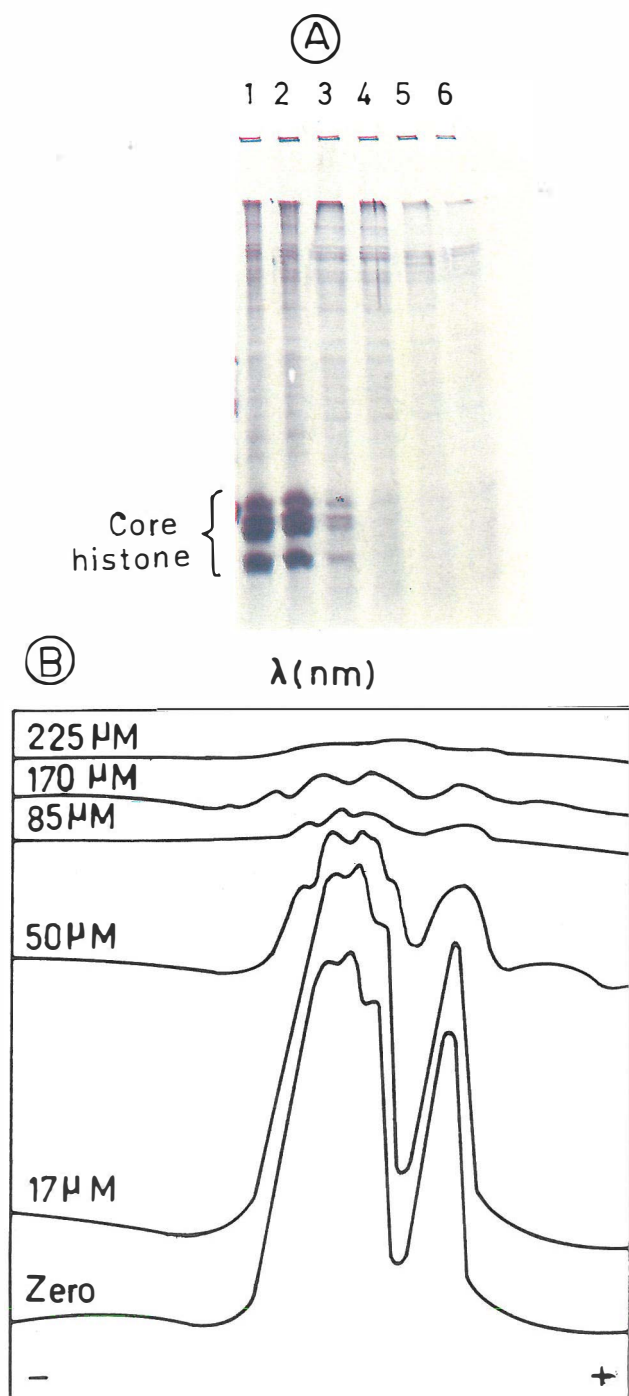
The proteins were analyzed on SDS polyacrylamide gel as described by Laemmli<sup>18</sup> with some modifications as follows: 15% acrylamide in separating gel; 4% acrylamide in stacking and 1.8% glycine in the electrode buffer were used. Calf thymus histone was used as a standard. Electrophoresis was carried out for 2 hours at 180 V. The gel was stained with Coomassie B.B. (1 mg/mL) and destained gels were scanned on a quick scan densitometer Beckman model R-112.

DNA samples were analyzed on 1.6% agarose gel system prepared in 0.01 M phosphate buffer (pH= 7.0). The gels were run at a constant voltage of 80-100 V for 30-40 min at ambient temperature. The gels were stained with ethidium bromide (5 μg/mL) in distilled water. EcoR<sub>1</sub>-HindIII digested DNA was used as a DNA molecular weight marker.

## RESULTS

Thymus nuclei were digested with micrococcal nuclease (MNase) and fractionated by a low salt procedure described in the *Methods* section. Three main fractions were obtained designated S<sub>1</sub> (chromatin released by MNase in the

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**Fig. 5.** A: 15% SDS polyacrylamide gel electrophoresis pattern of control and adriamycin treated supernatant fraction  $S_2$ . Lane 1: control with no drug. Lanes 2-6 are 17, 50, 85, 170 and 225  $\mu\text{M}$  adriamycin, respectively. B: stained gel.

supernatant),  $P_2$  (EDTA insoluble chromatin) and  $S_2$  (EDTA soluble chromatin). Fraction  $S_1$  was enriched in high mobility group (HMG) nonhistone proteins when analyzed on SDS gels (see below). However fraction  $S_2$  contained core particle

histones, and  $P_2$  was bulk chromatin and some core particles. In this study fractions  $S_1$  and  $S_2$  are taken as euchromatin and  $P_2$  as heterochromatin fractions.<sup>16,20</sup>

