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Interaction between DNA and an *Escherichia coli* Protein ω

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An *E. coli* protein, designated ω, has been purified at least 1000-fold. Treatment of a covalently closed DNA duplex containing negative superhelical turns with ω results in the loss of most of the superhelical turns. The loss of superhelical turns follows a gradual course rather than a one-hit mechanism. This reaction does not require a cofactor. No other change in the physical properties of the DNA could be detected. The DNA remains covalently closed. Its ultraviolet absorption spectrum, circular dichroism, buoyant density in CsCl, sedimentation properties in neutral media containing varying amounts of ethidium and in an alkaline medium, and its susceptibility toward *Neurospora* endonuclease, are not significantly different from an untreated DNA containing the same number of superhelical turns. Thus it appears that ω is capable of introducing a "swivel" reversibly into a DNA. A plausible mechanism is postulated.

1. Introduction

In a previous communication on the degree of superhelicity of a number of closed cyclic DNA's from *Escherichia coli*, it was noted that there was a macromolecular species in an *E. coli* extract, which could convert a highly twisted superhelical DNA to a less twisted, covalently closed form (Wang, 1969*b*). This species has now been purified at least 1000-fold. All evidence indicates that it is a protein, and is designated ω. Some of its novel properties are described in this communication.

2. Experimental

(a) *Assay of ω*

Covalently closed cyclic *λb2b5c* DNA containing variable number of superhelical turns were prepared as described previously (Wang, 1969*a*). In most of the assays, a DNA sample with a superhelical density (Bauer & Vinograd, 1968; Wang, 1969*a*) of −0·039 (150 negative superhelical turns per DNA molecule) was used. Unless specified otherwise, superhelical density (or superhelical turns) refers to the quantity measured in 3 M-neutral CsCl at 20°C. A standard assay mixture contained 0·01 M-Tris (pH 8·0), 0·002 M-$MgCl_2$, 0·001 M-Na_3 EDTA, 0·05 mg of bovine plasma albumin (Armour) per ml., and 20 μg of DNA per ml. To 30 μl. of the assay mixture, 1 μl. of a fraction containing ω was added. After mixing at 0°C, the solution was incubated at 30°C for 30 min. Reaction was stopped by the addition of 20 μl. of 4 M-NaCl, 0·02 M-Na_3 EDTA. 40 μl. of the final solution was used in a band sedimentation run (Vinograd, Bruner, Kent & Weigle, 1963). The bulk sedimentation medium for the assay of ω contained 3 M-CsCl (Harshaw), 0·01 M-Na_3 EDTA, and $8{\cdot}88 \times 10^{-6}$ M-ethidium bromide (Calbiochem). Before treatment with ω, a superhelical *λb2b5c* DNA with a superhelical density of −0·039 had a sedimentation coefficient S of 26 s in this medium. After treatment, the reduction in superhelical turns

caused an increase in S to 33 S (see Fig. 1). Usually an An-K rotor, which could accommodate four 30-mm cells, was used. Double-sector type III centerpieces were used exclusively, as described previously (Wang, 1969*a*). The standard assay could only detect whether ω was present in sufficient quantity to convert the 26 S species to the 33 S species. To quantitate the relative amounts of ω present, it was necessary to utilize results shown in Fig. 3(b). Such measurements were very time consuming, and were done for only a few selected fractions.

(b) *Purification of* ω

(i) *Crude extract*

Frozen *E. coli* 1100 cells, grown from a slant obtained from Dr B. M. Olivera, were a generous gift of Dr M. Maestre. This strain was used in all but one of the preparations, in which *E. coli* B was used. To 100 g of frozen cells, 100 ml. of 0·1 M-glycylglycine, pH 7·0, was added. The mixture was homogenized in a Waring blender, and sonicated at maximal intensity with a Branson sonicator (model BP1). The temperature of the solution was controlled between 0 to 4°C. Sonic bursts of a total duration of 20 min were sufficient to break up the cells. Cell debris were removed by centrifugation at 20,000 *g* and 4°C for 30 min in a model L ultracentrifuge (no. 30 rotor).

(ii) *Streptomycin precipitation*

This step was carried out as described by Olivera & Lehman (1967) for the preparation of the polynucleotide joining enzyme.

(iii) *Ammonium sulfate precipitation*

Solid ammonium sulfate, either the enzyme grade (Mann), or the reagent grade (Mallinckrodt), was added to the supernatant of the previous step at 0°C. The protein fraction, precipitated out between 20 to 28% w/w of $(NH_4)_2SO_4$ was resuspended in 50 ml. of 0·1 M-glycylglycine. Approximately 1 g of protein was present in this fraction.

