Novobiocin precipitates histones at concentrations normally used to inhibit eukaryotic type II topoisomerase

Matt Cotten, Doherty Bresnahan, Sally Thompson, Linda Sealy and Roger Chalkley

Department of Biochemistry, University of Iowa, Iowa City, IA 52242, USA

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ABSTRACT

At concentrations normally used to inhibit eukaryotic type II topoisomerase activity (100-1000 $\mu\text{g/ml}$) novobiocin binds core histones. Approximately 15 moles of novobiocin bind per mole of histone resulting in histone precipitation from solution in either 0.15 M or 2 M NaCl. The interaction between novobiocin and proteins appears to involve arginine residues: histones H3 and H4 (13.5 and 14 mole percent arginine) are precipitated at lower novobiocin concentrations than histones H2A and H2B (9.5 and 6.5 mole percent arginine). Furthermore, polyarginine but not polyornithine competes for novobiocin in histone precipitation. Moreover, histones with arginine residues modified with 1,2-cyclohexanedione are soluble in 1000 $\mu\text{g/ml}$ novobiocin. Because novobiocin can remove histones from solution as well as inhibit topoisomerase activity, and because both of these events can alter DNA topology, novobiocin should be used with caution in experiments designed to implicate topoisomerase activity in chromatin dynamics.

INTRODUCTION

Novobiocin has been characterized as a potent inhibitor of bacterial DNA gyrase activity. The compound inhibits both the supercoiling activity of the enzyme (at a concentration of 1 $\mu\text{g/ml}$) (1) and the ATPase activity of the enzyme (at 20-60 $\mu\text{g/ml}$) (2,3). The strong affinity between novobiocin and the B subunit of DNA gyrase has been used as the basis for novobiocin-Sepharose affinity purification of the gyrase (4). This method has also been used to purify type II topoisomerase activities from mouse cells (5) and from algae (6).

More recently, the compound has been used in the study of chromatin dynamics in efforts to demonstrate the role of a gyrase in eukaryotic DNA packaging (7,8) and in the generation of altered chromatin structures (7,8,9,10). In our attempts to reproduce some of this work, we have observed that novobiocin inhibited chromatin assembly even from purified components which can be shown to lack type II topoisomerase activity. This observation suggested that novobiocin was interacting with some component

in the assembly system and upon further analysis, we observed that the presence of novobiocin causes histones to precipitate.

We have extended these observations and we show that novobiocin has the general property of interacting with arginine-rich proteins, and if a sufficient concentration of the antibiotic is present, precipitation of these proteins occurs. We demonstrate that the arginine content appears to be the major determinant of susceptibility to novobiocin precipitation although the total positive charge on the protein appears also to play a role.

METHODS

Protein Procurement

Hyperacetylated core histones were isolated by salt extraction of hydroxyapatite-bound Hepatoma Tissue Culture (HTC) cell chromatin (11). Acid-extracted histones (containing histone H1) were prepared from HTC chromatin (11) which was adjusted to 0.4 N H_2SO_4 at 4°C. The acid soluble material was dialyzed against 4 volumes of ethanol at 4°C overnight, and the precipitated material was collected and dried. Histones labeled with ^3H -lysine were prepared from HTC cells grown for 12 hours in lysine-free medium containing 1 $\mu\text{Ci}/\text{ml}$ ^3H -lysine. Core histones prepared from these cells had a specific activity of approximately 1200 cpm/ μg . All other proteins, including poly-L-arginine (MW 40,000) and poly-L-ornithine (MW 43,000) were obtained from Sigma Chemical Corporation.