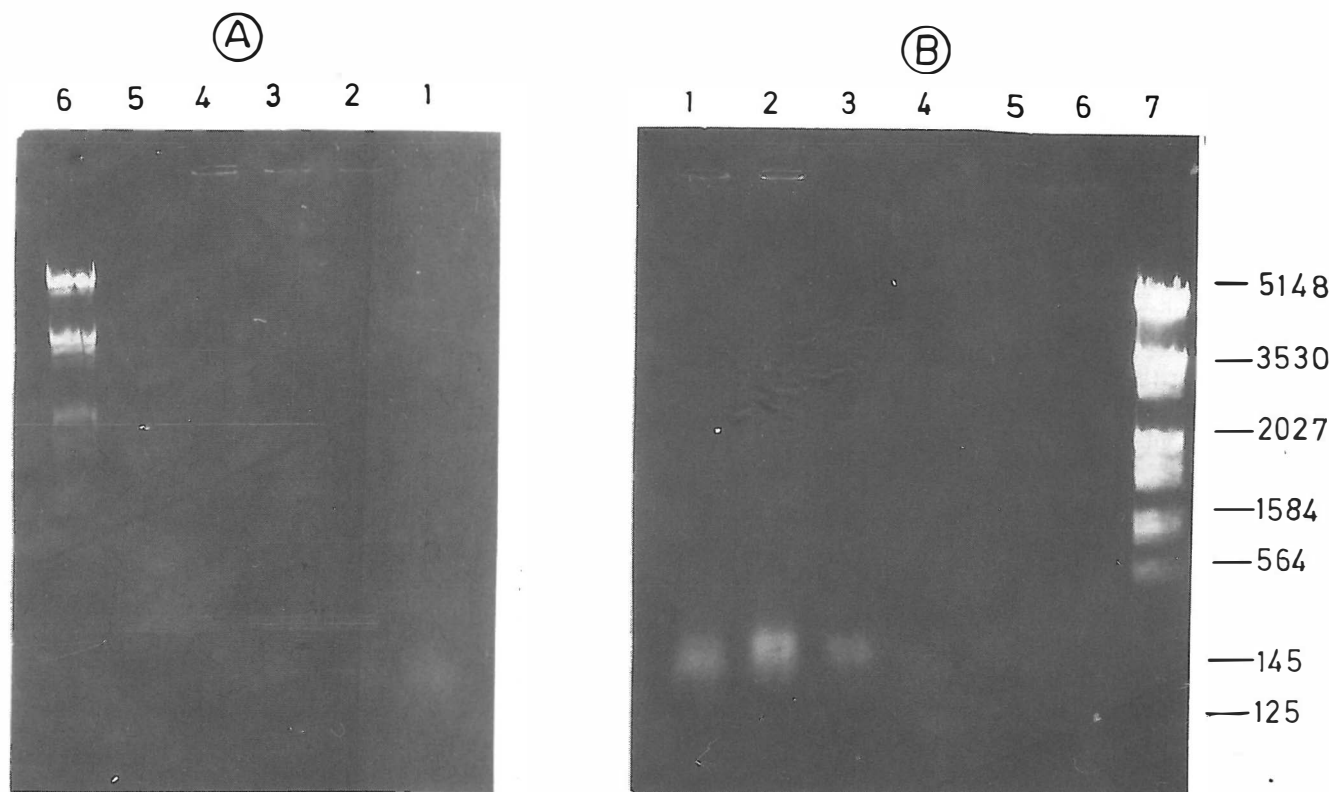
Fig. 1 shows the binding effect of adriamycin on fractions  $S_1$ ,  $S_2$  and  $P_2$  as determined by absorbance changes at 480 nm. Serial concentrations of adriamycin was made in buffer and used as control. From this Figure it is evident that the binding pattern differs between fractions:  $S_1$  shows a pattern similar to the drug absorbance pattern but in  $S_2$  the amount of drug in the supernatants is decreased so that at 170  $\mu\text{M}$  concentration nearly 50% reduction in absorbances is obtained. In  $P_2$  no drug has remained in the supernatants indicating that almost all drug has bound to the chromatin and precipitated along with it.

Fig. 2 gives the absorbance changes at 260 nm. In this case the absorbance at each point is the sum of drug and DNA absorbances at this wave length (drug wave lengths are 225, 250, 280 and 480 nm). It is seen that although  $S_2$  and  $P_2$  show the same binding pattern illustrated in Fig. 1,  $P_2$  shows a higher degree of absorbance reduction.

Fig. 3 shows the difference of  $S_1$  and  $S_2$ . As seen, in  $S_1$  no spectral changes are obtained as drug concentration is increased while in  $S_2$  the magnitude of the spectrum is decreased in a concentration dependent manner. Since  $P_2$  is bulk chromatin, after interaction with drug and centrifugation, chromatin is pelleted out; therefore it was not possible to draw the difference spectra against the same concentration of drug at each point.

