Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 3872–3876, November 1976
Biochemistry

DNA gyrase: An enzyme that introduces superhelical turns into DNA

(*Escherichia coli*/ATP-dependent reaction/superhelix density)

MARTIN GELLERT*, KIYOSHI MIZUUCHI*, MARY H. O'DEA*, AND HOWARD A. NASH†

* Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, and † Laboratory of Neurochemistry, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Gary Felsenfeld, August 6, 1976

ABSTRACT **Relaxed closed-circular DNA is converted to negatively supercoiled DNA by DNA gyrase. This enzyme has been purified from *Escherichia coli* cells. The reaction requires ATP and Mg^{++} and is stimulated by spermidine. The enzyme acts equally well on relaxed closed-circular colicin E1, phage λ, and simian virus 40 DNA. The final superhelix density of the DNA can be considerably greater than that found in intracellularly supercoiled DNA.**

In the course of studies on integrative recombination of phage λ DNA in a cell-free system from *Escherichia coli* (1, 2) we became aware that the process required a negatively supercoiled DNA substrate. This substrate could be replaced by relaxed closed-circular DNA only if the latter was incubated with an *E. coli* cell fraction and ATP (K. Mizuuchi, M. Gellert, and H. Nash, manuscript in preparation). The simplest interpretation of these results was that the *E. coli* extract contains an ATP-dependent activity capable of converting relaxed closed-circular DNA to the supercoiled form. We have obtained a considerable purification of this enzyme, which we call DNA gyrase, and report here the purification procedure and preliminary characterization of the enzyme.

MATERIALS AND METHODS

Chemicals. The four ribonucleoside and deoxyribonucleoside triphosphates were obtained from Sigma Chemical Co. Deoxyribonucleoside triphosphates were treated with periodate before use. Polymin P was a gift of BASF, WHOZ Hauptlaboratorium B9, Hochschullieferungen, 6700 Ludwigshafen/Rhein, Germany. Dilutions were made from a 10% (vol/vol) stock solution which had been neutralized to pH 7.9 with concentrated HCl and clarified by centrifugation (3). Purified *E. coli* ω protein (4) was a gift from J. C. Wang. Restriction endonuclease *Eco*RI was obtained from Miles Laboratories.

Preparation of Substrates. [^{3}H] Colicin E1 (Col E1) plasmid DNA was prepared from *E. coli* strain A745 *thy* as described by Sakakibara and Tomizawa (5). Unlabeled Col E1 DNA was prepared similarly, but with the thymine concentration in the medium increased to 20 μg/ml. DNA of the plasmid pNT1, a small variant (2.6×10^6 molecular weight) of Col E1 constructed by J. Tomizawa, was prepared in the same way, by starting from strain NT422 *recA*. Simian virus 40 DNA was prepared from infected cells (6); λ DNA was prepared from the purified virus by phenol extraction.

Relaxed closed-circular Col E1 DNA, pNT1 DNA, and simian virus 40 DNA were prepared by treating supercoiled DNA with a nicking-closing extract prepared from duck reticulocytes. (We are indebted to R. D. Camerini-Otero for generous gifts of this extract). Relaxed closed-circular λ DNA was prepared by sealing hydrogen-bonded circular DNA with DNA ligase (2). These DNA samples were deproteinized by shaking with chloroform–isoamyl alcohol (24:1 vol/vol) and repurified by centrifugation in cesium chloride-ethidium bromide density gradients. All DNA samples were dialyzed and stored in 0.01 M Tris·HCl at pH 8.0, 1 mM Na_3EDTA, at 4°.

Assay of DNA Supercoiling. The assay measures the conversion of relaxed closed-circular Col E1 DNA to the supercoiled form, as demonstrated by agarose gel electrophoresis. The standard reaction mixture (70 μl) contained 35 mM Tris·HCl at pH 7.5, 1.6 mM $MgCl_2$, 18 mM potassium phosphate at pH 7.5, 5 mM spermidine·HCl, 1.4 mM ATP, 90 μg/ml of *E. coli* tRNA (Calbiochem), 3.6 mg/ml of bovine serum albumin (Armour, crystalline), and 0.4 μg of relaxed covalently circular Col E1 DNA. Enzyme (1–5 μl) was diluted when necessary into 0.2 M potassium phosphate at pH 6.8, 1 mM Na_3EDTA, 1 mM dithiothreitol, 10% glycerol (wt/vol), and 3.6 mg/ml of bovine serum albumin.

