THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 26, Issue of June 25, pp. 18401–18406, 1999
Printed in U.S.A.

Daunomycin-induced Unfolding and Aggregation of Chromatin*

(Received for publication, January 28, 1999, and in revised form, April 2, 1999)

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Using equilibrium dialysis and sedimentation velocity analysis, we have characterized the binding of the antitumor drug daunomycin to chicken erythrocyte chromatin before and after depletion of linker histones and to its constitutive DNA under several ionic strengths (5, 25, and 75 mM NaCl). The equilibrium dialysis experiments reveal that the drug binds cooperatively to both the chromatin fractions and to the DNA counterpart within the range of ionic strength used in this study. A significant decrease in the binding affinity was observed at 75 mM NaCl. At any given salt concentration, daunomycin exhibits higher binding affinity for DNA than for linker histone-depleted chromatin or chromatin (in decreasing order). Binding of daunomycin to DNA does not significantly affect the sedimentation coefficient of the molecule. This is in contrast to binding to chromatin and to its linker histone-depleted counterpart. In these instances, preferential binding of the drug to the linker DNA regions induces an unfolding of the chromatin fiber that is followed by aggregation, presumably because of histone-DNA interfiber interactions.

Daunomycin is an anthracycline antibiotic widely used as a potent chemotherapeutic agent in the treatment of various cancers (1, 2). Studies on its mechanism of action indicate that nuclear DNA is an important target for daunomycin. The structure of this pharmacologically active drug consists of two distinct domains (Fig. 1): a planar aglycon chromophore that intercalates between adjacent base pairs of DNA and an amino sugar ring that lies in the minor groove of the DNA double helix (3, 4). Binding of daunomycin to DNA results in the inhibition of both DNA replication and RNA transcription (5–7).

The binding of daunomycin to DNA has been studied in detail in the past (8–11). However, in the cell, DNA does not exist as a naked structure but is associated with histones and other nuclear proteins in a complex that is known as chromatin (12–13). Thus, chromatin, not DNA, is the major target for daunomycin *in vivo*. How the presence of chromosomal proteins might modulate the binding of this drug to DNA and how in turn chromatin structure is affected by the binding of the drug are important questions that need to answered to understand the biological mechanism of action of daunomycin. Although several attempts in this direction have already been carried out using isolated nucleosome (14, 15) and crude chromatin fractions (16–18), the mechanism of daunomycin binding to chromatin and the conformational transitions involved are still poorly understood. In this paper we report several experiments designed to address these questions in more detail.

We have studied the ionic strength dependence of the binding of daunomycin to a well defined fraction of chicken erythrocyte chromatin consisting of an average number of 43 nucleosomes. The results are compared with those obtained with the same chromatin fraction upon depletion of linker histones and with the purified constitutive DNA counterpart.

EXPERIMENTAL PROCEDURES

Materials—Chicken blood was obtained from a slaughter house (Lilydale, Victoria, BC, Canada) and processed immediately after collection as described below. Micrococcal nuclease was obtained from Worthington. CM-Sephadex C-25 was obtained from Amersham Pharmacia Biotech. Daunomycin hydrochloride was purchased from Sigma. Stock solutions of this drug were prepared in sterile distilled water at a concentration of 5 mg/ml and stored at 20 °C until further use. Dilutions of the drug stocks in the appropriate buffers were prepared immediately before use, and the concentration of daunomycin was determined spectrophotometrically using an extinction coefficient of 11,500 M^{-1} cm^{-1} at 480 nm.

Preparation of Chromatin—Chicken erythrocyte nuclei were prepared as described elsewhere (19). The nuclear suspension at an A_{260} = 120 was digested with 6 units of micrococcal nuclease/ml at 37 °C for 5 min. After digestion, the suspension was brought to 10 mM ETDA on ice and was immediately centrifuged at 12,000 × *g* at 4 °C for 5 min. The pellet was resuspended in 0.25 mM EDTA (pH 7.5), and the nuclei were lysed by gently stirring at 4 °C for 1 h. The lysate was then centrifuged at 12,000 × *g* for 15 min, and the chromatin in the supernatant was fractionated by centrifugation using 5–20% (w/v) sucrose gradients in 25 mM NaCl, 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA. Centrifugation was carried out in a SW28 Beckman rotor at 82,000 × *g* for 3 h at 4 °C. One-ml fractions were collected, and their absorbance at 260 nm was determined. After analysis of the DNA size distribution of the different fractions by agarose gel electrophoresis, the fractions of interest were combined and dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA at 4 °C. A protease inhibitor mixture ("Complete" from Roche Molecular Biochemicals) was then added (1 tablet/100 ml), and the sample was stored on ice until further use.

