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# Characterization of purified DNA-relaxing enzyme from human tissue culture cells

**(supercoiled simian virus 40 DNA/DNA replication/ethidium bromide/gel electrophoresis)**

WALTER KELLER

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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**ABSTRACT** **Superhelical simian virus 40 DNA migrates more rapidly during electrophoresis in agarose gels than covalently closed DNA free of superhelical turns (relaxed DNA). The difference in electrophoretic mobility between superhelical and relaxed DNA was used to monitor the activity of a protein from human tissue culture cells which converts superhelical DNA into relaxed DNA. Purified DNA-relaxing protein removes both negative and positive superhelical turns and acts in a catalytic manner. The relaxation of DNA proceeds in a stepwise fashion and DNA intermediates with decreasing numbers of superhelical turns are seen during the course of the reaction.**

Proteins that can remove superhelical turns from circular DNA have been found in *Escherichia coli* (1), in extracts of mouse embryo cells (2), in eggs of *Drosophila melanogaster* (3), and in human and mouse tissue culture cells (4). These proteins are suspected to act as "swivels" during DNA replication (1, 2). The main obstacle to the purification of DNA-relaxing proteins has been the lack of a convenient and rapid assay procedure. Assays used until now have been based on the measurement of either a change in the sedimentation rate of DNA (1) or a shift in the buoyant density of a DNA/propidium iodide complex after incubation with an extract containing DNA-relaxing activity (2). Both methods are time-consuming and cannot be performed on many samples simultaneously.

Here I describe a way to use agarose gel electrophoresis to monitor changes in the tertiary structure of superhelical DNA. The method is based on the observation (5–7) that superhelical DNA migrates more rapidly than nicked-circular or linear molecules of the same molecular weight. (A nick is a broken phosphodiester bond.) With the aid of this technique as an assay, a DNA-relaxing protein was purified from human tissue culture cells and its properties were investigated. A preliminary report of this work has been published elsewhere (8). (For a general review of the properties of superhelical DNA, see ref. 9.)

## MATERIALS AND METHODS

Simian virus 40 (SV40) DNA was prepared from purified virions by standard methods (10). Radioactively labeled SV40 DNA was obtained by adding [$^3$H]thymidine or [$^{32}$P]orthophosphate to virus-infected cell cultures. The methods used to purify adenovirus type 2 DNA and to grow human cells (KB-3) in spinner cultures have been described previously (11, 12).

Abbreviations: EtdBr, ethidium bromide; SV40, simian virus 40; mol. wt., molecular weight.

**Assay of DNA-Relaxing Activity.** Reaction mixtures contained in a total volume of 0.05 ml: 0.01 M Tris·HCl, pH 7.9; 0.2 M NaCl; 0.2 mM EDTA; 0.1–1.0 μg of unlabeled or $^{32}$P-labeled SV40 DNA; and 5 μl of protein solution. The mixtures were incubated for 30 min at 37° and the reaction was stopped by adding 5 μl of 10% sodium dodecyl sulfate. The DNA was then analyzed by electrophoresis in gel slabs of 1.4% agarose as described by Sugden *et al.* (7) and examined after staining with ethidium bromide (EtdBr) (0.5 μg/ml in electrophoresis buffer) on a shortwave ultraviolet-light-plate (UV Products, San Gabriel, Calif.). Photographs were taken with Polaroid type 55 P/N film and a Kodak 23A red filter. For quantitation of the amount of radioactive DNA, fluorescent bands were cut from the gels with a razor blade and their radioactivity was measured in a scintillation spectrometer without addition of scintillation fluid (Cerenkov radiation). The counting efficiency was about 10%. In some cases DNA was analyzed by electrophoresis in cylindrical gel tubes as described in the appropriate figure legends.

One unit of DNA-relaxing activity is defined as the amount of protein that converts 1 μg of supercoiled SV40 DNA into the completely relaxed form I′ in 30 min at 37°.

**Purification of DNA-Relaxing Activity.** The purification of DNA-relaxing activity described below was carried out with 200 g of frozen KB cells as starting material. All procedures were conducted between 0° and 4°.