The solution was incubated at 25° for 60 min, and then extracted with an equal volume of chloroform–isoamyl alcohol (24:1 vol/vol). After brief centrifugation, 50 μl of the aqueous phase were added to 12.5 μl of a mixture of 5% sodium dodecyl sulfate, 25% glycerol, and 0.25 mg/ml of bromphenol blue, and the sample was loaded onto an agarose gel. Up to 30 samples at a time were electrophoresed in a 6 × 230 × 160 mm slab (E-C Apparatus Corp.) of 0.8% agarose (Type II, Sigma Chemical Co.) with Tris-borate-EDTA buffer (10.8 g of Tris base, 5.5 g of boric acid, and 0.93 g of Na_2EDTA per liter). After 16 hr of electrophoresis at 40 V, the slab was stained with 1 μg/ml of ethidium bromide in the electrophoresis buffer for 1 hr, and destained in electrophoresis buffer for at least an hour. The slab was photographed using two short wave ultraviolet lamps (Type S-68, Ultraviolet Products, Inc.) and Polaroid Type 105 film, with a Wratten 23A filter over the camera lens.

Growth of *E. coli* Cells. A 300-liter culture of *E. coli* N99 *rec*B_{21} aerated at 31° in LB medium [10 g of Bacto-tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of NaCl per liter] until the OD_{650} reached 0.65. After chilling, the cells were collected by centrifugation and resuspended in 10% sucrose, 0.05 M Tris·HCl at pH 7.5, with 66 ml of buffer per 100 g of packed cells. The suspension was stirred at low speed in a Waring blendor until homogeneous; aliquots were frozen in liquid nitrogen and stored at −70°.

Enzyme Purification. All steps were carried out at 0–4°. Centrifugation was at 15,000 × *g* for 10 min unless otherwise indicated. All buffers contained 1 mM Na_3EDTA and 1 mM dithiothreitol.

Fraction I: the frozen cells were thawed in a 25° water bath and chilled to 0°. To 200 ml of cells, we added 10 ml of a freshly-made 20 mg/ml solution of lysozyme (Worthington)

Abbreviation: Col E1, colicin E1.

in 0.25 M Tris·HCl at pH 7.5 and 2 ml of 0.2 M Na_3EDTA. After gentle mixing, the solution was incubated at 0° for 30 min, and then centrifuged at 17,000 rpm for 30 min in a Sorvall SS-34 rotor. The supernatant lysate was further centrifuged at 40,000 rpm in a Spinco 60Ti rotor for 3 hr. The supernatant extract (fraction I, about 100 ml and 0.6 g of protein) was frozen in liquid nitrogen and stored at −70°. DNA gyrase can be assayed in this fraction, although there is evidence of inhibitory activities.

Fraction II: fraction I was precipitated with polymin P, with minor variations from the method of Burgess and Jendrisak (3). To 100 ml of fraction I, 7 ml of 5% (vol/vol) polymin P were added dropwise, with stirring. After 15 min of additional stirring, the suspension was centrifuged. The tubes were drained and the pellets resuspended in 40 ml total volume of 0.5 M NaCl, 0.05 M Tris·HCl at pH 7.5, by using a stirring rod to disperse the pellets. After stirring for 15 min, the suspension was centrifuged, the supernatant discarded, and the pellets were resuspended in 40 ml of 1 M NaCl, 0.05 M Tris·HCl at pH 7.5. After stirring for 15 min, the suspension was again centrifuged and the supernatant solution was collected. Solid ammonium sulfate (0.31 g/g of supernatant) was added, and the suspension was stirred for 15 min. The precipitate was collected by centrifugation, resuspended in 5 ml of 0.1 M KCl, 0.05 M Tris·HCl at pH 7.5, and dialyzed for 4 hr against 2 liter of the same buffer. A precipitate which formed during dialysis was removed by centrifugation. The supernatant, fraction II, was about 5 ml and contained 5 mg of protein.