Preparation of Histone H1-depleted Chromatin—Histone H1 depletion was carried out as described previously (20). Briefly, chromatin was prepared as described above and dialyzed overnight at 4 °C against 50 mM NaCl. 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, concentrated with a Centriprep 50 concentrator (Amicon Inc.) and loaded onto a (3 × 15 cm) CM-Sephadex C25 column that had been pre-equilibrated with the same buffer. The column was eluted at a flow rate of 6 ml/h at 4 °C, and the fractions of the H1-stripped chromatin peak were pooled together and dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The dialysate was treated with a mixture of protease inhibitors ("Complete") as described above and stored on ice.

Isolation of DNA—DNA was obtained by proteinase K digestion (1 μg of enzyme/10 μg of DNA) of the chromatin samples described above. After digestion at 37 °C for 3–4 h, the DNA was extracted first with an equal volume of phenol/chloroform/isoamylic alcohol (25:24:1 v/v) followed by a chloroform:isoamylic alcohol (24:1 v/v) extraction. The DNA from the aqueous phase was then ethanol-precipitated at −20 °C, and the precipitate was rinsed with 70% ethanol, centrifuged, air-dried, and resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The concentration was determined spectrophotometrically using a molar extinction coefficient of 12,824 M^{-1} cm^{-1} at 260 nm.

Gel Electrophoresis—Agarose (1–1.2% w/v) gel electrophoresis was

* This project was supported by Medical Research Council of Canada Grant MT-13104 (to J. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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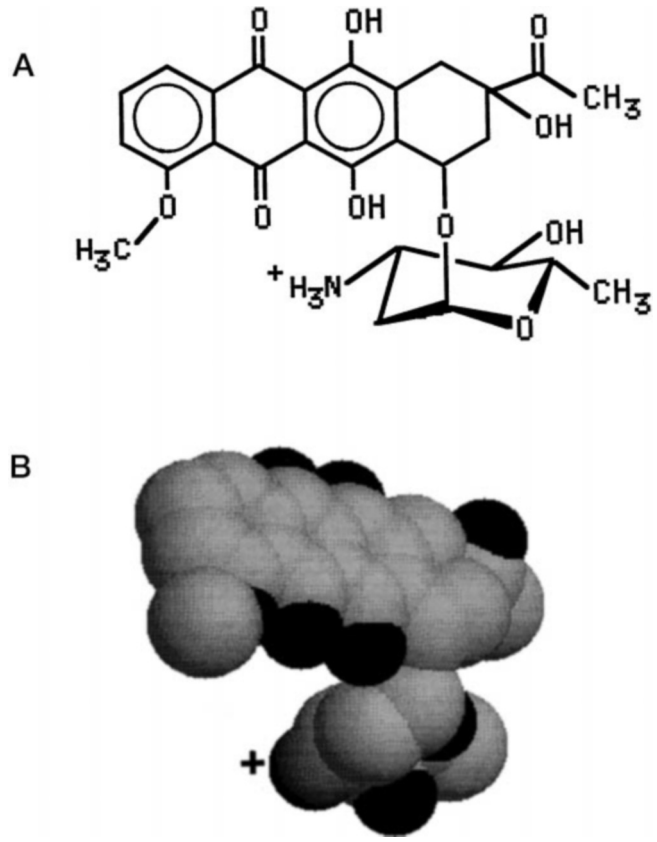

FIG. 1. **Structure of daunomycin.** *A*, structural formula. *B*, space-filling model.

carried out in 45 mM Tris-borate, 1 mM EDTA (pH 8.0) buffer according to Maniatis *et al.* (21). Native 4% polyacrylamide gels (PAGE)[1] for DNA analysis were performed as described by Yager and van Holde (22). Electrophoretic analysis of proteins was carried out on SDS-PAGE according to Laemmli (23).