Lysis of cells, extraction of the proteins, removal of DNA, and ammonium sulfate precipitation were carried out as described previously (12). The material was dialyzed exhaustively against 0.05 M Tris·HCl, pH 7.9, 1 mM EDTA, 1mM dithiothreitol, and 15% (v/v) glycerol (buffer A). A precipitate of insoluble protein that formed during dialysis was removed by centrifugation for 1 hr at 25,000 rpm in an SW27 rotor. The supernatant was applied to a column (5 × 25 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with buffer A. The column was washed with 5 volumes of buffer A and the adsorbed protein was eluted with buffer A containing 0.2 M KCl, dialyzed against 0.02 M potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol (buffer B), and applied to a column (3.5 × 25 cm) of phosphocellulose (Whatman P11) equilibrated with buffer B. The column was washed with 5 volumes of buffer B and subsequently developed with 2 liters of a linear gradient of 0.02–0.8 M potassium phosphate in buffer B. Fractions of 20 ml were collected and assayed for DNA-relaxing activity. The activity binds strongly to phosphocellulose and elutes at a phosphate concentration of approximately 0.5 M, whereas the bulk of the protein elutes at lower salt concen-

Table 1. Purification of DNA-relaxing activity from 200 g of KB cells

| Purification step | Total protein (mg) | Total activity (units × $10^{-6}$) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| 1. High-speed supernatant of crude extract | 12,600 | 5.04 | 400 | 100 |
| 2. Ammonium sulfate precipitate | 7,800 | 3.98 | 510 | 79 |
| 3. DEAE-cellulose chromatography | 4,100 | 3.16 | 770 | 63 |
| 4. Phosphocellulose chromatography | 95 | 0.91 | 9,580 | 18 |
| 5. CM-Sephadex chromatography | 32 | 0.83 | 25,900 | 16 |
| 6. DNA-cellulose chromatography | 12 | 0.58 | 48,000 | 11 |
| 7. Sucrose gradient | 0.6 | 0.31 | 510,000 | 6 |

DNA-relaxing activity was assayed as described in *Materials and Methods*. Protein concentrations were determined as described earlier (12).

trations. Fractions containing relaxing activity were combined, dialyzed against buffer B, and applied to a column (3 × 15 cm) of carboxymethyl (CM)-Sephadex (Pharmacia C50). After washing with buffer B, adsorbed protein was eluted with a 400-ml gradient of 0.02–0.6 M potassium phosphate in buffer B. Fractions of 6 ml were collected and assayed for DNA-relaxing activity. As in the phosphocellulose step, most protein elutes at lower salt concentrations than the DNA-relaxing activity, which elutes at approximately 0.3 M potassium phosphate. Fractions containing activity were combined, dialyzed against buffer B, and applied to a column (1.5 × 10 cm) of DNA-cellulose prepared as described by Alberts and Herrick (13) with phage T4 DNA. After washing, the column was eluted with a 140-ml linear gradient of 0.02–0.6 M potassium phosphate in buffer B. Fractions of 2 ml were collected. The DNA-relaxing activity eluted at a phosphate concentration of approximately 0.25 M. For concentration of the activity, the material was dialyzed against buffer B, adsorbed to a 0.7 × 2 cm column of DNA-cellulose, and eluted with buffer B containing 0.5 M potassium phosphate. This fraction was dialyzed for 4 hr against buffer B containing 0.2 M potassium phosphate. Aliquots of 0.4 ml were layered onto gradients of 5–20% sucrose in buffer B containing 0.2 M phosphate, and centrifuged at 40,000 rpm in a Spinco SW41 rotor for 85 hr. Fractions of 0.5 ml were collected from the bottom of the centrifuge tubes. Fractions containing DNA-relaxing activity were combined, and the solution was stored in liquid nitrogen in the presence of 0.1 mg/ml of bovine serum albumin.

## RESULTS AND DISCUSSION

The purification of DNA-relaxing activity from KB cells is summarized in Table 1. The DNA-relaxing activity is contained in a protein as judged by the following criteria: *(a)* preincubation with Pronase (0.3 mg/ml) or with sodium dodecyl sulfate (1%) or heating to 50° for 5 min destroys the activity; *(b)* the ratio of absorbance at 280/260 nm of fraction 6 (Table 1) is 1.9–2.0, indicating the absence of a major nucleic acid component; *(c)* the activity is completely inhibited by the presence of 0.3 mM *p*-chloromercuribenzoate or 0.3 mM *N*-ethylmaleimide. This latter effect not only suggests that the DNA-relaxing activity is contained in a protein but also indicates that functional sulfhydryl groups are required for its action.