(iv) *DEAE column chromatography*

The ammonium sulfate fraction was dialyzed against two changes (2 hr per change) of 0·1 M-NaCl–0·005 M-potassium phosphate, pH 7·5, in the cold room. The protein was loaded on a 2·5 cm × 20 cm DEAE column. After washing with 60 ml. of the same buffer, a linear gradient (500 ml.) of 0·1 to 0·5 M-NaCl was applied. Potassium phosphate buffer, 0·005 M with a pH of 7·5, was present across the salt gradient. Fractions of 5 ml. each were collected. Under these conditions, ω was only weakly adsorbed on the column and was eluted very early. This step separated ω from the polynucleotide joining enzyme.

(v) *First phosphocellulose column chromatography*

Active fractions from the DEAE column fractionation were pooled and dialyzed overnight against 0·05 M-potassium phosphate, pH 6·5. The solution was loaded on a 2·2 cm × 10 cm phosphocellulose column and eluted successively with the following potassium phosphate solutions, all of a pH of 6·5: 0·05 M, 60 ml.; 0·1 M, 40 ml.; 0·2 M, 60 ml.; 0·5 M, 40 ml. All activity was eluted between 0·1 and 0·2 M phosphate. Fractions of 5 ml. were collected.

(vi) *Second phosphocellulose column chromatography*

Twenty ml. of the pooled active fractions from the above step were dialyzed against dry sucrose to reduce the volume to about 5 ml. It was then dialyzed against 0·05 M-potassium phosphate, pH 6·5, for 20 hr (3 changes). After loading on a 0·8 cm × 10 cm phosphocellulose column and washing with 10 ml. of the same buffer, a linear gradient (30 ml.) of 0·1 to 0·2 M-potassium phosphate, pH 6·5, was applied. Fractions of 2 ml. were collected. The peak fractions were pooled, concentrated by dialysis against sucrose, and then dialyzed against 0·1 M-NaCl, 0·005 M-potassium phosphate, pH 7·5.

Steps (i) through (iii) above were usually carried out without interruption. The ammonium sulfate fraction was stable for a few weeks, and the DEAE fraction and the phosphocellulose fraction after concentration were stable for at least a few months. Starting with 100 g of frozen cells, only 0·5 mg of protein were obtained in the peak

fractions of the second phosphocellulose chromatography. The specific activity of the purified protein was approximately 1000 times that of the ammonium sulfate fraction. The crude extract showed no ω activity, presumably due to the large amount of sonicated *E. coli* DNA present, which would inhibit ω (see Results). In connection with this, it should be noted that ω activity could be detected in cell lysate obtained by the lysozyme–Brij procedure, following the removal of the membrane attached DNA (Wang, 1969*b*).

(c) *Other enzymes*

DNA polymerase activity was kindly assayed for me by Mr J. Carlson, using the d(A-T) primed assay procedure, as described by Richardson (1966). RNA polymerase activity was assayed as described by Chamberlein & Berg (1962). The polynucleotide joining enzyme was assayed by incubation with cyclized *λb2b5c* in 0·01 M-Tris (pH 8), 0·002 M-$MgCl_2$, 0·001 M-Na_3 EDTA, and 10^{-5} M-DPN, at 30°C for 30 min. The reaction mixture also contained 0·05 mg bovine plasma albumin/ml. After incubation, part of the solution was used in a band sedimentation run, with 3 M-CsCl, 0·1 M-KOH, 0·01 M-Na_3 EDTA as the bulk medium. Since the absence of covalently closed *λb2b5c* DNA after incubation could be due to a nicking enzyme, a second assay was performed by dialyzing the rest of the DNA solution against 0·1 M-NaCl, 0·01 M-Na_3 EDTA. The DNA solution was heated at 75°C for 5 min, quickly cooled to 0°C, and a band sedimentation run on neutral 3 M-CsCl was performed. The joining of either one, or both, of the two original breaks in cyclized λ DNA would give a cyclic species. If none of the breaks were joined, a linear species would be obtained. The *Neurospora* endonuclease, originally prepared by Linn & Lehman (1965), was a gift of Mr D. Hinckel. The assay medium was 0·1 M-Tris (pH 7·5), 0·01 M-$MgCl_2$, 0·004 M-2-mercaptoethanol and 0·05 mg bovine plasma albumin/ml. Incubation was done at 37°C. The fd DNA used was a gift of Professor S. Linn. Band sedimentation in 3 M-CsCl, 0·1 M-KOH, 0·001 M-Na_3 EDTA showed no detectable amount of linear DNA in this sample. Pronase (Calbiochem) stock solution (1 mg/ml. in 0·01 M-Tris, pH 8) was autolyzed by incubating at 37°C for 2 hr. It was then heated at 80°C for 2 min and quickly cooled to 0°C before use.

3. Results

(a) *Some properties of ω*

The activity of ω is retained after dialysis against 1 M-NaCl, 0·005 M-potassium phosphate (pH 7·5) for 24 hours, followed by dialysis against 0·1 M-NaCl, 0·005 M-potassium phosphate (pH 7·5). Heating above a temperature of 50°C for 10 minutes, or incubation with 100 μg pronase/ml. at 37°C, leads to inactivation of ω. These results suggest that ω is a protein. The purified ω does not have the Kornberg DNA polymerase activity, RNA polymerase activity, or the polynucleotide joining enzyme activity.