Equilibrium Dialysis—Chromatin, H1-depleted chromatin, and their corresponding DNA at 40–45 μg/ml in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA plus NaCl were dialyzed against the same buffer containing increasing amounts of daunomycin. Three concentrations of NaCl were analyzed: 5, 25, and 75 mM. Dialysis was carried out at 4 °C using Spectra/Por 2 (Spectrum companies). Equilibrium was reached within 72 h with no detectable binding of drug to the membrane. The concentration of free drug (c_f) in the dialysate was measured directly from the absorbance at 480 nm. Binding parameters were determined from the plot of r/c_f *versus* r according to Scatchard method (24), where r is the ratio of bound drug to total base pair concentration, and c_f is the amount of free drug. In this representation, n (the apparent number of binding sites), is the intercept of the linear region of the binding curve with the horizontal axis, and K (apparent binding constant) corresponds to the negative value of the slope of the curve.

Sedimentation Analysis—For the sedimentation velocity analysis, the chromatin, linker histone-depleted chromatin, and DNA samples at the desired salt concentration (5, 25, or 75 mM NaCl) in Tris-HCl (pH 7.5) 0.2 mM EDTA were mixed with different amounts of drug in the same ionic strength buffer, so that in all cases the same final concentration corresponded to 43 ± 2 μg/ml, as determined from the absorbance at 260 nm (A_{260} = 0.86 ± 0.04). The input ratio r_i (mol of drug/mol of bp) can then be calculated from $r_i = c_D \cdot M_{bp}/c_s \cdot M_D$, where c_D = concentration of daunomycin (μg/ml), c_s = concentration of sample (μg/ml), m_b = molecular weight of a base pair (663), and M_D = molecular weight of daunomycin (527). Sedimentation velocity experiments were carried out in a Beckman XL-A analytical ultracentrifuge using an aluminum An-55 rotor and double sector aluminum-filled Epon centerpieces. All runs were routinely performed at 20 °C. The boundaries were analyzed according to the method of van Holde and Weischet (25) using an XL-A Ultra Scan-origin version 2.93 sedimentation data analysis software (B. Demeler, Missoula, MT). To estimate the average number of nucleosomes of the chromatin samples, the sedimentation coefficient distribution of the corresponding DNA was determined as described elsewhere (26), and the molecular weight was estimated using the following empirical equation $s_{20,w} = 2.68 + 1.263 \times 10^{-2}$ $M^{0.455}$ (27). To calculate the average number of nucleosomes, an average nucleosome repeat length of 210 bp was used for chicken erythrocyte chromatin (13).

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; bp, base pair; S, Svedberg units; $s_{20,w}$, coefficient corrected to standard conditions (20 °C and water).

RESULTS

Chromatin—The major goal of this paper is the characterization of the interactions of daunomycin with chromatin. We have specifically looked at the individual role played by the core and linker histones. To this purpose, we used a well defined chromatin fraction from chicken erythrocytes that either contained the full native complement of linker histones (histones H1 and H5) or had been artificially depleted of them. The binding of daunomycin to either fraction under different ionic strengths was analyzed and compared with that of their constitutive DNA under the same conditions. Fig. 2 shows the biochemical composition of these chromatin samples in terms of their constitutive DNA (Fig. 2*A*) or protein (Fig. 2*B*) counterparts. Fig. 2*C* displays the salt-dependent folding characteristics of the chromatin fraction used in comparison to its constitutive DNA, as analyzed by sedimentation velocity analysis in the ultracentrifuge. The sedimentation coefficients of chromatin and linker histone-depleted chromatin increase with the ionic strength. Such an increase is because of the folding of the chromatin fiber (20, 26). The extent of this increase depends on the presence (26) or the absence (20) of linker histones. The salt dependence of the sedimentation coefficient observed for the chromatin fraction before and after stripping of the linker histones is in agreement with earlier results (26, 28, 29).

The average sedimentation coefficient of the constitutive DNA at low salt $s_{20,w}$ = 17.9 S, and the integral distribution of the sedimentation coefficients (26) corresponds to a chromatin fraction with an average nucleosome number of N_w = 43.