Polyacrylamide Gel Electrophoresis

Histone samples were analyzed by electrophoresis through Triton-acid-urea polyacrylamide gels containing 15% acrylamide, 0.4% bisacrylamide, 0.9 N acetic acid, 8 M urea and 0.44% (vol/vol) Triton X-100 with a stacking gel of 5% acrylamide, 0.15% bisacrylamide, 0.9 N acetic acid and 8 M urea. A 16 volt/cm potential was applied to the gel for at least 6 hours before applying samples. Forty minutes before sample application, free radicals in the system were scavenged by applying 0.6 M cysteamine, 20% sucrose, 0.9 N acetic acid (10 $\mu\text{l}/\text{well}$) and continuing the electrophoresis. The electrophoresis buffer was 8 M urea, 0.9 N acetic acid (bottom tray) and 0.9 N acetic acid (top tray). Samples contained 8 M urea, 0.9 N acetic acid, 10 % glycerol and 0.005% (wt/vol) methyl green. Electrophoresis was at 14 volts/cm for 18 hours.

SDS polyacrylamide gel electrophoresis was performed in 18% acrylamide, 0.09% bisacrylamide gels as previously described (12).

Gels were stained with 0.25% (wt/vol) Coomassie blue (Bethesda Research Laboratories) in 10% acetic acid, 40% methanol. Where indicated, gels were then washed extensively with 50% methanol before staining with silver (13).

Novobiocin Precipitation

Proteins were precipitated with the amounts of novobiocin indicated in the figure legends. Samples to be precipitated with novobiocin were incubated with the novobiocin for 45 minutes. The precipitation reaction appears to be relatively insensitive to temperature, with identical results obtained when the incubation is performed at room temperature. We routinely perform the incubation on ice to limit proteolysis by enzymes which often contaminate histone preparations. Because novobiocin is virtually insoluble below pH 7, precipitations were performed in 10 mM Tris, pH 8.0 and 150 mM NaCl (unless indicated otherwise). The precipitated material was collected with a 3 minute spin in an Eppendorf centrifuge (15,000 X g). The supernatant was removed and the pellet was washed with an equal volume of 150 mM NaCl, 10 mM Tris, pH 8.0 containing the same amount of novobiocin as the original sample. The sample was centrifuged again and this wash was discarded. Upon addition of electrophoresis sample buffer containing 8 M urea or 1% SDS, the samples were ready for analysis by gel electrophoresis.

Novobiocin was obtained from either Sigma or Boehringer Mannheim with both preparations yielding identical results. A 10 mg/ml solution of novobiocin in water was prepared and stored in small aliquots at -20°C in the dark. We have observed that novobiocin undergoes a light-induced cleavage that appears to interfere with its ability to precipitate histones (M.C., unpublished data).

Modification of Histones with 1,2-cyclohexanedione

Cyclohexanedione modification has been described in detail (14,15). Histones at a concentration of 0.03 mg/ml were incubated with 0.015 M 1,2-cyclohexanedione (Aldrich Chemical Corporation) in 20 mM sodium borate, pH 8.5, at 37°C for 20 hours. After reaction, the sample (1 ml) was dialyzed against 20 mM sodium borate, pH 8.5 (4 changes of 1000 ml) for 24 hours at 4°C.

RESULTS

Novobiocin Precipitates Histones

Core histones were incubated in the presence of various concentrations of novobiocin, in 150 mM NaCl and 10 mM Tris, pH 8.0 for 30 minutes on ice. After centrifugation for 3 minutes at 15,000 X g, the material in the

Figure 1

Novobiocin precipitation of core histones in 150 mM NaCl. Histone samples (6 µg, hyperacetylated HTC core) were incubated with the indicated concentrations of novobiocin in 50 µl of 150 mM NaCl, 10 mM Tris, pH 8.0, for 45 minutes on ice. The samples were centrifuged for 3 minutes in an Eppendorf centrifuge (15,000 X g, room temperature) the supernatant was removed and the pellet was suspended in an equal volume of 150 mM NaCl, 10 mM Tris, pH 8.0 containing the indicated concentration of novobiocin. The sample was centrifuged again, this second supernatant was discarded and the pellet and first supernatant were resolved on a Triton-acid-urea gel as described in the Methods section. The values at the top of the figure indicate novobiocin concentration in µg/ml. P and S refer to pellet and supernatant.