(b) *Interaction between ω and negative superhelical DNA*

A pronounced reduction in the number of superhelical turns was observed when a negative superhelical DNA was incubated with ω. Figure 1 depicts the dependence of the sedimentation coefficient S on the free ethidium concentration c_f in the sedimentation medium for a superhelical *λb2b5c* DNA before and after treatment with ω. Before incubation with ω, the S *versus* c_f curve showed a minimum at $c_f = 1{\cdot}73 \times 10^{-5}$ M, corresponding to an initial superhelical density of $-0{\cdot}039$, or 150 negative superhelical turns (Wang, 1969*a*). After incubation with ω followed by phenol extraction, the S *versus* c_f curve gave a minimum at $c_f = 2{\cdot}92 \times 10^{-6}$ M, corresponding to an initial superhelical density of $-0{\cdot}011$ or 42 negative superhelical turns. Therefore, 110 negative superhelical turns per DNA molecule were lost.

The reduction in the number of superhelical turns was also evident from electron micrographs of the DNA sample before and after treatment with ω. As depicted in

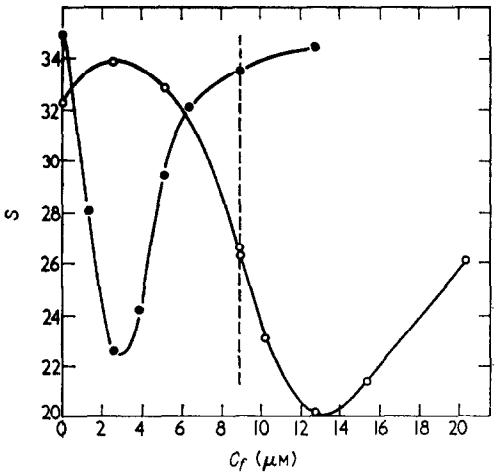


FIG. 1. Sedimentation coefficient, S, as a function of free ethidium concentration c_f in the sedimentation medium. —○—○—, Untreated *λb2b5c* DNA; —●—●—, the same sample after treatment with ω. All sedimentation runs were done in 3 M-CsCl, 0·01 M-Na_3EDTA, at 24,630 rev./min and 20°C. The dotted line shows the ethidium concentration at which assays of ω were done. At this ethidium concentration, nicked *λb2b5c* circles has an S of 22 S, and is distinguishable from the 26 S untreated DNA.

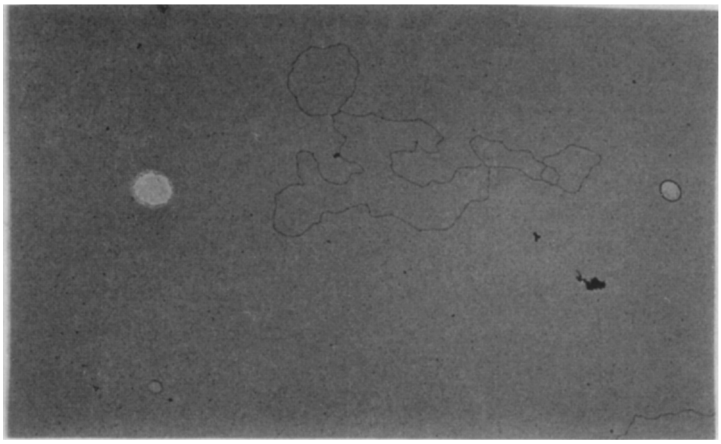
Plate I, before treatment the highly twisted DNA appeared as a branched molecule. Such a branched structure is always seen for a *λb2b5c* DNA molecule containing more than 100 superhelical turns, using the sample fixation procedure described in the legend of Plate I (Baase & Wang, unpublished results). Each branch shown in the Plate undoubtedly consists of two DNA double helices twisted together. After treatment with ω, the branched structure typical of a highly twisted molecule was no longer observed. The DNA molecule appeared much more open, and crossovers could be observed.

Treatment of a superhelical *λb2b5c* DNA containing 100 negative superhelical turns with ω gave a product indistinguishable from that resulting from the DNA sample with 150 negative turns. The physical properties of an ω treated negative superhelical DNA will be described in a later section.