Equilibrium Dialysis—The binding isotherms obtained from equilibrium dialysis experiments under different ionic conditions for the binding of daunomycin to chicken erythrocyte chromatin containing or lacking linker histones, in comparison to those of its constitutive DNA, are shown in Fig. 3. In 5 mM sodium chloride, all samples exhibit a cooperative binding behavior, as illustrated by the positive slope observed in the low r regions of the binding isotherms (see Fig. 3*A*). The curves reach a maximum at a value r = 0.1 (DNA), r = 0.22 (linker histone-depleted chromatin), and r = 0.34 (linker histone-containing chromatin). A decreasing slope is observed at higher r values. DNA and linker histone-depleted chromatin exhibit similar, albeit not identical, binding isotherms in contrast to chromatin that shows lower r/c_f values and a higher r_{max} and which are indicative of a much lower binding affinity of daunomycin in the later case. Indeed, the binding constant (K) of chromatin 4.6×10^4 M^{-1} is relatively lower than that of the linker histone-depleted ($K = 6.4 \times 10^4$ M^{-1}) or DNA ($K = 7.7 \times 10^4$ M^{-1}) counterparts. The higher binding affinity of daunomycin by free DNA is consistent with early data (14, 30). When the ionic strength is increased to 75 mM sodium chloride (Fig. 3*C*), the Scatchard plots still retain their positive cooperativity; however, the r/c_f is reduced considerably compared with 5 mM (Fig. 3*A*) or 25 mM (Fig. 3*B*) salt conditions, indicating an overall decrease in the binding affinity of daunomycin at this ionic strength. Indeed, the binding constants for daunomycin experience a significant decrease, which affects the chromatin fractions. The values calculated were $K = 5 \times 10^4$ M^{-1} for

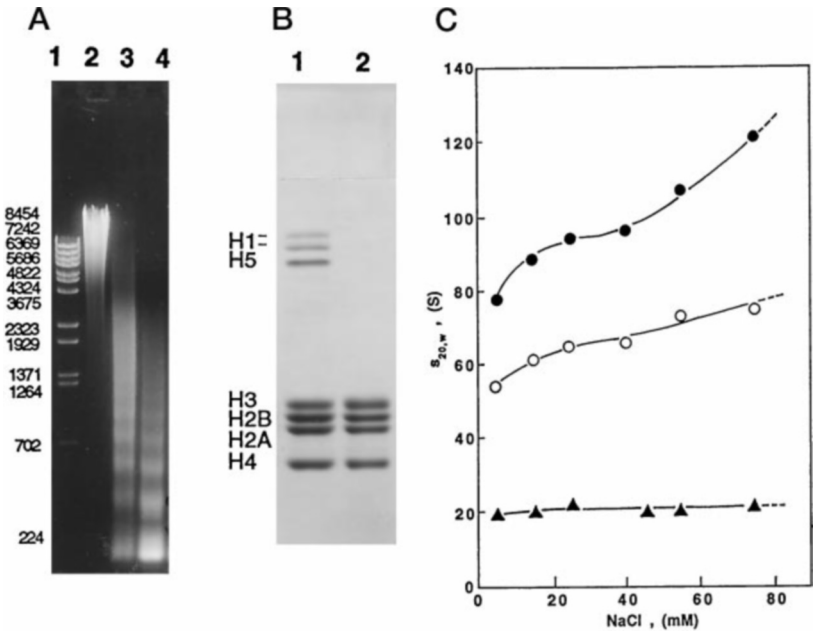

FIG. 2. **Compositional and structural characterization of the DNA and chromatin used in this work.** *A*, 1% agarose gel electrophoresis of λDNA *Bst*EII digest, used as a marker (*lane 1*) is shown. DNA from the chromatin fraction was analyzed (*lane 2*). DNA from the linker histone-stripped counterpart exhibited the same electrophoretic mobility (results not shown). DNA fragments obtained from a micrococcal nuclease digestion of the chromatin fraction used before (*lane 3*) and after (*lane 4*) stripping of the linker histone . These two *lanes* are shown to reveal the nucleosome organization of the native and linker histone-depleted chromatin fractions. Notice that the DNA fragments of the linker histone-depleted fraction exhibit a slightly higher electrophoretic mobility because they are digested by micrococcal nuclease to a smaller size as a result of the linker histone depletion. The number of base pairs corresponding to the fragments of the λDNA marker are indicated on the left hand side of the figure. *B*, SDS-PAGE (23) of the chromatin fraction before (*lane 1*) and after (*lane 2*) stripping of the linker histones. *C*, salt dependence of the average sedimentation coefficient of chromatin (●), linker-histone-stripped chromatin (○), and corresponding DNA (▲). The average number of nucleosomes of the chromatin fraction was $N_w = 43$. Sedimentation velocity runs were carried out at 20 °C in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer with different NaCl concentrations. The rotor speeds were 16,000 rpm for (●,○) and 32,000 rpm for (▲).