supernatant and pellet was analyzed by electrophoresis on an Triton-acid-urea gel. Core histones associate predominately as H3-H4 tetramers and H2A-H2B dimers at physiological ionic strengths and these complexes are clearly soluble (figure 1, lanes 1 and 2). We find that in the presence of 200 µg/ml novobiocin, a substantial proportion of the histone H3 and H4 becomes insoluble. Increasing the novobiocin concentration to 300 µg/ml causes all of histone H3 and H4 and approximately 40% of H2A and H2B to precipitate leaving essentially pure H2A and H2B in solution. Novobiocin concentrations greater than 500 µg/ml cause all four core histones to precipitate. It is also apparent that the level of acetylation of the histone has no effect on the susceptibility of

Figure 2

Novobiocin binds histones. Salt extracted core histones were incubated with 1000 $\mu\text{g/ml}$ novobiocin in 150 mM NaCl, 10 mM Tris, pH 8.0, for 45 minutes on ice. The precipitated material was collected by centrifugation for 3 minutes in an Eppendorf centrifuge. After removing the supernatant, the pellet was dissolved in 150 mM NaCl, 10 mM Tris, pH 8.0, 1% (wt./vol.) SDS and the absorbance at 300 nm was measured. Novobiocin has an absorbance maximum at 300 nm with a molar absorptivity of $2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in this buffer. Histones have virtually no absorbance at this wavelength, therefore the absorbance of the solubilized pellet at 300 nm was used to determine the novobiocin content. The molar concentration of histones was determined using an average molecular weight value of 13,560 for the four core histones. The amount of novobiocin present (in moles $\times 10^9$) is plotted as a function of core histone present in the sample (in moles $\times 10^{10}$).

the histone type to precipitation. For example, we find that tetracetylated H4 is precipitated to the same extent as parental H4. We have routinely assayed for precipitation at 4°C as this allows us to avoid problems with proteolysis of the histones. However we have observed similar amounts of precipitation when the materials were held at room temperature for thirty minutes.

Histones Bind Novobiocin

Increasing concentrations of core histones were incubated with a constant (excess) amount of novobiocin (at 1000 $\mu\text{g/ml}$). The precipitate

Figure 3

The effects of various agents on novobiocin precipitation of histones. Novobiocin precipitation of 6 µg samples of hyperacetylated core histones were performed as described in the legend to figure 1 except for the addition of the indicated agents. Lanes 5 and 6, 0.1% (wt/vol) SDS; lanes 7 and 8, 2.0 M urea; lanes 9 and 10, 4.0 M urea; lanes 11 and 12, 0.5 M NaCl; lanes 13 and 14, 1.0 M NaCl; lanes 15 and 16, 2.0 M NaCl. The novobiocin concentration used was 500 µg/ml. All samples contained 10 mM Tris pH 8.0 and samples 1 through 10 contained 150 mM NaCl. The samples in lanes 5 and 6 were acidified and extracted twice with butanol to remove the SDS before electrophoresis on a Triton-acid-urea gel.

which formed was collected and redissolved in the presence of SDS. The amount of novobiocin associated with the precipitated histones was quantitated spectrophotometrically and is displayed graphically in figure 2. We find that there is a linear relationship between the amount of histone present in each sample (and completely precipitated by this concentration of novobiocin) and the amount of novobiocin associated with the precipitated material. The slope of this line indicates that there are approximately 15 moles of novobiocin bound per mole of histone.

Agents that Interfere with Novobiocin Precipitation of Histones

The precipitation of histones by novobiocin is blocked by the presence of SDS (0.1%, figure 3) and by urea (2 M and 4 M, figure 3). These agents can also be used to solubilize precipitated histones in preparation for

Figure 4
Novobiocin precipitation of histones in 2 M NaCl. Novobiocin precipitation of hyperacetylated core histones was performed as described in the legend to figure 1 except that all samples contained 2.0 M NaCl. The values at the top of the figure indicate novobiocin concentrations (µg/ml). 6 µg samples of histones were used for the precipitation. After fractionation the samples were diluted fourfold to lower the NaCl concentration to a level permissible for electrophoresis and aliquots corresponding to 1.5 µg of histone were applied to the gel. After electrophoresis and staining with Coomassie blue, the gel was stained with silver. Note that histone H2B stains with silver much more intensely than the other core histones and thus appears to be present in lane 7 to a greater extent than H2A.

electrophoresis. However, we have found that precipitation occurs in the presence of 0.5 M NaCl (figure 3, lane 6), 1 M NaCl (figure 3, lane 7) and 2 M NaCl (figure 3, lane 8).