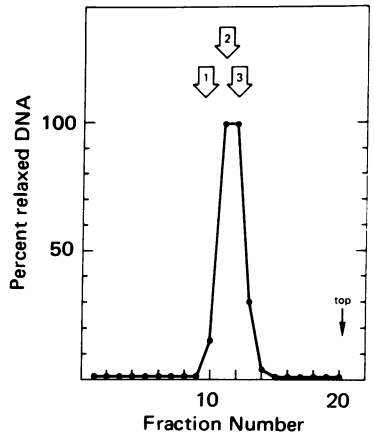


FIG. 1. Sucrose gradient centrifugation of purified DNA-relaxing activity. Fraction 6 (0.18 ml) (Table 1), containing 1800 units of activity, was mixed either with 0.02 ml of purified phage-T4-induced DNA polymerase [mol. wt. 114,000 (18)], or with 0.02 ml of the large fragment of DNA polymerase I from *E. coli* [mol. wt. about 75,000 (19)], or with 0.02 ml of bovine serum albumin (mol. wt. 68,000), and layered on gradients containing 5–20% sucrose, 0.2 M potassium phosphate, pH 7.5, 1 mM EDTA, and 1 mM dithiothreitol, and centrifuged for 16 hr at 50,000 rpm in a Spinco SW56 rotor at 2–3°. DNA-relaxing activity was assayed as described in *Materials and Methods*, with $^{32}$P-labeled SV40 DNA (60,000 cpm/μg). The numbered arrows indicate the position of the marker proteins in the gradients. 1 = T4 DNA polymerase; 2 = large fragment of *E. coli* DNA polymerase I; 3 = bovine serum albumin.

**Molecular Weight and Purity.** The molecular weight of DNA-relaxing protein was estimated by zone sedimentation through a sucrose gradient in the presence of markers of known molecular weight (Fig. 1). The activity sedimented as a single peak with a sedimentation coefficient of 4.4–4.5 S. From the relationship $S_1/S_2 = (MW_1/MW_2)^{2/3}$ (14), this corresponds to a molecular weight of approximately 70,000. Sodium dodecyl sulfate/polyacrylamide electrophoresis of material from fraction 7 (Table 1) revealed one major and three minor protein bands (Fig. 2). Comparison of the electrophoretic mobility of the major protein band with that of marker proteins (15) showed the major protein to have a molecular weight of 60,000 ± 3,000. The molecular weights of the minor proteins were about 90,000, 100,000, and 120,000, respectively. The protein with a molecular weight of 60,000 coincided exactly with the peak of DNA-relaxing activity, whereas the three minor proteins were present in greater quantity in fractions sedimenting slightly faster than the relaxing activity in sucrose gradients. It is likely, therefore, that the protein of 60,000 molecular weight actually represents relaxing activity; at least 40% of the protein in fraction 7 consists of the species with a molecular weight of 60,000. The sedimentation coefficient of native DNA-relaxing protein is slightly higher than expected for a spherical protein of 60,000 daltons (14). The native protein could, therefore, consist of a highly globular, compact polypeptide chain of 60,000 daltons or it could be an asymmetric molecule containing two subunits of 60,000 daltons. At present, we cannot