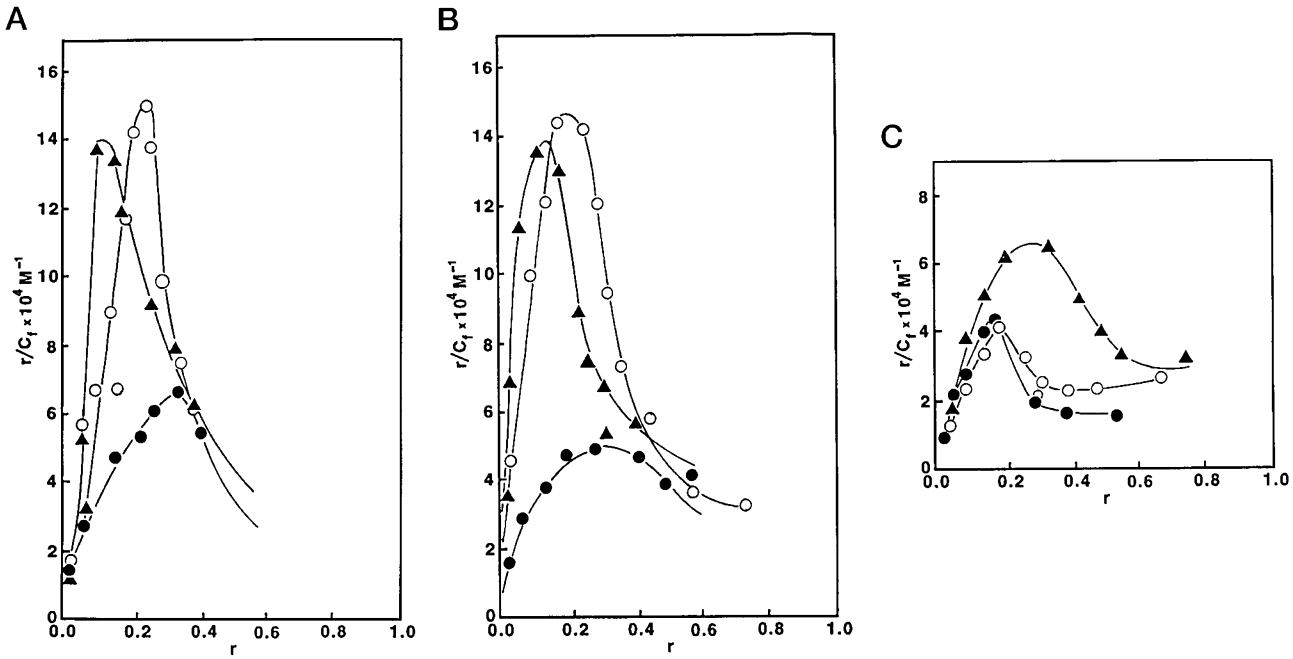

FIG. 3. **Scatchard plot analysis of the binding of daunomycin to chicken erythrocyte DNA and chromatin in the presence and absence of histone H1.** A, 5 mM NaCl; *B*, 25 mM NaCl; *C*, 75 mM NaCl in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer. ▲, DNA; ●, chromatin; ○, linker histone-depleted chromatin.

naked DNA, 1.98×10^4 M^{-1} for linker histone-depleted chromatin, and 1.76×10^4 M^{-1} for chromatin.