Novobiocin Precipitation of Histones in 2 M NaCl

The interaction of novobiocin with serum albumin is influenced by ionic strength, with high ionic strengths enhancing the binding affinity (16). Thus, we have tested the novobiocin concentration dependence of histone precipitation at pH 8.0 in the presence of 2 M NaCl (figure 4) to determine if high ionic strengths accentuate the precipitation reaction. We find there is detectable precipitation of histone H3 and H4 at 50 µg/ml novobiocin and that substantial precipitation of histone H3 and H4 occurs

Figure 5

Other proteins precipitated by novobiocin. Samples containing 1 µg of each of the listed proteins plus 5 µg of acid-extracted HTC histones were incubated with either 500 µg/ml novobiocin (lanes 7 and 8) or 1000 µg/ml novobiocin (lanes 9 and 10) in 150 mM NaCl, 10 mM Tris, pH 8.0 for 45 minutes on ice. The samples were then fractionated by centrifugation as described in the legend to figure 1, and resolved by SDS-PAGE. Lanes 1-6 contain 1 µg samples of the indicated proteins (5 µg of the histones). Caa, carbonic anhydrase, 2.7 mole percent arginine, pI 5.8; Sti, soybean trypsin inhibitor, 5.2 mole percent arginine, pI 4.6; Myo, myoglobin, 1.9 mole percent arginine, pI 6.8; Lys, lysozyme, 9.0 mole percent arginine, pI 10.5-11; Cyc, cytochrome c, 2.2 mole percent arginine, pI 10.6; histone H1, 1.5 mole percent arginine; histone H2A, 9.5 mole percent arginine; histone H2B, 6.5 mole percent arginine; histone H3, 13.5 mole percent arginine, histone H4, 14 mole percent arginine. The pIs of the four core histones and H1 are all greater than 9.5. P and S indicate pellet and supernatant.

at 100 µg/ml novobiocin in 2 M NaCl (figure 4, lane 7) while the bulk of the H2A and H2B remains in solution (figure 4, lane 8). Furthermore, precipitation of all four histone classes is complete at 300 µg/ml novobiocin. This is in contrast to the precipitation that occurs at 150 mM NaCl, where histones H3 and H4 begin to precipitate at 200 µg/ml and the precipitation of all four histone classes is not complete until 500 µg/ml. In 2 M NaCl, histones interact as heterotypic octamers (17). The observation that 100 µg/ml novobiocin causes histones H3 and H4 to precipitate from solutions containing 2 M NaCl, while histones H2A and H2B remain in solution suggests that the novobiocin-histone interaction disrupts the histone octamer.

Other Proteins Precipitated by Novobiocin

We were interested in determining the types of proteins that are precipitated by novobiocin. We have examined the effects of novobiocin on the solubility of a number of purified proteins with known charge and amino acid composition (carbonic anhydrase, soybean trypsin inhibitor, lysozyme, cytochrome c, myoglobin and core histones plus histone H1, see the legend to figure 5 for a listing of arginine content and pIs). As shown above, the core histones are completely precipitated by 500 $\mu\text{g/ml}$ novobiocin (figure 5, lane 8) while at 1000 $\mu\text{g/ml}$, lysozyme and histone H1 are precipitated (figure 5, lane 9). The presence of soybean trypsin inhibitor in the pellet at some of these novobiocin concentrations is due to an ionic interaction between the protein and histones because, when the precipitation of trypsin inhibitor is assayed from a pure solution of the protein, it remains soluble in 1000 $\mu\text{g/ml}$ novobiocin.