In an attempt to analyze the possible displacement and nature of chromosomal proteins released into the supernatants after drug binding, the proteins in the supernatants were precipitated with TCA and an equal volume of each sample was loaded on to the SDS polyacrylamide gel. Fig. 4 shows the protein composition of fractions  $S_1$  and  $P_2$  as analyzed on SDS gels.  $S_1$  and  $P_2$  differ in that  $S_1$  contains mainly HMG proteins and there is no difference between the samples with various concentrations of adriamycin (Fig. 4A), but  $P_2$  shows a trace amount of core histone released by drug action and as the amount of drug is increased the amount of histones remaining in the supernatants is decreased (Fig. 4B). It should be noted here that histones in  $P_2$  fractions are mostly sedimented by drug action and only a small amount which is nearly 5% of the total protein remains in the supernatants.

Fig. 5 shows the SDS gel and densitometric scan pattern of fraction  $S_2$  in the presence and absence of adriamycin. It can be seen that core histones are the strongest bands seen in the control (with no drug). Following addition of various concentrations of adriamycin to the samples the chromatin tends to precipitate, therefore as drug concentration is increased the amount of core histone remaining in the supernatants is decreased. Furthermore the densitometric scan of the gel was drawn (Fig. 5B) and the percent of proteins released by drug binding was determined by calculating the area under the peaks. The results show that



**Fig. 6.** 1.6% agarose gel of samples extracted from the supernatant of  $S_2$ (A) and  $P_2$ (B). Lanes 1-5 from A and lanes 1-6 from B are 0, 17, 50, 85, 170 and 225  $\mu\text{M}$  adriamycin, respectively. Lanes 6(A) and 7(B) are ECo  $R_1$ -Hind III standard DNA with a molecular weight range of 125-5148 bp.

50  $\mu\text{M}$  of adriamycin reduces the content of core histone by 10% and by increasing the concentration up to 85  $\mu\text{M}$ , the precipitate reaches its maximum and is almost completed.

The nature of the DNA released into the supernatants after drug treatment was also analyzed on 1.6% agarose gel. In this case DNA was isolated by a phenol extraction procedure explained in the *Methods* section and then 15  $\mu\text{g}$  of each was run parallel to a DNA marker (Fig. 6). It is shown that the amount of DNA in the supernatants of  $S_2$  and  $P_2$  fractions with increasing drug concentrations is decreased, and in  $P_2$  only DNA with 125 bp remained in the supernatants.

## DISCUSSION

Numerous studies on the binding of antitumor agents such as anthracycline antibiotics to DNA have been undertaken and all indicate that these drugs exert their biological function via intercalation.<sup>7,13,19</sup> While the effects of these drugs may be understood in terms of their interaction with naked DNA, it is obviously necessary to understand what further effects the drugs might have at the chromatin level.

In this study we attempted to examine the effect of adriamycin as a potent antitumor agent on salt fractionated chromatin to provide further insight into the action of this drug on active and inactive parts of chromatin. Fractions

obtained from salt fractionation of macrococcal nuclease digested thymus chromatin are designated  $S_1$  and  $S_2$  as active chromatin and  $P_2$  as inactive chromatin as reported by Levy and Dixon<sup>16</sup> and Rocha et al.<sup>20</sup>

Results obtained from the effect of various concentrations of adriamycin on  $S_1$ ,  $S_2$  and  $P_2$  fractions revealed that adriamycin affects the chromatin fractions with different magnitude. It shows a very slight effect on  $S_1$  fraction which is enriched in nonhistone chromosomal proteins (SDS gel, Fig. 4) and is known as active chromatin. On the other hand, results obtained from the behaviour of the drug on the  $P_2$  fraction (inactive chromatin) is similar to the results of crude chromatin reported previously,<sup>21</sup> thus addition of increasing concentrations of adriamycin to  $P_2$  samples changes both spectroscopic and SDS gel patterns considerably. It seems that the effect at low concentrations of drug (< 17  $\mu\text{M}$ ) is negligible but at higher concentrations (> 50  $\mu\text{M}$ ) induces aggregation and compaction of chromatin possibly via cross links between the chromatin components. Therefore the drug is mostly coprecipitated with the chromatin and the absorbance at both 260 and 480 nm are decreased and the bands on SDS and agarose gels disappear. To date several reports have shown the condensation of nucleosomes and chromatin by several anthracyclines.<sup>22, 24</sup> This effect is in fact dose and ionic strength dependent.

In the case of the  $S_2$  fraction, which is also known as

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active chromatin and consists of mainly core particles, although with adriamycin the nucleosomes tends to become aggregated, the extent of precipitation is less than in  $P_2$ . Furthermore absorption and difference spectra clearly demonstrate that fraction  $P_2$  is more influenced by drug action than fraction  $S_2$ .

From these results it is concluded that although the effect of adriamycin on the  $S_1$  fraction is negligible, probably cross linking of chromatin components and its condensation mostly occurs in both  $S_2$  and  $P_2$  fractions by action of the drug. However it is difficult to be certain that the condensation of chromatin is the fundamental effect of the drug which subsequently inhibits DNA replication and transcription; but possibly, the drastic cytological effects of adriamycin reported on cells<sup>25,26</sup> might be due to drug induced condensation of chromatin in both active and inactive regions to which the aggregation reported would contribute.

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