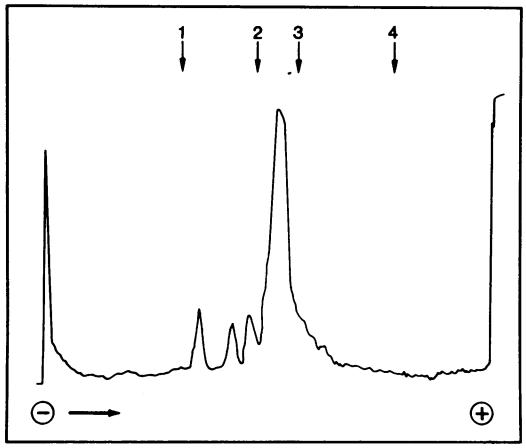


FIG. 2. Densitometer tracing of a sodium dodecyl sulfate/polyacrylamide gel of purified DNA-relaxing activity. An aliquot of the peak fractions from the sucrose-gradient step (fraction 7, Table 1) containing 50,000 units of activity was dialyzed against 1 mM Tris·HCl, pH 7.5, and then lyophilized. The protein was dissolved in 0.1 ml of 0.1% dodecyl sulfate, 10% (v/v) glycerol, 1% 2-mercaptoethanol, incubated at 60° for 20 min, and analyzed by electrophoresis through a gel containing 5% polyacrylamide (15). The protein was stained with Coomassie blue and the gel was traced with a Joyce-Loebl Chromoscan at 620 nm. The total amount of protein was estimated by measuring the areas underneath the peaks and comparing them to the area from gel tracings containing known amounts of marker proteins as standards. The numbered arrows indicate the positions of molecular weight markers run in a separate gel. 1 = β-galactosidase from *E. coli* [mol. wt. 130,000 (15)]; 2 = bovine serum albumin [mol. wt. 68,000 (15)]; 3 = γ-globulin, H chain [mol. wt. 50,000 (15)]; 4 = γ-globulin, L chain [mol. wt. 23,500 (15)].

distinguish between these alternatives. Fraction 7 is not contaminated by deoxyribonuclease, ribonuclease, ribonuclease H, DNA ligase, DNA polymerase, RNA polymerase, or polynucleotide kinase.

**Interaction with DNA Containing Negative Superhelical Turns.** As illustrated in Fig. 3, DNA-relaxing activity

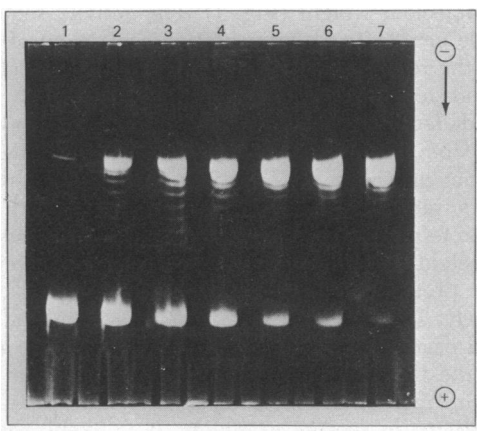


FIG. 3. Agarose slab gel electrophoresis of SV40 DNA treated for various times with DNA-relaxing activity. Reaction mixtures containing 2 μg of SV40 DNA and 10 units of DNA-relaxing activity (fraction 6, Table 1) were incubated at 37° for the time indicated below, stopped by adding 5 μl of 10% sodium dodecyl sulfate, and the DNA was analyzed by electrophoresis in a 1.4% agarose gel slab as described in *Materials and Methods*. Electrophoresis was carried out for 12 hr at 40 V. Incubation times were as follows: 1 = 0 min; 2 = 0.5 min; 3 = 1 min; 4 = 3 min; 5 = 6 min; 6 = 12 min; 7 = 30 min.

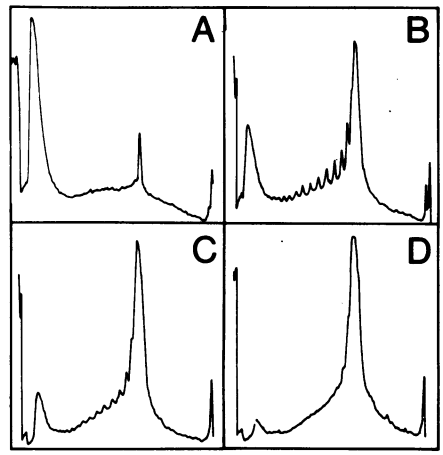


FIG. 4. Relaxation of SV40 DNA at 37°. Reaction mixtures contained 2 μg of SV40 DNA and 3 units of DNA-relaxing activity (fraction 6, Table 1). Samples were layered onto cylindrical gels containing 1.4% agarose in 16 cm × 6 mm (internal diameter) glass tubes. Electrophoresis was carried out for 22 hr at 30 V in a conventional electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The gels were stained and photographed as described in *Materials and Methods*. The photographic negatives were scanned with a Joyce-Loebl microdensitometer. Reaction times were: A = 0 min; B = 0.5 min; C = 3 min; D = 10 min. The direction of electrophoresis was from right to left.