If one examines the amino acid content of the precipitated proteins however, one finds that (except for histone H1) only those proteins with greater than 6.5 mole percent arginine are susceptible to precipitation by this range of novobiocin concentrations. The observation that histones H3 and H4 (arginine content 13 and 14 mole percent) are precipitated at lower novobiocin concentrations than histones H2A and H2B (arginine content 6.5 and 9.5 mole percent) is consistent with this interpretation. Furthermore, the solubility of cytochrome c (pI 10.5, 2.2 mole percent arginine) at these high novobiocin concentrations suggests that it is not merely the charge that determines susceptibility to novobiocin precipitation.

Novobiocin Precipitates Nonhistone Chromosomal Proteins

We have also assayed the ability of novobiocin to precipitate a more general group of proteins. The proteins released from hydroxyapatite-bound chromatin between 0.3 M NaCl and 0.6 M NaCl (11) were incubated with various concentrations of novobiocin and the soluble and precipitated proteins were analyzed by SDS-PAGE (figure 6). We find that there is a substantial amount of precipitation in the presence of 500 $\mu\text{g/ml}$ novobiocin, including the precipitation of histones H2A and H2B (figure 6, lanes 7 and 8). In 1000 $\mu\text{g/ml}$ novobiocin, nearly all of these chromosomal proteins are precipitated (figure 6, lanes 15 and 16).

Polyarginine Competes with Histones for Novobiocin Precipitation

The unusual susceptibility of arginine-rich proteins to precipitation by novobiocin suggests that novobiocin might selectively interact with arginine residues during the precipitation process. To test this

Figure 6

Precipitation of nonhistone chromosomal proteins by novobiocin. The proteins that are eluted from hydroxyapatite-bound HTC chromatin between 0.3 M and 0.6 M NaCl (11) were exposed to various concentrations of novobiocin in 150 mM NaCl, 10 mM Tris, pH 8.0. The precipitated material was collected as described in the legend to figure 1 and supernatant (S) and pellet (P) samples were resolved by SDS-PAGE and the gel was stained with Coomassie blue followed by silver. The numbers at the side of the figure indicate molecular weights in kilodaltons. Also indicated are the positions of histones H1, H2A and H2B which are present in this protein preparation.

possibility, radiolabeled core histones were incubated with 1000 µg/ml novobiocin in the presence of increasing amounts of polyarginine. The concentrations of polyarginine (MW 40,000) ranged from 0.1 mg/ml (equal to 0.5 mM arginine) to 1 mg/ml (5 mM arginine). After fractionating the material by centrifugation, the radioactivity in the pellet and supernatant was quantitated. We find that with the highest polyarginine concentration tested there is a decrease in the precipitation of histones from the control value of 89% to a value of 33% suggesting that polyarginine is absorbing novobiocin and preventing the precipitation of histones (figure 7). The highest concentration of polyarginine tested (1 mg/ml) corresponds to an arginine concentration of 5 mM which is in molar excess to the amount of novobiocin in the system (1.6 mM). In order to test the possibility that the polycation is merely absorbing novobiocin through nonspecific ionic interactions, we tested the ability of polyornithine (molecular

Figure 7

Polyarginine competes with novobiocin for histone precipitation. HTC core histones (5 μ g, approximately 6000 cpm samples) labeled with ^3H -lysine were precipitated with 1000 μ g/ml novobiocin in 50 μ l of 150 mM NaCl, 10 mM Tris, pH 8.0 as described in the legend to figure 1. The histones were mixed with the amounts of polycation indicated on the abscissa, in 150 mM NaCl, 10 mM Tris, pH 8.0 before adding novobiocin to a final concentration of 1000 μ g/ml. The samples were then incubated for 45 minutes on ice, fractionated by centrifugation as in figure 1 and the radioactivity in the supernatant and pellet (after solubilization in 1% SDS) was determined by liquid scintillation counting. The percentage of the total sample (pellet plus supernatant) radioactivity present in the pellet of each sample is indicated on the graph.

weight 43,000) to compete in the precipitation assay. We find that at the molar concentrations of polyornithine equal to those tested for polyarginine, polyornithine has no effect on the precipitation of histones by novobiocin (figure 7).