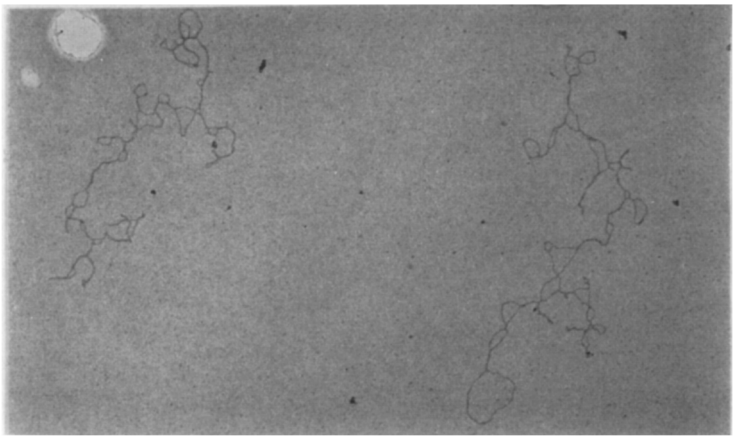
(c) *Interaction between ω and covalently closed* λb2b5c *DNA containing no superhelical turns or positive superhelical turns*

Figure 2 depicts S as a function of c_f for a *λb2b5c* DNA sample before and after ω treatment. The original DNA sample contained 22 negative superhelical turns in 3 M-CsCl at 20°C. In the salt medium used for incubation with ω the DNA should have zero superhelical turns (Wang, 1969*a*). The S *versus* c_f curves of this sample before and after incubation with ω showed no difference.

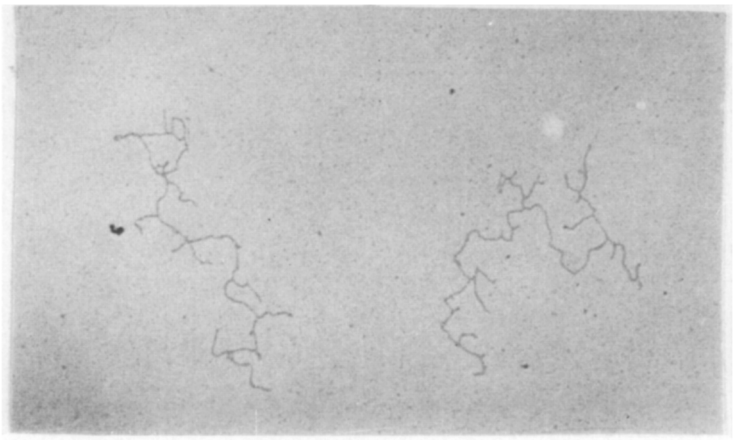
For a *λb2b5c* DNA sample with 8·5 negative superhelical turns in 3 M-CsCl at 20°C, incubation with ω also causes no change in the number of superhelical turns (Fig. 2). Under incubation conditions with ω, this sample should contain 14 positive superhelical turns (Wang, 1969*a*). At the present time it is not possible to prepare a superhelical DNA containing more positive turns. Therefore, in order to study whether ω had an effect on a superhelical DNA with higher number of positive turns, addition of ethidium to the incubation mixture was necessary.



(a)



(b)



(c)

PLATE I. Electron micrographs of twisted *λb2b5c* DNA before and after treatment with ω. (a) Before treatment. The sample had a superhelical density of $-0{\cdot}039$ (or 150 negative superhelical turns per DNA). (b) After treatment with ω. (c) A nicked *λb2b5c* DNA shown for comparison. All grids were prepared as described previously (Martin & Wang, 1970). The freshly mixed DNA (approx. 2 μg/ml.) and cytochrome *c* (approx. 0·2 mg/ml.) solution in 0·45 M-ammonium acetate was layered onto 0·3 M-ammonium acetate. The protein–DNA film was picked up on carbon stabilized Parlodion covered grids within one minute after layering.

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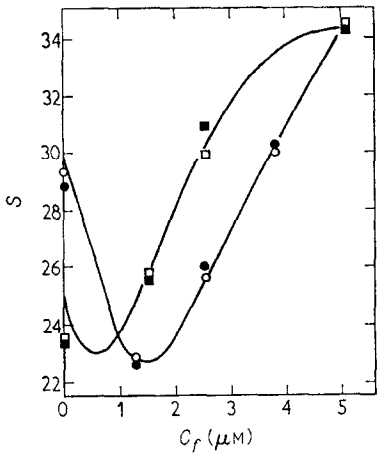


FIG. 2. Dependence of the sedimentation coefficient, S, on free ethidium concentration for two covalently closed cyclic *λb2b5c* DNA samples before (open symbols) and after (filled symbols) treatment with ω. The two samples contained 8·5 and 22 negative superhelical turns, respectively. The curves drawn were taken from Fig. 1 of Wang (1969*a*) for samples prepared the same way as the samples used here. All runs were performed in 3 M-CsCl, 0·01 M-Na_3EDTA, at 24,630 rev./min and 20°C.

To test whether ethidium inhibits the reaction, 0·025 mole of ethidium per mole of DNA nucleotide were added to a superhelical DNA sample originally containing 150 negative superhelical turns. Assuming quantitative binding of ethidium in the incubation mixture, the dye–DNA complex contained approximately 85 negative superhelical turns. If ethidium inhibits the activity of ω, treatment with ω would not change the number of superhelical turns. On the other hand, if there is no inhibition, the DNA–dye complex would relax to a form with approximately 40 negative superhelical turns (see section (b) above). In other words, a loss of approximately 45 negative superhelical turns would result. The latter was found experimentally to be the case.