converts supercoiled SV40 DNA (form I) into a new form (designated form I′) which exhibits the same low electrophoretic mobility as open-circular, nicked (form II) DNA. As will be demonstrated below, this form I′ DNA corresponds to closed-circular DNA containing no superhelical turns. When the concentration of sodium chloride in the reaction mixture was lowered from 0.2 M to 0.05 M, no activity could be detected. Likewise, raising the salt concentration above 0.4 M abolished the activity. A similar effect was observed with potassium chloride. The monovalent salt could be replaced by 10 mM magnesium chloride. However, the activity was reduced under these conditions to about half the values observed with 0.2 M sodium chloride. Fig. 3 shows that after a short incubation, some DNA migrated as distinct bands between forms I and I′; the quantity of DNA present in these intermediate bands decreased with increasing time of incubation (Fig. 4). However, when the reactions were performed at 0° instead of 37°, the rate of conversion was reduced about 60-fold and more DNA was seen in bands with intermediate mobilities (Fig. 5).

Treatment with Pronase and extraction with dodecyl sulfate/phenol after the reaction had no effect on the electrophoretic mobility of the DNA; this indicates that the conversion of DNA from form I to form I′ by relaxing activity is irreversible.

**Analysis of the Reaction Products by the Buoyant Separation Method.** If the reduction in electrophoretic mobility of supercoiled DNA after treatment with relaxing protein results from a progressive removal of superhelical turns caused by temporary nicking of one DNA strand, rotation of the two ends at the nick in relation to the helix axis, and subsequent resealing events, relaxed DNA should have a reduced superhelix density, σ (9). In the experiment shown in Fig. 5 (panel 3) $^{32}$P-labeled SV40 DNA was treated at 0° with relaxing protein and subjected to electrophoresis. The gel was sliced and the DNA was extracted from the pooled slices (a) to (d) as indicated. The superhelix density of sam-

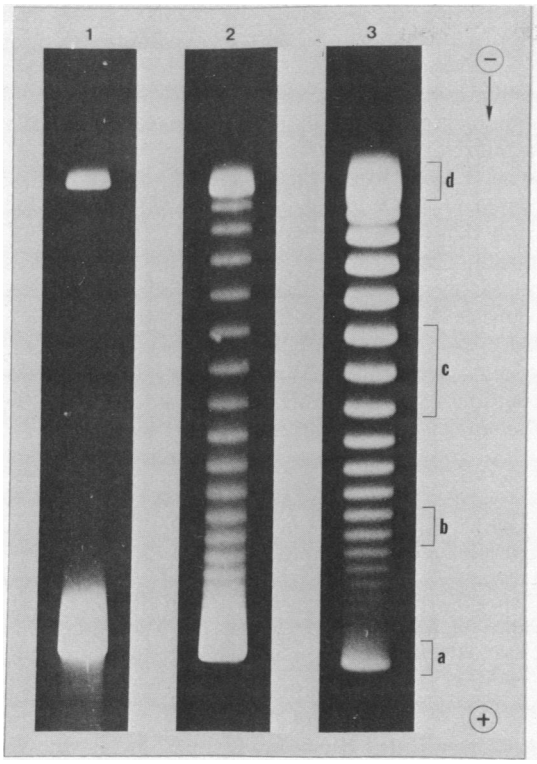


FIG. 5. Relaxation of SV40 DNA at 0°. Reaction mixtures with a total volume of 0.03 ml contained 1.2 μg of SV40 [$^{32}$P]DNA (400,000 cpm/μg) and 30 units of relaxing activity (fraction 6, Table 1). Electrophoresis was carried out in cylindrical gels as described in the legend of Fig. 4 for 17 hr at 60 V. Incubation times: 1 = 0 min; 2 = 5 min; 3 = 30 min. Gel no. 3 was sliced into 1 mm segments and the DNA in the fractions corresponding to the brackets (a) to (d) was extracted by homogenization with a Potter-Elvehjem homogenizer in 1 ml of 0.02 M Tris·HCl, pH 8, 1 mM EDTA. Agarose was removed by centrifugation at 1000 × *g* for 5 min and the DNA was analyzed as described in the legend of Fig. 6 (see *text* for explanation).