Arginine Modification Prevents Histone Precipitation

We have also attempted to directly test the idea that there is a direct interaction between novobiocin and arginine residues, by assaying the effects on novobiocin precipitation of histones with arginine residues modified by 1,2-cyclohexanedione. This reagent has been used in other studies to specifically modify the arginine residues of a protein thereby limiting trypsin cleavage to lysine residues (14,15). If the guanidino moiety of arginine residues is crucial to the novobiocin-protein interaction, we reasoned that attaching a bulky cyclohexane group would probably interfere with this interaction.

Figure 8

Arginine modification prevents histone precipitation by novobiocin. Hyperacetylated HTC core histones were reacted with 1,2-cyclohexanedione as described in the Methods section. Unmodified or cyclohexanedione-modified (Dionated) histones were incubated with the indicated concentrations of novobiocin in 20 mM sodium borate, pH 8.5 for 45 minutes on ice. The samples were then fractionated by centrifugation as described in the legend to figure 1 and analyzed by electrophoresis on a Triton-acid-urea gel. P and S refer to pellet and supernatant.

Core histones are normally completely precipitated by 500 µg/ml novobiocin (figure 8, lanes 3 and 4; see also figure 1, lanes 13 and 14). However, if these same histones are first modified with cyclohexanedione, (subsequently dialyzed extensively to remove unreacted dione) and then exposed to novobiocin, they remain soluble in concentrations of novobiocin up to 1000 µg/ml (figure 8, lanes 8-12).

DISCUSSION

We have demonstrated that novobiocin interacts with core histones with approximately 15 moles of novobiocin binding per histone molecule resulting in precipitation of the protein. This process is so efficient that it can

be used to precipitate histones from solutions as dilute as 0.1 $\mu\text{g/ml}$ (M.C. unpublished data). Furthermore, the precipitation process is accentuated by high NaCl concentrations, providing a useful method of recovering histones from high salt solutions.

The interaction between novobiocin and basic proteins appears to have some specificity for arginine residues. Of the proteins which we have found to be precipitated by novobiocin, all except histone H1 possess greater than 6.5 mole percent arginine. Furthermore, modification of arginine residues on core histones interferes with the precipitating effects of novobiocin. Moreover, polyarginine but not polyornithine competes with histones for novobiocin precipitation. On the other hand, we have observed that the presence of free arginine (in 3-fold molar excess to novobiocin) has no effect on histone precipitation (M.C., unpublished data). It appears then, that there must be various degrees of novobiocin-arginine binding, depending on the local environment of the arginine residue. Indeed, in this regard, we note that the most efficiently precipitated proteins contain sets of adjacent arginine residues.

The observation that histone H1 is moderately efficiently precipitated by novobiocin (it is completely precipitated by 750 $\mu\text{g/ml}$ novobiocin in 150 mM NaCl) despite its low arginine content (1.5 mole percent) probably reflects the protein's unusually high positive charge content (29 mole percent lysine) and the development of interactions between the negative charge of novobiocin and this cation. We have observed that polyarginine can be precipitated by 250 $\mu\text{g/ml}$ novobiocin while polyornithine is not completely precipitated until concentrations of novobiocin in excess of 750 $\mu\text{g/ml}$ are reached (M.C. unpublished data).

The competitive inhibition of *E. coli* gyrase by novobiocin occurs with a K_i of 6.0×10^{-9} M (2). This strong affinity of novobiocin for the gyrase B subunit has been used as the basis for a purification of gyrase with a novobiocin-Sepharose affinity column (4). We have found that when *Xenopus laevis* oocyte extracts are fractionated on novobiocin-Sephadex, the elution profile indicates that histones and many other proteins are differentially retained. However, histones are not strongly bound to the resin indicating that the histone interaction with novobiocin is weak relative to that of the gyrase B subunit (M.C., unpublished data). Evidently there is a spectrum of novobiocin-protein interactions ranging from the strong interaction demonstrated by the B subunit of DNA gyrase to the moderate interaction with arginine-rich proteins such as the core

histones to a weak interaction with basic proteins in general, demonstrated by the nature of the interaction with histone H1 and lysozyme.