Since ethidium does not seem to inhibit the reaction, 0·025 mole of ethidium per mole of DNA nucleotide were added to a superhelical DNA originally containing 14 positive superhelical turns under incubation conditions with ω. Again assuming quantitative binding of ethidium, the DNA–dye complex contained approximately 80 positive superhelical turns. Surprisingly, no reduction in the number of superhelical turns was observed upon incubation with ω.

Unfortunately, while the dye–DNA nucleotide ratio was the same in the two experiments with negative and positive superhelical DNA's as substrates, these experiments are not directly comparable in a strict sense. This is because the binding of ethidium by a superhelical DNA is dependent on both the number and the sense of the superhelical turns (Bauer & Vinograd, 1970). The concentration of free ethidium is approximately a factor of two higher in the experiment with positive superhelical DNA as the substrate, as estimated from the free energy of superhelicity of *λb2b5c* DNA (Wang, unpublished results).

(d) *Interaction between ω and some other nucleic acids*

With the exception of negative superhelical DNA, no physical change could be detected when a DNA was treated with ω. Nevertheless, it is possible to study the

effect of a nucleic acid on the reaction between ω and a negative superhelical DNA. A number of such competition reactions were carried out. It was found that single-stranded DNA, either denatured sonicated *λb2b5c* DNA or the cyclic fd DNA, was very effective in inhibiting the reduction of negative superhelical turns by ω. Approximately 0·5 μg/ml. of a single-stranded DNA would inhibit the reaction of ω on a negative superhelical DNA. Sonicated native *λb2b5c* DNA and calf thymus DNA also inhibited the reaction, although they were somewhat less effective than single-stranded DNA. Native *λb2b5c* DNA was much less effective, by at least a factor of 50 compared with the same DNA after sonication, in inhibiting the reaction. In none of these experiments was there evidence of appreciable endonucleolytic activity. No reduction in size of the DNA substrates was detectable. *E. coli* transfer RNA, at concentrations as high as 100 μg/ml. in the incubation mixture, did not inhibit the reduction of negative superhelical turns by ω.

(e) *Dependence of the rate of reduction of superhelical turns by ω on temperature and the concentration of ω*

Figure 3(a) depicts the sedimentation coefficient at 20°C of the superhelical DNA in 3 M-CsCl, $8{\cdot}88\times10^{-6}$ M-ethidium as a function of the incubation time at several temperatures. In the presence of this amount of ethidium, all of the points shown in the Figure correspond to DNA containing positive superhelical turns. The increase in S for each curve reflected an increase in the number of positive superhelical turns in the sedimentation medium or a reduction of the number of negative superhelical turns in the incubation medium. The data indicate two important features of the reaction. Firstly, the reduction of the number of superhelical turns by ω follows a gradual course rather than a one-hit mechanism. The sedimentation coefficient gradually changed from that of the initial sample to that of the final product, as the incubation time was increased. The superhelical DNA sedimented as a sharp band in each case, indicating a sharp distribution in the number of superhelical turns at any time during the course of the reaction. Secondly, the reaction is very temperature sensitive. It can

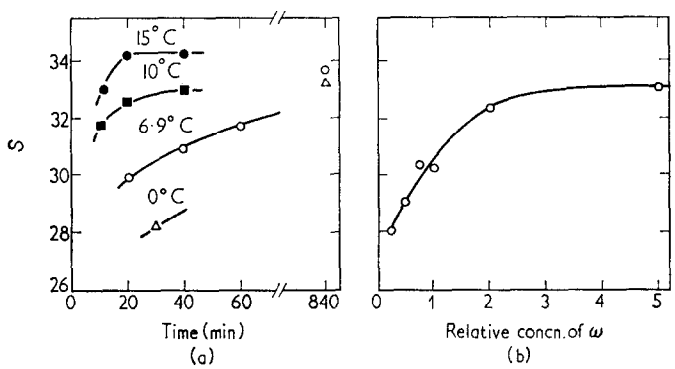


FIG. 3. (a) Sedimentation coefficient, S, of a *λb2b5c* DNA sample initially containing 150 negative superhelical turns, as a function of incubation time (in min) with ω. The incubation temperature for each set of data is indicated in the Figure. The concentration of ω in each incubation mixture was the same. Sedimentation runs were done in 3 M-CsCl, 0·01 M-Na_3EDTA, $8{\cdot}88\times10^{-6}$ M-ethidium, at 24,630 rev./min and 20°C. (b) Sedimentation coefficient, S, in the same medium as described in (a), as a function of the relative concentration of ω in the incubation mixture. Incubation was done at 10°C for 30 min. At a relative concentration of 1, approx. 1 μg/ml. of the ω fraction of the highest specific activity was present in the incubation mixture.