ples (a) to (d) was measured by the buoyant separation technique (9) as described in the legend of Fig. 6, with untreated SV40 [$^{3}$H]DNA as density marker. The slow-migrating DNA band (pool d) showed a reduction of $\sigma$ as compared to a marker of untreated DNA [$\sigma$ = 0.039 in 2.85 M CsCl (16)] corresponding to a value of $\Delta\sigma$ = -0.034. Since the initial superhelix density of SV40 DNA in 0.2 M NaCl (the ionic strength of the reaction mixture) is about 13% lower than in 2.85 M CsCl (17), the superhelix density of the DNA from pool (d) had been reduced to zero by incubation with relaxing protein. The DNA in pool (b) had a value of $\Delta\sigma$ = 0.012 and that from pool (c) a value of $\Delta\sigma$ = 0.025. These values are characteristic for DNA that has undergone partial relaxation. This experiment thus corroborates the correlation of superhelix density and electrophoretic mobility of closed-circular DNA. The finding of partially relaxed DNA at early times of reaction indicates that the relaxing activity does not act by a one-hit mechanism but causes a stepwise relaxation of superhelical DNA.

**Interaction with DNA Containing Positive Superhelical Turns.** To test the effect of DNA-relaxing activity from KB cells on positively supercoiled DNA, we added ethidium bromide (EtdBr) to the reaction mixtures in an amount sufficient to bring the superhelix density of form I DNA to a value of $\sigma$ = +0.04. The concentration of EtdBr required to induce this change of conformation was calculated with the

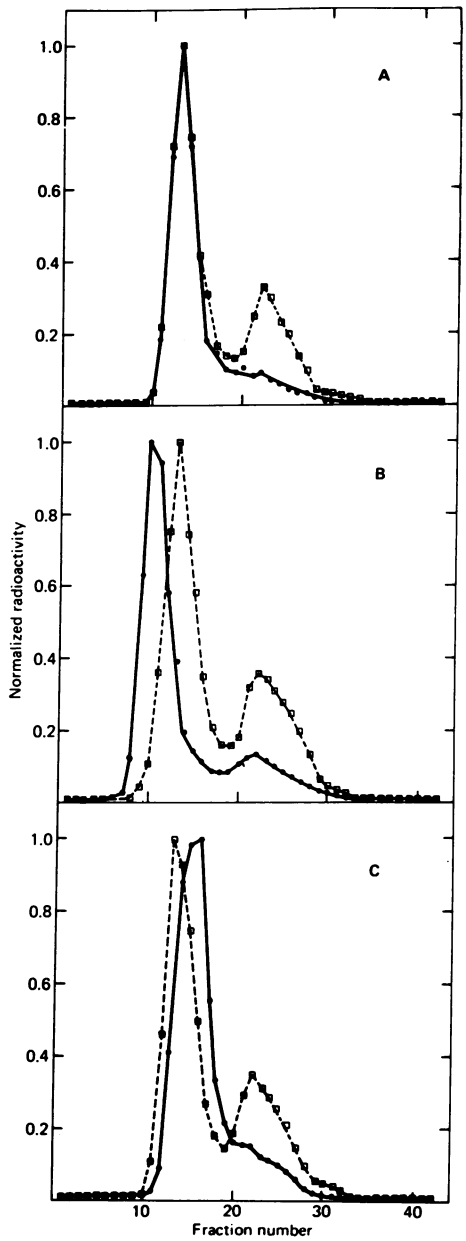


FIG. 6. Removal of negative and positive superhelical turns; analysis by the buoyant separation technique. 0.1 μg of SV40 [$^{32}$P]DNA (2·10$^{6}$ cpm/μg) was incubated for 30 min at 37° in a standard reaction mixture containing 5 units of DNA-relaxing activity (fraction 6, Table 1). A second reaction mixture contained in addition 3 μg/ml of EtdBr. A control mixture was incubated without relaxing activity. After the incubation, the reaction mixtures were diluted to 3.25 ml with 0.01 M Tris·HCl, pH 7.9, 1 mM EDTA, and 3.40 g of CsCl was added. After the addition of 20 μl of $^{3}$H-labeled SV40 DNA (2000 cpm/μl) as density marker and 1.0 ml of propidium diiodide (2.5 mg/ml; Calbiochem), the mixtures were centrifuged at 32,000 rpm in a SW50.1 rotor at 20° for 40 hr. Fractions were collected from the bottom of the centrifuge tubes and the DNA was precipitated with cold 5% trichloroacetic acid and collected on Whatman GF/C glass fiber filters. After drying, the filters were analyzed for radioactivity in Liquifluor. Panel A, control without DNA-relaxing activity; panel B, density separation of DNA treated with relaxing activity in the absence of EtdBr; panel C, density separation of DNA treated in the presence of EtdBr. (●, [$^{32}$P]DNA; □, [$^{3}$H]DNA). The density shifts compared to marker DNA were determined according to the equation given by Bauer and Vinograd (9). The $\sigma$-values thus obtained are based on a DNA unwinding angle for EtdBr of 12°.