The observation that novobiocin can precipitate arginine-rich proteins at low concentrations and many proteins at higher concentrations has important implications. This antibiotic has recently been used in experiments designed to implicate type II topoisomerase in chromatin structure alterations. It has been reported that treatment of Drosophila cells with 150 $\mu\text{g/ml}$ novobiocin leads to the blockage of a heat shock-induced chromatin rearrangement (10). There has also been a report of 1000 $\mu\text{g/ml}$ novobiocin causing the alteration of globin gene chromatin structure (9). It is possible that the basis for these observations may indeed be an alteration in topoisomerase activity, but certainly the general interaction with (and possibly even precipitation of) histones and other proteins by these concentrations of novobiocin must be considered in the interpretation of these experiments. We have observed that 1000 $\mu\text{g/ml}$ novobiocin in 0.15 M NaCl can precipitate nearly all of the proteins dissociated from chromatin at 0.6 M NaCl. One wonders what profound effects this concentration of novobiocin has when it is used to treat whole cells.

There have been several intriguing experimental results reported recently which have been interpreted as being due to the inhibitory action of novobiocin on topoisomerase activity, that could be readily ascribed to the precipitation reactions described in this paper. For instance, one group injected a 5S rRNA gene into Xenopus oocytes, allowed chromatin assembly to occur and then coinjected novobiocin (to a final concentration approaching 1000 $\mu\text{g/ml}$) and labeled GTP (7). The novobiocin injection inhibits transcription of the 5S gene. In light of the results of this paper, one cannot ignore the likelihood that this inhibition is a consequence of protein precipitation rather than reflecting a putative role for a gyrase activity in maintaining the 5S gene chromatin in a transcriptionally-active state.

In a second set of experiments, Glikin, et al., have developed a Xenopus laevis oocyte extract which is capable of assembling chromatin onto added DNA (8). Novobiocin interferes with the assembly reaction at 250 $\mu\text{g/ml}$, certainly a concentration that is capable of altering histone solubility. This sensitivity to novobiocin is interpreted as an indication that a gyrase activity is required for the chromatin assembly process (8). Our data would indicate that at present, one cannot exclude the possibility that novobiocin is merely removing histones from the assembly system.

There are some indications that ATP and magnesium may be able to counter the inhibitory effects of novobiocin in the chromatin assembly system of Glikin et al. (8). This is consistent with the effects of novobiocin on bacterial gyrase activity, where novobiocin behaves as a competitive inhibitor for ATP. However, in several attempts, we have been unable to detect any effect of ATP or magnesium on novobiocin precipitation of histones in purified systems.

The results of our experiments clearly dictate that the antibiotic novobiocin should be used with caution in experiments designed to detect eukaryotic type II topoisomerase activity and the roles of this type of enzyme in nuclear functions. Because novobiocin can remove histones from solution as well as interfere with topoisomerase activity, and because both of these events can cause changes in DNA topology, both effects must always be considered. On the other hand, the interaction of novobiocin with arginine-rich proteins, especially histones, can be exploited. We have found that novobiocin precipitation can be used as a convenient histone isolation procedure, capable of precipitating histones from solutions as dilute as 100 ng/ml (M.C. unpublished data). Furthermore, the observation that histones H3 and H4 are precipitated by lower novobiocin concentrations than histones H2A and H2B suggests a simple method of purifying these two subsets of histones.

Finally, we wish to stress that the experiments reported in this paper were not designed to test, and in no way disprove, the notion that there is a role for topological stress in transcription or replication. We simply wish to draw attention to the serious drawbacks associated with the use of novobiocin as a means to test hypotheses concerning torsional strain in eukaryotic DNA molecules.

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