Fraction III: fraction II (5 ml) was applied to a column with a 1 ml bed volume of DEAE-cellulose (type DE-52, Whatman), previously equilibrated with 0.1 M KCl, 0.05 M Tris·HCl at pH 7.5. The column was washed with several column volumes of the same buffer and then eluted with 0.25 M KCl, 0.05 M Tris·HCl at pH 7.5. Fractions with high activity were pooled (fraction III, 1.5 ml containing a protein total of 3.3 mg).

Fraction IV: fraction III was dialyzed for 2½ hr against 1 liter of 0.05 M potassium phosphate at pH 6.8, 10% (wt/vol) glycerol. The dialyzed fraction was diluted with an equal volume of 10% glycerol (containing Na_3EDTA and dithiothreitol), and immediately applied to a column (3 ml bed volume) of phosphocellulose (type P-11, Whatman) previously equilibrated with 0.025 M potassium phosphate at pH 6.8, 10% (wt/vol) glycerol. The column was washed with several volumes of the same buffer, then with 0.05 M potassium phosphate at pH 6.8, 10% glycerol, and finally the enzyme was eluted with 0.2 M potassium phosphate at pH 6.8, 10% glycerol. The active fractions were pooled and brought to 25% (vol/vol) glycerol before storage (fraction IV, 2.5 ml and 0.3 mg total of protein).

Fraction IV represents about 1/2000 of the total starting protein of the extract, with an apparent recovery of activity well above 100%. More precise quantitation of the purification procedure is not now possible because of nonlinearity of the assay (see below). Fractions I through III can be frozen in liquid nitrogen and stored for several months at −70° without loss of activity. Fraction IV can be stored at −70° in small aliquots, with less than 30% loss of activity in a month; it is not stable to repeated freezing and thawing.

All experiments described below were done with Fraction IV enzyme.

RESULTS

Supercoiled structure of the DNA product

When relaxed closed-circular Col E1 DNA was incubated with high levels of DNA gyrase, most of the DNA was converted to a form whose mobility in agarose gel was indistinguishable from that of naturally supercoiled Col E1 DNA (Fig. 1a and j). With less enzyme (Fig. 1c–i), DNA species with intermediate mobility were seen, which could be identified (7) with closed-circular DNA forms of lower degrees of supercoiling, which differ by one superhelical turn per band. The identification of the product of the DNA gyrase reaction as supercoiled Col E1 DNA was confirmed by its response to various enzymatic digestions. When the DNA product was treated with restriction endonuclease *Eco*RI, a single DNA species was formed that migrated to the same position as *Eco*RI-digested Col E1 DNA (Fig. 2a–f). Limited DNase I digestion produced a species with the mobility of open-circular Col E1 DNA, while treatment of the DNA product with 100 μg/ml of proteinase K (E. Merck) prior to the standard deproteinization by chloroform-isoamyl alcohol did not alter its electrophoretic mobility (data not shown).