Hydrodynamic Analysis of Daunomycin Binding to Chromatin—In parallel with the equilibrium experiments, we also carried out a sedimentation velocity analysis of the binding of daunomycin to chromatin. We have chosen the range of ionic strength from 5 to 75 mM NaCl because chromatin and its linker histone-depleted counterpart exhibit a well established pattern of folding under these conditions (26, 20), as monitored by sedimentation velocity analysis. Therefore any perturbation in the hydrodynamic parameters of the chromatin sample arising from titration with the drug under these conditions must

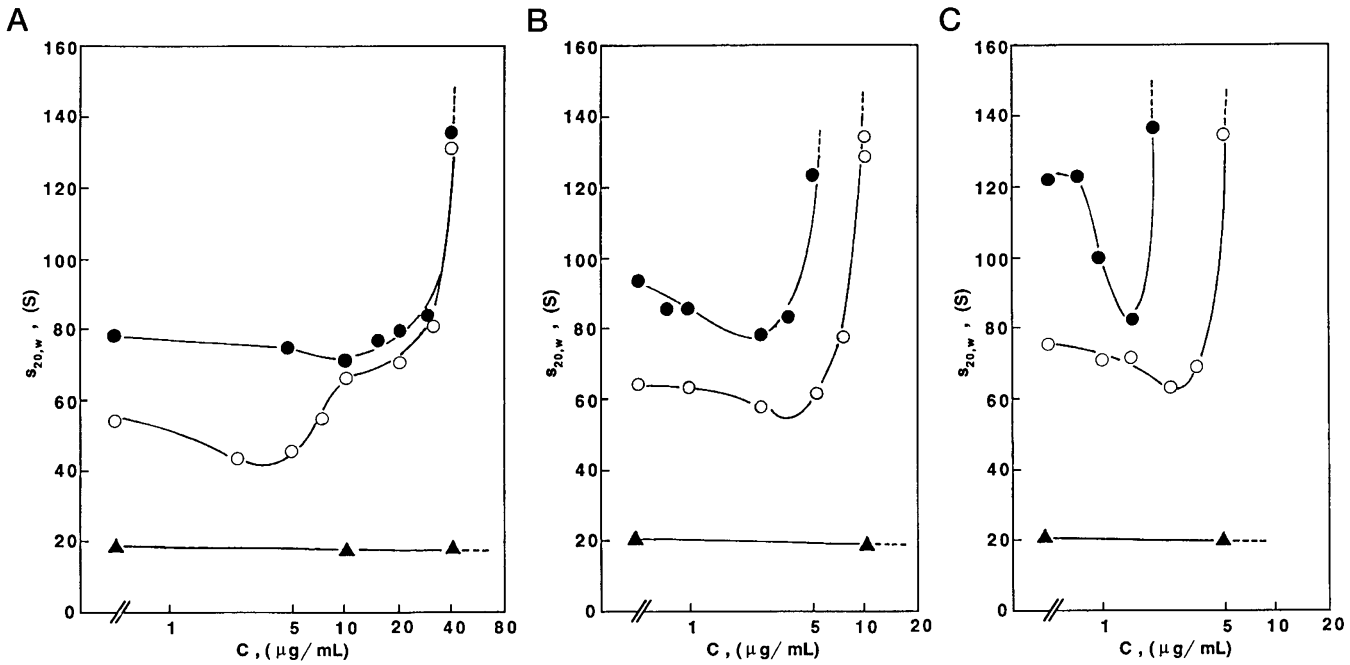

FIG. 4. **Change in the sedimentation coefficient of chromatin (●), linker histone-depleted chromatin (○), and corresponding DNA (▲) as a function of daunomycin concentration in 5 mM NaCl (*A*), 25 mM NaCl (*B*) and 75 mM NaCl (*C*).** The buffer was 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. The speed of the rotor was 16,000 rpm on (○, ●) and 32,000 rpm (▲). All experiments were carried out at 20 °C.

reflect changes in their intrinsic conformation and folding. The results are shown in Fig. 4. The sedimentation behavior of DNA seems to be affected relatively little by the binding of daunomycin under any of the ionic strengths analyzed. Upon increasing the drug concentration, a consistent small decrease in $s_{20,w}$ is observed that is almost within the experimental error limits of the technique. In contrast, the chromatin counterparts exhibit a bimodal trend in which the sedimentation coefficients first decrease upon initial binding of the drug followed by an increase and aggregation upon further binding. Thus it appears that histones and their hierarchical folding of DNA in the chromatin fiber are responsible for this hydrodynamic and aggregation behavior. In the case of chromatin containing the full complement of linker histones this bimodal transition is more pronounced at the highest salt concentration studied (75 mM NaCl) (see Fig. 4*D*), *i.e.* when chromatin, to be titrated with the drug, is in its naturally folded state. A decrease in the sedimentation coefficient of the sample without changes in its molecular/chemical composition reflects a conformational transition in which the sample becomes more extended (unfolded) as the sedimentation coefficient decreases, or it becomes more compact (folded) as this value increases.