equation given by LePecq and Paoletti (20). Control reactions were run in the absence of EtdBr. After incubation with relaxing activity from various stages of purification, the DNA was analyzed by the buoyant separation technique (9). The results obtained with fraction 6 (Table 1) are shown in Fig. 6. SV40 DNA treated with relaxing activity in the absence of EtdBr exhibited a heavier buoyant density than marker DNA, corresponding to a reduction of the superhelix density of $\Delta\sigma = 0.035$ (Fig. 6, panel B). Thus, all negative turns initially present in the DNA had been removed. By contrast, when the DNA initially contained positive superhelical turns, owing to the presence of EtdBr, its reaction product had lighter density than marker DNA in the buoyant separation gradient (Fig. 6, panel C), corresponding to a change in superhelix density of $\Delta\sigma = 0.04$. Therefore, the relaxing activity had removed all positive superhelical turns. The activities acting on negatively and positively supercoiled DNA co-purified at all stages of the fractionation; it is likely, therefore, that a single protein is responsible for both activities.

**Stoichiometric Versus Catalytic Action.** The interaction of relaxing protein with superhelical DNA results in an irreversible change in the tertiary structure of the DNA. There are two possible ways in which this might occur.

*(a)* DNA-relaxing protein might bind to superhelical DNA in a reversible manner, as for example histones bind to DNA. Upon binding, the average rotation angle per base pair could be slightly decreased, thereby unwinding the helix and consequently reducing the number of superhelical turns. The topological constraint (9) in cyclic DNA requires that the number of superhelical turns ($\tau$) plus the number of helix turns per ten base pairs ($\beta$) be constant ($\tau+\beta$ = constant). Thus, binding of protein to DNA would result in a change of $\beta$. *(b)* The second possible mechanism requires that the topological constraint be temporarily absent during the reaction. The relaxing protein might transiently introduce a nick into one of the polynucleotide chains, allowing unwinding. If one molecule of DNA-relaxing activity can perform this nicking and resealing of DNA only once, a stoichiometric interaction would be observed. If more than one molecule of superhelical DNA can be converted into the relaxed form by one molecule of relaxing protein, its action must be catalytic.

The first mechanism requires the binding of many molecules of relaxing protein per DNA molecule and the effect would be reversible. As shown above, the action of relaxing protein is irreversible. The observation that the relaxing protein is able to remove both negative and positive superhelical turns is also incompatible with such a model.

As far as the second mechanism is concerned, a simple calculation using the specific activity of fraction 7 (Table 1) and assuming a molecular weight of DNA-relaxing protein of 60,000 shows that one molecule of protein can relax about 10 molecules of SV40 DNA. This is a minimal estimate because fraction 7 does not consist of pure relaxing protein and because the relaxation reaction does not proceed with a one-hit mechanism.

Most probably therefore, DNA-relaxing activity acts by the catalytic nicking and re-sealing of DNA chains. Thus, the activity can be called "DNA-relaxing *enzyme*." The detailed molecular mechanism of the relaxing reaction remains to be elucidated. Attempts to demonstrate nicked reaction intermediates have so far been unsuccessful, presumably because the nicking and sealing reactions are much faster than the overall relaxation.

The relaxation reaction proceeds without an external energy donor (1, 2). Wang (1) has proposed for the $\omega$-protein of *E. coli* that the protein remains bound to the DNA after the introduction of a nick and that the energy gained by cleaving a phosphodiester bond is stored in this DNA–protein complex. After relaxation of the DNA, this energy could be used to restore a phosphodiester bond. Alternatively, the free energy of the superhelix could be utilized for the sealing reaction. However, such a mechanism would require some means of transducing mechanical into chemical energy.

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