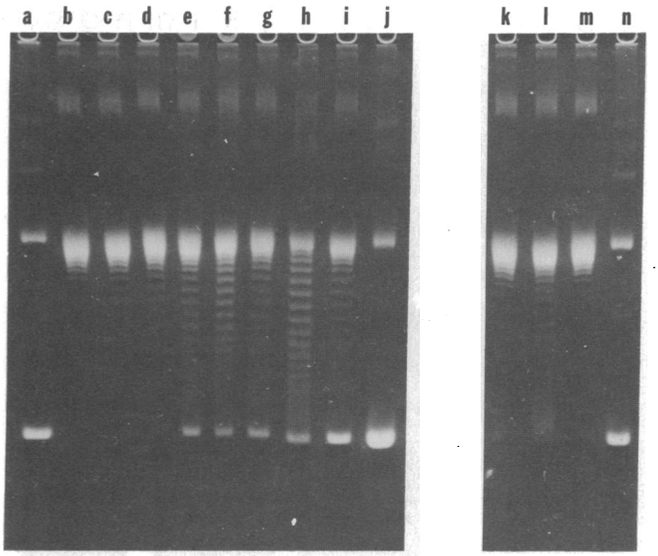

FIG. 1. Assay and reaction requirements of DNA gyrase. (a) Intracellularly supercoiled Col E1 DNA, no enzyme. (b–j) Relaxed Col E1 DNA incubated with varying amounts of DNA gyrase enzyme: (b) no enzyme; (c) 24 ng; (d) 48 ng; (e) 72 ng; (f) 96 ng; (g) and (h) 120 ng; (i) 240 ng; (j) 360 ng. (k–m) Relaxed Col E1 DNA incubated with 240 ng of DNA gyrase with: (k) ATP omitted; (l) spermidine omitted; (m) Mg^{++} omitted. (n) Supercoiled Col E1 DNA incubated with 240 ng of DNA gyrase in the absence of ATP. Reactions were carried out as described in *Materials and Methods*.

Because the gel analysis does not distinguish between positively and negatively supercoiled DNA, and because all highly twisted DNA of a given size forms a band at the same position regardless of its exact superhelix density (8), an independent measurement of superhelix density was necessary. This was carried out by equilibrium centrifugation in a CsCl–ethidium bromide mixture (9). The limit product at a high level of DNA gyrase (Fig. 3D) contained only negatively supercoiled DNA with a superhelix density about 1.5 times that of intracellularly supercoiled Col E1 DNA [by the analysis of (9)]. The sharpness of the band in Fig. 3D indicated that the product was quite homogeneous in superhelix density.

Characterization of the enzyme reaction

The enzyme catalyzes supercoiling of relaxed Col E1, λ, and simian virus 40 DNA; other DNAs have not been tested. The reaction is relatively specific for ATP. Half-maximal activity was obtained with about 15 μM ATP, while 0.5 mM dATP was required to produce similar activity. The other ribonucleoside

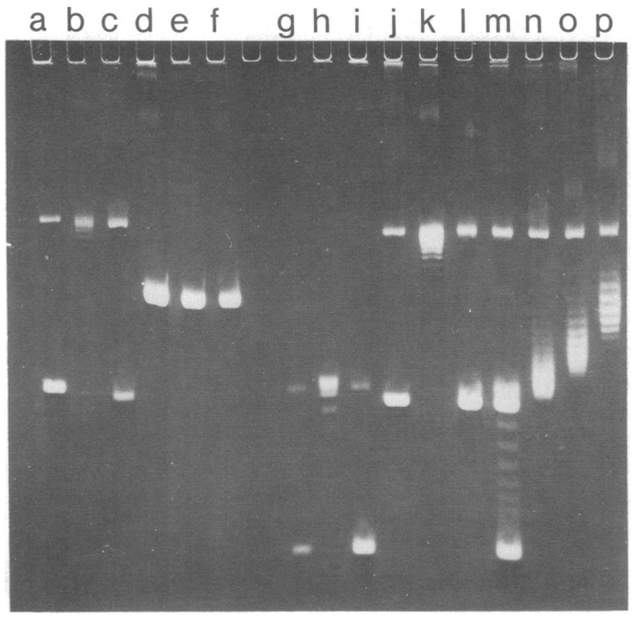

FIG. 2. Characterization by agarose gel electrophoresis of the product of the DNA gyrase reaction. (a) Col E1 DNA; (b) relaxed Col E1 DNA; (c) relaxed Col E1 DNA incubated with 0.60 μg of DNA gyrase; (d–f) the same three DNA samples digested with endonuclease *Eco*RI [see ref. (2) for digestion method]; (g) pNT1 DNA; (h) relaxed pNT1 DNA; (i) relaxed pNT1 DNA incubated with DNA gyrase; (j) Col E1 DNA; (k) relaxed Col E1 DNA; (l) relaxed Col E1 DNA incubated with DNA gyrase; (m–p) same as (l), but the incubation was continued for a second hour after the addition of: 0.4 μg relaxed pNT1 DNA or 5, 17, or 50 ng of ω protein, respectively. Reactions were carried out as described in *Materials and Methods*, except that in samples (a–f) the DNA concentration was decreased by 50%.