be seen that it took 70 minutes at 6·9°C to give a product with an S of 32 s. At a temperature 3 deg. C higher, the same change took only 11 minutes. From the sedimentation coefficients measured, the number of superhelical turns of the DNA samples in the absence of ethidium can be estimated. For three *λb2b5c* DNA samples with 42, 76, and 116 negative superhelical turns, the sedimentation coefficients in 3 M-CsCl, $8{\cdot}88\times10^{-6}$ M-ethidium were measured to be 33·5, 26·8 and 21·0 s, respectively. Interpolating from these points, it can be estimated that the number of negative superhelical turns dropped from 150 to about 60 after a 20-minute incubation at 6·9°C. This number was reduced to about 56 after 40 minutes incubation, and to about 52 after 60 minutes incubation.

At the same temperature, the rate is dependent on ω concentration, as shown in Figure 3(b). These results provide a way to assay the relative concentration of ω. A detailed study of the reaction kinetics will be presented elsewhere.

(f) *Dependence of the rate of reduction of superhelical turns by ω on ionic species*

The standard assay mixture used contained 0·01 M-Tris buffer (pH 8·0), 0·002 M-$MgCl_2$, and 0·001 M-Na_3 EDTA. If Mg(II) was omitted, or if the EDTA concentration exceeded the Mg(II) concentration, no activity was detectable, Ca(II) or Mn(II) were not as effective as Mg(II) in supporting the reduction of negative superhelical turns by ω. The divalent ion requirement was not absolute, however, and could be replaced by a monovalent cation at a higher concentration. For example, ω was active in 0·05 M-potassium phosphate without Mg(II). On the other hand, addition of monovalent salt to the standard assay medium was inhibitory to the reaction. The rate of the reaction was progressively lowered as increasing amounts of NaCl was added. No reaction was detectable when the sodium ion concentration exceeded 0·3 M. A similar effect was observed for potassium ion.

Due to the difficulty in the quantitative assay of ω, no attempt was made to determine the optimal pH for the reaction. It was only known that ω was active at least in the pH range 6·5 to 8·0. Finally, no cofactor was required by ω.

(g) *Physico-chemical properties of ω treated negative superhelical DNA*

After incubation with ω, the DNA sample which contained 150 negative superhelical turns was phenol extracted. Approximately 85% of the DNA was in the covalently closed form, and was isolated by an ethidium bromide CsCl density gradient centrifugation. The small amount of nicked DNA could not be closed by the polynucleotide joining enzyme, and it was not clear whether these nicks were introduced enzymically. Ethidium was removed by extraction with n-butanol as usual (Wang, 1969*a*).

A number of physical measurements were carried out on this product. Its sedimentation coefficients as a function of ethidium concentration in neutral 3 M-CsCl is presented in Figure 1. In 3 M-CsCl, 0·1 M-KOH, the DNA has an S of 112 s, the same as an untreated covalently closed *λb2b5c* DNA. The ultraviolet spectrum was identical to that of an untreated *λb2b5c* DNA. Its circular dichroism spectrum in 0·1 M-NaCl, 0·001 M-Na_3EDTA was identical to that of a superhelical *λb2b5c* DNA containing approximately the same number of superhelical turns as the ω-treated product. With d(A-T) as a marker, the buoyant density of the ω-treated sample in CsCl was the same as the untreated DNA (within an experimental error of ±0·0002 g/ml.).

The S *versus* c_f curve shown in Figure 1 was not significantly altered by digesting the

ω-treated DNA with pronase before phenol extraction or annealing in 0·1 M-NaCl, 0·01 M-Na_3 EDTA at 60°C for two hours.

Therefore, with the exception of the number of negative superhelical turns, no other change in the physical properties of the DNA could be detected.

(h) *Possibility of short single-stranded regions in ω treated DNA*

For reasons to be discussed in a later section, the possibility of the existence of single-stranded regions in an ω-treated negative superhelical DNA was tested. Two experiments were performed.

The first experiment was intended to answer the question, that given a superhelical λDNA containing 150 negative superhelical turns, if a small segment was denatured, would the DNA renature to give the original species containing 150 negative superhelical turns?

In order to disrupt a helical segment, the DNA (in 3 M-CsCl) was titrated with KOH and its sedimentation coefficient was measured as a function of pH. The *S versus* pH curve was similar to that reported by Vinograd & Lebowitz (1966) for polyoma DNA. Below a pH of 11·40, the DNA with 150 negative superhelical turns had an S of 39 S (1·6 times that of the nicked cyclic DNA). Above pH 11·4, the sedimentation coefficient began to decrease. A minimal value of 24 S was reached at a pH of 11·65. Further increase in pH raised the sedimentation coefficient. At pH 11·80, $S = 44$ S. At pH 12·0, $S = 48$ S. The interpretation for the dependence of S on pH is the same as the one given by Vinograd & Lebowitz (1966) for polyoma DNA. The decrease and subsequent increase in S around pH 11·6 results from the disruption of helical segments, which caused a reduction of negative superhelical turns and then an increase in the positive superhelical turns. The disruption of helical segments at high pH before complete strand separation was also demonstrated by the electron microscopic studies of Inman & Schnös (1970). Assuming that the change in the number of superhelical turns was solely due to the disruption of helical segments, then at a pH around 11·6 where the number of superhelical turns was zero, approximately 1500 base pairs per *λb2b5c* DNA molecule were disrupted.