Linker histones are dynamically associated to chromatin and are known to be very mobile at ionic strengths above 25 mM NaCl (31). To ensure that no histone H1/H5 was lost during the drug titration, we analyzed the histone composition of some of the chromatin precipitates, see Fig. 5. As seen in Fig. 5*A*, the stoichiometric amount of linker histones (histone H1 and H5) relative to the core histones remained the same at each salt concentration and at the drug concentration(s) responsible for the aggregation. Furthermore, the relative stoichiometry of the core histone (histones H2A, H2B, H3, and H4) also stayed constant both in chromatin (Fig. 5*A*) and in the linker histone-depleted counterparts (Fig. 5*B*). This indicates that no significant selective displacement of histone H2A-H2B dimers by the drug (32) had taken place before aggregation occurred. It is important to point out here that the input ratio r_i (mol of drug/mol of bp) at which the folding transition and aggregation occurred, decreased with the salt and with the presence of

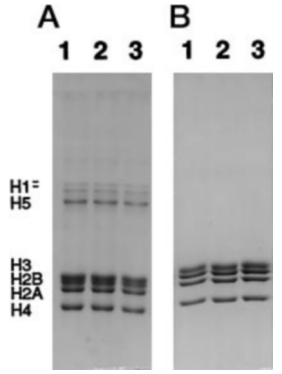

FIG. 5. **SDS-PAGE analysis (23) of the proteins from the chromatin precipitates induced by daunomycin at different salt concentrations.** Native chicken erythrocyte chromatin (*A*) and chicken erythrocyte chromatin stripped from linker histone (H1 + H5) (*B*) (see Fig. 2) at approximately 40 μg/ml (DNA weight equivalent) in 5 mM NaCl (*lane 1*), 25 mM NaCl (*lane 2*), or 75 mM NaCl (*lane 3*) were completely precipitated with 2.5 μg/ml (*lane 1*), 10 μg/ml (*lane 2*), 40 μg/ml (*lane 3*) (*A*) and 5 μg/ml (*lane 1*), 10 μg/ml (*lane 2*), 50 μg/ml (*lane 3*) daunomycin (*B*) (see Fig. 4).

linker histones (see Fig. 4). However, even at the lowest salt (5 mM) and in the absence of linker histone, precipitation took place at $r_i \leq 1$.

DISCUSSION

Daunomycin is an anthracycline antibiotic that binds to the DNA by forming a complex in which the aglycon chromophore intercalates between DNA base pairs (4, 33, 34) and the amino sugar (see Fig. 1) that lies in the minor groove without forming any bonds to DNA (4), although eventually it may also form covalent adducts with DNA (36, 37).

The results of the equilibrium dialysis experiments in Fig. 3 are in agreement with earlier data on the binding of daunomycin to different DNAs using phase-partitioning techniques (9). They are in disagreement with the work from Crothers and co-workers, who found no cooperativity of binding for either DNA (8) or calf thymus nucleosomes (14); however, different

techniques of analysis and different experimental conditions were used. Consistent with our data, however, their studies have also shown that binding of daunomycin to 175 bp (histone H1-depleted) of nucleosomes is strongly reduced relative to its affinity for free DNA (14). Their findings also agree with our results that the affinity of daunomycin for different nucleohistone complexes increases in the following order: nucleosome core particle < 175 bp nucleosome < DNA (38). Thus it appears as if the affinity of the drug for the chromatin complexes decreases with the decrease of the number of base pairs in the DNA region flanking the DNA coils protected by the histone octamer. A salt-dependent decrease on the affinity of binding was also observed in earlier chromatin studies (16).