triphosphates (rGTP, rCTP, rUTP) and deoxyribonucleoside triphosphates (dGTP, dCTP, dTTP) were totally inactive when assayed at 1.4 mM, as were (α,β-methylene)ATP and (β,γ-methylene)ATP. In the absence of ATP, no reaction took place (Fig. 1k). Magnesium ion was required (Fig. 1m); the optimum concentration in the standard assay was 1–2 mM. Manganous ion (1.0 mM) and calcium ion (5 mM) gave lower levels of activity. The reaction was stimulated by spermidine (Fig. 1l), although with purified DNA gyrase the requirement was not absolute. With fraction IV, the spermidine requirement could be partly replaced by doubling the concentrations of potassium phosphate and $MgCl_2$. The final superhelix density of the product made in the absence of spermidine was close to that of intracellularly supercoiled DNA (data not shown). The enzyme is sensitive to *N*-ethylmaleimide. It is also 50% inactivated by incubation at 55° for 5 min.

Quantitative assay of the supercoiling reaction is complicated by several features. As already mentioned, the gel assay superposes all DNA species whose superhelix density reaches or exceeds a critical value. In addition, the reaction kinetics are strongly non-linear. Fig. 3 shows that a 2.5-fold increase in amount of enzyme (from 0.24 to 0.60 μg) resulted in about a 10-fold increase in amount of twisted product. The time course of the reaction at a single enzyme level showed a similar sigmoidal character (data not shown). Until these nonlinearities are better characterized, it does not seem useful to define a unit of DNA gyrase activity.

Fraction IV enzyme has been tested for a number of contaminating enzyme activities. Incubation under the conditions of Fig. 3D produced 2% additional nicked DNA (in addition to that present in the substrate) and, by gel analysis, no detectable double-strand breakage. The preparation contained no detectable *E. coli* DNA ligase [<5 pmol of bound AMP per mg of protein by the adenylylation assay of (10)], ω-protein activity (Fig. 1n), or RNA polymerase activity (<1 nmol/mg of protein). There was a low level of DNA polymerase activity [4 nmol/mg of protein by the assay of (11)], and some contaminating DNA-dependent ATPase (150 nmol/mg of protein under DNA gyrase assay conditions).

An approximate molecular weight of native DNA gyrase has been determined by sedimentation in a glycerol gradient in 0.25 M potassium phosphate at pH 7.0, using catalase, aldolase, and malate dehydrogenase as markers. A molecular weight of 140,000 was found.

Reaction mechanism

Our preferred model for the DNA gyrase reaction is that the enzyme directly introduces superhelical turns, and thus mechanical strain energy, into closed-circular DNA in an ATP-dependent reaction. In an alternate model, DNA gyrase activity would reflect the co-purification of a DNA-binding protein capable of introducing positive superhelical turns and a nicking-closing enzyme which would relax those turns. Deproteinization of such a binding protein–DNA complex would result in negatively superhelical DNA. One test to distinguish these models is to ask whether enough DNA gyrase is added to the reaction to supply the large amount of such a hypothetical binding protein that would be needed. In an experiment to estimate the maximum amount of DNA which could be supercoiled by the action of a given quantity of DNA gyrase, a 10-fold increased amount of relaxed Col E1 DNA (4 μg) was incubated for 18 hr with 0.1 μg of DNA gyrase. About 35% of the DNA became supercoiled as assayed by isopycnic centrifugation. This corresponds to roughly one molecule of supercoiled DNA per two molecules of enzyme, if we assume all the protein in fraction IV to be DNA gyrase. This is too small an amount of protein to make the binding protein model plausible. The question of whether a single enzyme molecule can catalyze the supercoiling of more than one molecule of DNA must await further purification of the enzyme.