Five DNA solutions titrated to pH values of 11·21, 11·40, 11·62, 11·82 and 12·00, respectively were neutralized and their sedimentation coefficients were measured as a function of ethidium concentration c_f in neutral 3 M-CsCl. In each case, the S *versus* c_f curve was not significantly different from that of the original DNA shown in Figure 1. These results showed that with a total of several thousands of base pairs per *λb2b5c* DNA molecule disrupted, the answer to the question posed at the beginning of this section was affirmative. This agreed with the results of Rush & Warner (1970) that ϕX174 replicative form I titrated to a high pH below a certain critical value could renature to a species of the same sedimentation coefficient in a neutral medium as the original molecule.

In a second experiment, the sensitivity of ω-treated negative superhelical *λb2b5c* DNA toward the *Neurospora* endonuclease was studied. This enzyme was known to be specific for single-stranded nucleic acids (Linn & Lehman, 1965). Therefore, if a DNA contained single-stranded regions of appreciable length, it would be expected to be more susceptible to digestion by this enzyme. Four DNA's were used as substrates: the single-stranded cyclic DNA fd, the *λb2b5c* DNA containing 150 negative superhelical turns before and after treatment with ω, and a *λb2b5c* DNA containing 22 negative superhelical turns. The average number of single-chain scissions per DNA

molecule $<i>$ was calculated by assuming that the fraction of intact molecules left p was related to $<i>$ by the Poisson distribution relation $p = e^{-<i>}$. Since the fd DN was much more susceptible, $<i>$ for this sample was calculated from digestion with enzyme concentrations 5 to 25 times lower than the enzyme concentration used for the other DNA samples. It was further assumed that $<i>$ was proportional to the enzyme concentration in this concentration range (0·0002 to 0·005 unit/ml.). This assumption was substantiated using denatured ^{32}P-labeled λDNA as a substrate and assaying for acid precipitable counts after digestion.

Under conditions 30 hits were introduced in each fd DNA, each ω-treated λDNA encountered only 0·3 hit. The *λb2b5c* sample with 22 negative superhelical turns in 3 M-CsCl at 20°C, which should have a negligible number of superhelical turns under the endonuclease digestion conditions, had 0·2 hit per molecule under the same conditions.

These results indicated that if there were single-stranded regions in ω-treated negative superhelical λDNA, the total number of nucleotides in such regions per λ molecule was less than 1% of the number of nucleotides per fd DNA, or approximately 50 bases.

4. Discussion

For a closed cyclic DNA duplex, the topological constraint requires:

$$\tau + \beta = \text{constant}$$

where τ and β are the superhelical turns and the right-handed turns of the double helix, respectively (Vinograd & Lebowitz, 1966; Glaubiger & Hearst, 1967). Since treatment with ω changes τ, there are only two possibilities: (1) β is simultaneously changed; (2) The topological constant is absent during the reaction. That is, a swivel point or points is introduced during treatment with ω.

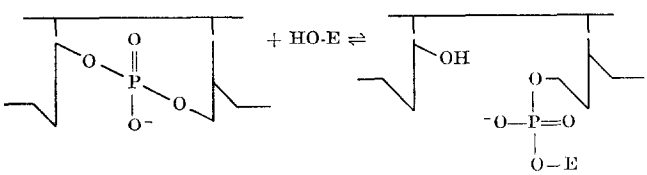
If β is changed, there are two important possibilities: (1(a)) the DNA helix is modified by ω in such a way that the helix rotation angle per base pair, $\theta°$, is decreased. For the sample with 150 negative superhelical turns, 110 turns were lost by treatment with ω. This requires a decrease of one degree in $\theta°$. (1(b)) The helix rotation angle remain unchanged. However, treatment with ω disrupts segments of the helix. The total length of such regions is estimated to be of the order of 2200 nucleotides, assuming that the topological constraint is still applicable to a covalently closed DNA duplex with single-stranded regions of this length.