At low salt (5 mM NaCl) and in the absence of drug (see Fig. 4*A*), the linker histone-depleted fraction sediments with an $s_{20,w}$ = 53 S as compared with $s_{20,w}$ = 78 S for the chromatin-containing linker histones. The former corresponds to a polynucleosome fiber in which approximately 145 bp of DNA are wrapped around each histone octamer, and these are interconnected by extended DNA linker regions of approximately 65 bp (20). As the ionic strength of the medium increases, the nucleosome particles come close together with an important part of the linker DNA regions sequestered between the closely packed histone octamers.

In the case of the chromatin fraction containing a full complement of linker histones, the sedimentation coefficient at low salt is close to that of the linker histone-depleted counterpart at high salt (39). However, in this instance, the polynucleosome fiber at low salt has a three-dimensional zigzag organization (40, 41) in which approximately 145 bp of DNA interact with the histone octamer, and an extra 20–30 bp adopt a stem-like (42) structure as a result of interaction with the linker histones. This zigzag arrangement persists as the ionic strength increases to yield a compact fiber (40, 41) in which the extended linker DNA regions and the nucleosomes are closely packed together.

Upon interaction with daunomycin, the sedimentation coefficient of the linker histone-depleted chromatin exhibits a similar pattern in all three ionic strengths (5, 25, and 75 mM NaCl) analyzed (see Fig. 4). At each salt concentration, the sedimentation coefficient initially decreases as the drug concentration increases. However, at a certain drug concentration, which is smaller at higher ionic strength, the trend changes, and the sedimentation coefficient increases in anticipation of the aggregation and precipitation of the sample, that also takes place at higher drug concentrations for the lower ionic strengths. This fact is indicative of a preferential interaction of daunomycin with linker DNA, as the amount of exposed linker DNA decreases with increasing ionic strength because of the concomitant folding of the chromatin fibers described above. This result is in agreement with the strong preference of daunomycin for free DNA *versus* nucleosomal DNA (14). The initial decrease in $s_{20,w}$ observed at the different ionic strengths corresponds to the intercalation of the drug with this linker region. Such intercalation results in alterations of the linker twist, which affect the way in which the fiber is folded (43), bringing it to a more extended open conformation, in which some of the histone domains are able to establish cross-links with adjacent fibers (18) (reflected by a small increase in $s_{20,w}$), and this eventually leads to a complete aggregation of the sample. The decrease in the sedimentation coefficient as a result of the drug interactions with DNA could also be partly ascribed to an unfolding of the nucleosome core particle (14), which can occur at extremely low ionic strengths (see Ref. 13 for a review). It could also be that stiffening of the linker DNA because of the drug intercalation leads to partial dissociation of the DNA-flanking regions at the entry and exit points to the nucleosome core particle. Although our data does not allow us to distinguish between either model, we prefer the last, as subnucleosome core particles with DNA smaller that 125 bp tend to aggregate (44).

In the linker histone-containing chromatin, the number of binding sites are reduced, as part of the linker DNA is occupied by linker histones. Furthermore, the extent of unfolding of the fiber is even more dramatic and occurs over a narrower range at 25 and 75 mM NaCl. This is consistent with the highest compaction of these chromatin fibers at these salt concentrations and also with the more rigid nucleosomal structure organization imposed by the interaction of the linker histones with the linker DNA (42). In contrast with previous chromatin studies, our results do not need to invoke any salt-induced alteration in the DNA conformation (16), which is very unlikely to occur at these relatively low ionic strengths. Our results also indicate that daunomycin has a destabilizing rather that inhibiting (17) effect on chromatin folding.

Our results are consistent with the early interpretation by Chaires *et al.* (14) on the *in vivo* significance of the *in vitro* data. Accordingly, it is probable that daunomycin recognizes and exerts its primary action on those regions of chromatin that are more unfolded and have a reduced number of nucleosome structures, such as found in transcription or replication active domains. Also, the aggregation behavior observed *in vitro* could account for the *in vivo* cytological effects of the drug observed in HeLa cells (14, 35).

Acknowledgment—We thank Susan Moore for her careful reading of the manuscript and John Lewis for his skillful computer assistance. We are also grateful to Claire Tugwell and Melinda Powell for typing the paper.

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