A more critical distinction between the two models can be obtained by the deliberate addition to the reaction mixture of a nicking-closing enzyme such as *E. coli* ω protein. DNA which is relaxed in the presence of the reaction components should be unaffected, and should continue to yield superhelical DNA of unchanged superhelix density after deproteinization. However, if DNA gyrase produces negatively supertwisted DNA directly, then this DNA will be relaxed by the added ω protein and will display a reduced superhelix density.

Such an experiment is shown in Fig. 2g–p. After an incubation with enough DNA gyrase to supercoil most of the input DNA (Fig. 2l), varying amounts of ω protein were added and the incubation was continued (Fig. 2n–p). The ensuing reduction in superhelix density implies that the DNA was supercoiled in the reaction mixture. To show that DNA gyrase was still active during such a second incubation, we added plasmid pNT1 DNA in place of ω protein after the first incubation with Col E1 DNA, and the incubation was continued (Fig. 2m). Both DNA species became supercoiled. Thus, the final superhelix density in the presence of both DNA gyrase and ω protein is presumably determined by a balance of the two activities.

DISCUSSION

DNA gyrase appears to be an enzyme of a new class. Previously discovered nicking-closing enzymes (4, 12) are able to decrease

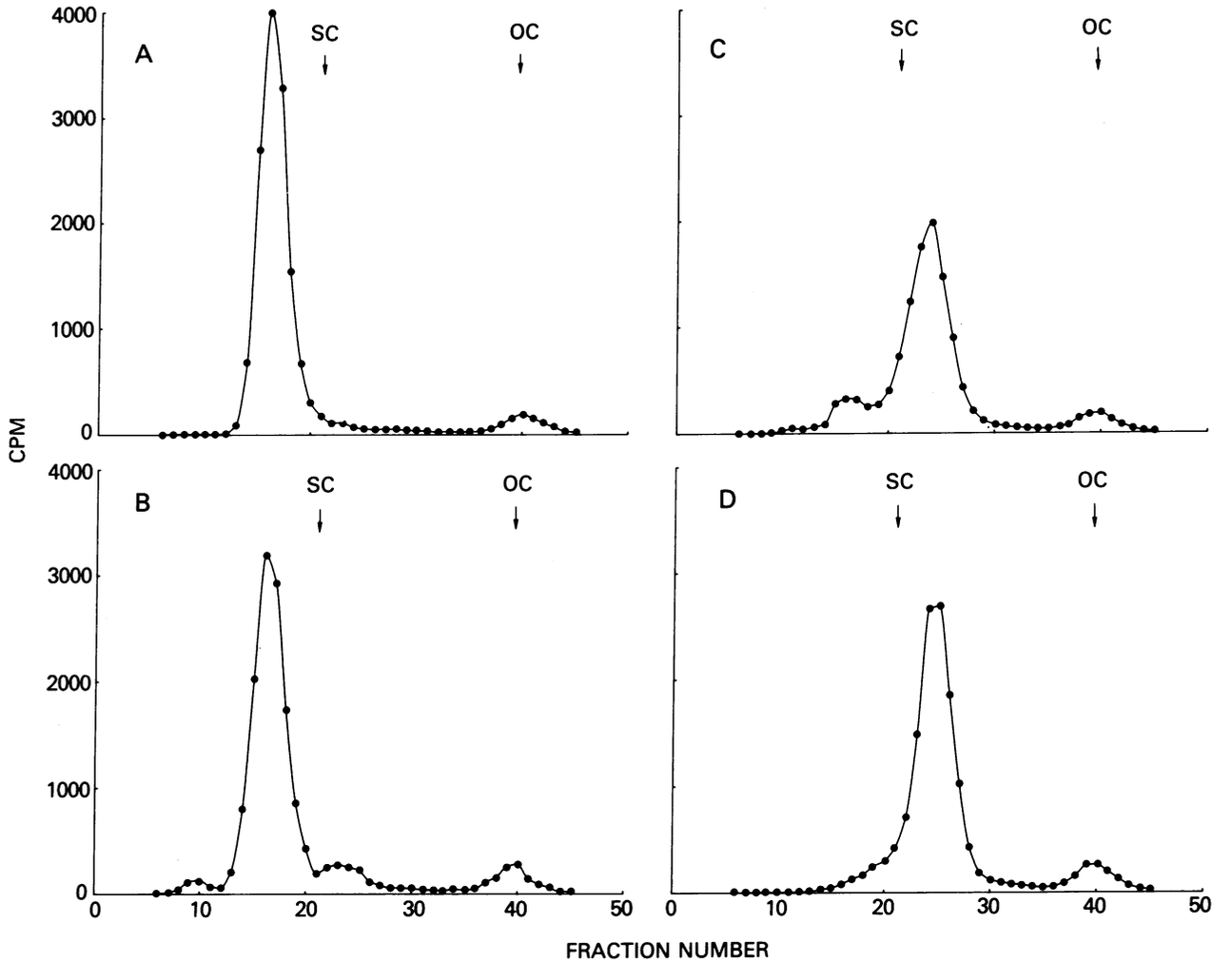

FIG. 3. Equilibrium density gradient centrifugation in CsCl-ethidium bromide of relaxed Col E1 [^{3}H]DNA incubated with varying amounts of DNA gyrase. (A) no enzyme; (B) 0.24 μg; (C) 0.60 μg; (D) 3.0 μg. Reactions were carried out as described in *Materials and Methods* but on a 2-fold volume scale, and were stopped by the addition of Na_3EDTA (0.01 M). The samples were mixed with a CsCl solution (final density of 1.55 g/ml) containing 0.33 mg/ml of ethidium bromide and a small amount of intracellularly supercoiled Col E1 [^{14}C]DNA, containing some open circles, as a density marker. The final volume was 7.4 ml. The samples were centrifuged at 35,000 rpm for 60 hr at 15° in a Spinco 65 rotor. Approximately 60 fractions were collected and the radioactivity was measured. The arrows show the position of supercoiled (SC) and open-circular (OC) marker Col E1 [^{14}C]DNA.