Both (1(a)) and (1(b)) are not supported by experimental results. A decrease of one degree in $\theta°$ is a fairly large perturbation of the DNA helix, yet both the absorption spectrum and circular dichroism do not show any difference comparing with an untreated DNA. Furthermore, the buoyant density (in CsCl) of ω-treated DNA agrees with that of the untreated DNA to within ±0·0002 g/ml. This renders modifications such as methylation improbable. If 1% of the nucleotides are methylated, the buoyant density would change by 0·001 g/ml. (Szybalski, 1968). With regard to the possibility of single-stranded regions, the renaturability of alkaline-generated single-stranded regions suggested that such regions, if existing, should have renatured under conditions for which measurements on the degree of superhelicity of ω-treated DNA were carried out. If one postulates that ω molecules remained bound to the single-stranded regions and therefore renaturation was inhibited, one would have to make the improbable assumptions that, (i) such bound molecules were not removed by phenol extraction and Pronase digestion, and (ii) the number of bound ω-molecules necessary

for the inhibition of renaturation (for single-stranded regions approx. 2000 bases long) were sufficiently small so that no shift in buoyant density of the ω–DNA complex could be detected. Furthermore, the susceptibility of ω-treated negative superhelical DNA to *Neurospora* endonuclease digestion indicates that no appreciable single-stranded regions exist in such molecules.

These results leave one with possibility (2), i.e. the topological constraint was temporarily removed during treatment with ω. One straightforward mechanism is that ω has two activities: a nicking activity which catalyzes the hydrolysis of a phosphodiester bond to give an adjacent pair of phosphoryl and hydroxyl groups, and a joining activity which catalyzes the reformation of the phosphodiester bond. This mechanism is unlikely. First, ω does not catalyze the joining of ligase-joinable phosphoryl-hydroxyl groups. Second, if a pair of phosphoryl-hydroxyl groups were formed, the reformation of a phosphodiester bond from such groups in an aqueous solution would require an energy source, yet ω does not require such a cofactor. It cannot be ruled out however, that ω catalyzes the formation of a 5′-hydroxyl-3′-phosphoryl pair and that the rejoining of such a pair is carried out by an ω-molecule which has been "activated" *in vivo* to a higher energy state.

A second mechanism, which circumvents the difficulties discussed in the preceeding paragraph, is the following. Instead of the generation of a nick of the phosphoryl-hydroxyl type as a swivel point, a nick is formed with ω directly attached to one of the groups at the nicking point. This reaction is reversible, and the departing of ω is accompanied by the reformation of the backbone bond. One specific possibility of this type of mechanism is illustrated below:



where HO—E represents ω. The hydroxyl group in HO—E above is shown solely as an illustration. While chemically reasonable, it is not an essential part of the hypothesized mechanism.

If the function of ω is to introduce a swivel in a double-stranded DNA, there remain two questions: (1) why is it not active on a positive superhelical DNA? (2) Data in Figure 1 showed that the DNA after treatment with ω contained 42 negative superhelical turns in 3 M-CsCl at 20°C. This corresponded to 20 negative superhelical turns under incubation conditions with ω. Why was this number of superhelical turns left in the molecule?

With respect to the second question, there might be two contributing factors. It is possible that at the end of the reaction the DNA contains no superhelical turns, but some ω molecules are bound on single-stranded regions to prevent the renaturation of such regions. Removal of ω after the reaction introduces some negative superhelical turns. A second plausible contributing factor is kinetic rather than thermodynamic in nature. As noted in Results, the rate of loss of negative superhelical turns decreases as

the number of superhelical turns in the DNA molecule decreases, presumably due to the reduction of the magnitude of the driving torque. For measurements done at 6·9°C, 90 superhelical turns were lost in the first 20 minutes, while in the next 20 minutes only four superhelical turns were lost. Therefore the 20 or so negative superhelical turns left might be due to the slow rate when the degree of superhelicity is low.

With respect to the first question, there are a number of possibilities. (i) Since the presence of ethidium is necessary to give a DNA containing a large number of positive superhelical turns, the experiment demonstrating the lack of activity of ω on twisted DNA containing a large number of positive superhelical turns might not be valid. This point has already been discussed. Therefore the only definitive conclusion on positively twisted DNA is that ω does not affect the number of turns of a *λb2b5c* DNA containing 14 positive superhelical turns. This can easily be attributed to the kinetic effect discussed in the above paragraph. (ii) Assuming that the small amount of ethidium does not inhibit the reaction, there are two possibllities. (ii(a)) ω is capable of recognizing the handedness of the superhelical turns. (ii(b)) The fact that single-stranded DNA inhibits the action of ω on negative superhelical DNA suggests that ω binds strongly to single-stranded DNA. If the disruption of a small segment of double-strand helix is necessary for the binding of ω, then the free energy of superhelicity favors such a reaction for a negative superhelical DNA, while the opposite is true for a positive superhelical DNA.

Among these three possibilities, (i), (ii(a)) and (ii(b)), I feel that (ii(a)) is the least likely and (ii(b)) is the most likely explanation.

As a final remark, the swivel mechanism suggested immediately leads one to ponder the possibility that ω is part of the DNA replication apparatus. Its function as a swivel, and its ability to rejoin the nick would make it an attractive candidate for the replication team.

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