the superhelix density of covalently circular DNA, and thus reduce the strain energy stored in the molecule (13). Because this is the thermodynamically favored direction, these reactions do not require any added energy-yielding cofactor. DNA gyrase, on the other hand, catalyzes a reaction which increases the negative superhelix density and thus stores mechanical strain energy in the DNA. We presume that this reaction is driven by the hydrolysis of ATP although, at the present stage of purification, it has not been possible to demonstrate this coupling directly. Further purification is also needed to decide whether DNA gyrase activity is produced by a single protein or by the interaction of several protein species.

There is preliminary evidence that DNA gyrase functions in DNA replication, in addition to its role in the recombination reaction which led to its purification. In recent experiments (in collaboration with J. Tomizawa and T. Itoh) we found that DNA gyrase is strongly inhibited by novobiocin at a concentration of 3 μg/ml, while the enzyme from a novobiocin-resistant mutant of *E. coli* is entirely resistant to this level of the drug. Novobiocin is a specific inhibitor of DNA replication in toluene-treated *E. coli* cells (14), and in cell-free systems for Col E1 DNA replication (15) and for the duplication of phage ϕX 174 replicative form DNA (16). It thus appears that DNA gyrase supplies an essential function for the replication of double-stranded circular DNA. DNA gyrase could either act as a swivel to counteract the positive superhelical turns introduced by replication, or could pre-tension the DNA to produce a state of negative supercoiling strain. In the latter case, there would be a persistent torsional stress tending to unwind local regions of the DNA double helix, as for instance at replication forks.

We thank Sue Wickner for expert advice and criticism on questions of enzymology, and for help with some experiments. We are also indebted to Jun-ichi Tomizawa and Robert Bird for valuable discussions, and to James Wang for a generous gift of purified ω protein.

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